Factors important to the secretion and matrix deposition of tissue transglutaminase

Kamila Malgorzata Pytel

Doctor of Philosophy

Aston University
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Factors important to the secretion and matrix deposition of tissue transglutaminase

Kamila Malgorzata Pytel

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2011

Thesis summary

Data suggest that for TG2 to be secreted, an intact N-terminal FN binding site (for which TG2 has high affinity) is required, however interaction of TG2 with its high affinity binding partners presents both in the intracellular and extracellular space as well as with specific cell surface receptors may also be involved in this process.

Using a site-directed mutagenesis approach, the effects of specific mutations of TG2 on its translocation to the cell surface and secretion into the ECM have been investigated. Mutations include those affecting FN binding (FN1), HSPGs binding (HS1, HS2) GTP/GDP binding site (GTP1, 2) as well as N-terminal and C-terminal domains (TG2 deletion mutants N, and C). By performing transglutaminase activity assays, cell surface protein biotinylation and verifying distribution of TG2 mutants in the ECM we demonstrated that one of the potential heparan sulfate binding site mutants (HS2 mutant) is secreted at the cell surface in a much reduced manner and is less deposited into the ECM than the HS1 mutant. The HS2 mutant showed a low affinity for binding to a heparin sepharose column demonstrating this mutation site may be a potential heparin binding site of TG2. Analogous peptides to this site were shown to have some efficiency in the inhibition of the binding of the FN-TG2 complex to cell surface heparan sulfates in a cell adhesion assay indicating the peptide to be representative of the novel heparin binding site within TG2.

The GTP binding site mutants GTP1 and GTP2 exhibited low specific activity however, GTP2 showed more secretion to the cell surface in comparison to GTP1. The FN1 binding mutant did not greatly affect TG2 activity nor did it alter TG2 secretion at the cell surface and deposition into the ECM indicating that fibronectin binding at this site on the enzyme is not an important factor. Interestingly an intact N-terminus (Δ1-15) appeared to be essential for enzyme externalisation. Removal of the first 15 amino acids (N-terminal mutant) abolished TG2 secretion to the cell surface as well as deposition into the ECM. In addition it reduced the enzymes affinity for binding to heparin. In contrast, deletion of the C-terminal TG2 domain (Δ594-687) increased enzyme secretion to the cell surface.

Consistent with the data presented in this thesis we speculate that TG2 must fulfill two requirements to be successfully secreted from cells. The findings indicate that the closed conformation of the enzyme as well as intact N-terminal tail and a novel HS binding site within the TG2 molecule are key elements for the enzyme’s localisation at the cell surface and its deposition into the extracellular matrix. The importance of understanding the
interactions between TG2, heparan sulfates and other TG2 binding partners at the cell surface could have an impact on the design of novel strategies for enzyme inhibition which could be important in the control of extracellular TG2 related diseases.
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Abstracts:

Pytel, K., Collighan, R., and Griffin, M. Factors important in the secretion and localisation of tissue transglutaminase. The poster was presented at the 2nd EMBO Conference on Cellular Signaling & Molecular Medicine, 2010, May 21-26, Cavtat (Dubrovnik), Croatia.

Pytel, K., Collighan, R., and Griffin, M. Factors important to the non-conventional export of tissue transglutaminase. The poster was presented at the Workshop of immunology and related techniques organised on the 26-28th August, 2009, by University of Tampere, Finland.

Pytel, K., Collighan, R., and Griffin, M. Factors important to the non-conventional export of tissue transglutaminase to the cell surface. The presentation was presented at the Marie Curie meeting, organised on the 9-10th February, 2008 in Rome, Italy.
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<table>
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine-5’-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Biotin-X-cadaverine</td>
<td>5(((N-(Biotinoyl)amino)hexanoyl) amino) pentylamine trifluoroacetate salt</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C</td>
<td>TG2 C-terminal deletion mutant (Δ594-687)</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Free calcium ion</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CUB7402</td>
<td>Translutaminsae II Ab-1 monoclonal antibody</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunoabsorbant assay</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>FN</td>
<td>Fibronectin</td>
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<td>GDP</td>
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<td>gpl tTGase</td>
<td>Guinea pig liver transglutaminase</td>
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<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<td>Guanosine-5’-triphosphatase</td>
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<td>TG2 mutant (S171E)</td>
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<td>GTP2</td>
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<td>HEK293</td>
<td>Human embryonic cells</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSPGs</td>
<td>Heparan sulfate proteoglycans</td>
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<td>HS2</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IgG</td>
<td>Immunoglobulin</td>
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<td>kDa</td>
<td>Kilodaltons</td>
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<td>LTBP-1</td>
<td>Latent TGF-β1 binding protein-1</td>
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<td>ml</td>
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<td>µ</td>
<td>Micro</td>
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<td>Symbol</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>N</td>
<td>TG2 N-terminal deletion mutant (Δ1-15)</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Mouse embryo fibroblasts</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>Negative log of hydrogen ion concentration</td>
</tr>
<tr>
<td>PKCα</td>
<td>Protein Kinase Cα</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N´-tetramethylene diamine</td>
</tr>
<tr>
<td>TGase</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>TG1</td>
<td>Keratinocyte transglutaminase</td>
</tr>
<tr>
<td>TG2</td>
<td>Tissue transglutaminase</td>
</tr>
<tr>
<td>TG3</td>
<td>Epidermal transglutaminase</td>
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<td>TG4</td>
<td>Prostate transglutaminase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-aminoethane</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>t-Octylphenoxypolyethoxyethanol</td>
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Figure 6.2 TG2 sequence alignment. Alignment is showing conserved (green) positively charged amino acids and non-conserved (red) residues.
Chapter I: Introduction
1.2 Transglutaminases and post-translational modification of proteins

Transglutaminases are multifunctional enzymes that possess more than one enzymatic feature acting on a wide range of different substrates. The first data reporting a TG were obtained from guinea pig liver by Clarke and his collaborators in 1957 (Clarke et al., 1957). Two years later an official name of ‘transglutaminase’ was used to describe this enzyme in a scientific report of Mycek (Mycek et al., 1959). All together, nine proteins have been characterised within the transglutaminase family. Eight of them are functionally and structurally connected with the TGase’s functions (the TGase 1-7 and Factor XIIIa). However the ninth one which is band 4.2 lacks a catalytic active site and remains only structurally related with the rest of the group (Griffin et al., 2002; Lorand and Graham, 2003).

Transglutaminases are particularly interesting enzymes because of their ability to function in many physiological processes. It was reported that TGases can catalyse a number of different reactions which may affect either protein structure or the signal transduction process across the cell membrane. Some of these enzymatic reactions are chemically similar. Although there can be a difference in terms of substrate specificity or the kinetics of the reaction (Facchiano and Facchiano, 2009).

The main function of TGases which are characterised as Ca\(^{2+}\) dependent thiol enzymes is to catalyse the post-translational modification between proteins resulting in their cross-linking (Griffin and Wilson, 1984; Griffin et al., 2002; Fesus and Piacentini, 2002; Lorand and Graham, 2003; figure 1; Zemskov et al., 2006). The transamidating activity involves forming an N\(^\epsilon\)(γ-glutamyl)lysine isopeptide bond between the glutamine γ-carboxamide group of one polypeptide (acceptor) and the lysine ε-amino group of another polypeptide (donor) (Griffin et al., 2002; Zemskov et al., 2006) (Figure 1.1a). The reaction is strictly dependent on the presence of calcium which exposes a cysteine in the TGases active site resulting in the formation of cross-linked proteins which are highly protected against proteolytic degradation resulting in increased tissue strength (Folk and Finlayson, 1977; Griffin and Wilson, 1984). The transamidating activity can also lead to the amine incorporation (putrescine, spermine, histamine) which is based on replacing neutral
glutamine (Gln) residues by a positively charged one resulting in the formation of (γ-glutamyl)-polyamine bonds (Folk and Finlayson, 1977) (Figure 1.1b). Another intriguing property of TGases is to create ester bonds. In particularly TGase 1 enzyme has been shown to mediate an esterification reaction between specific glutaminyl residues of human involucrin and a synthetic analogue of ω-hydroxyceramides (Nemes et al., 1999) (Figure 1.1d). This specific function plays an important role in maintaining the epidermal barrier of the skin. It has also been reported that TGases can modify protein structure by catalysing the hydrolysis of peptide bound glutamine residues to glutamic peptide bond glutamate (Mycek and Waelsch, 1960). It was shown that deamidation of glutamine residues, catalysed by transglutaminases can influence the mechanism of the autoimmune diseases e.g. celiac disease (Aleanzi et al., 2001; Shan et al., 2002; Falini et al., 2008). This specific function of TGases is due to its ability to modify gluten proteins (Shan et al., 2002; Mazzeo et al., 2003). Briefly, TG2 mediated deamidation of gliadin favors the antigen presentation for activating T cells in celiac disease (Shan et al., 2002; Falini et al., 2008). Further analysis of TGases specificities, has revealed information about their potential in vitro to break the isopeptide bonds with high affinities for γ-branched peptides. TG2 as well as FXIIIa exhibit isopeptide activity and has been reported to hydrolyse Nε(γ-glutamyl)lysine isopeptide bonds indicating the transglutaminase involvement in cell biology by both catalysing the formation and breaking of Nε(γ-glutamyl)lysine isopeptide bonds (Parameswaran et al., 1997).

Another function that these multifunctional enzymes exhibit is acting as a protein disulphide isomerase which is totally independent on the presence of calcium as well as their transamidation activity (Hasegawa et al., 2003).

Although it seems easy to outline all reactions that transglutaminases catalyse, their enzymatic pathways are much more complex. When the acceptor carrying a glutaminyl residue is recognised by enzyme, the γ-glutamylthioester bond is formed with the cysteine residue of the TG active site which is known as the acylenzyme intermediate. This results in the release of ammonia (Figure 1.1a-e) or amine (Figure 1.1f). Meanwhile the donor amine reacts with the acyl-enzyme complex which attacks the thioester bond causing the cysteine residue of the enzyme to reverse back to its original form. Having an active site again, the enzyme is allowed to participate in another reaction and depending
Figure 1.1 Post-translational reactions catalysed by transglutaminases adopted from Lorand and Graham, 2003. Schematic illustration of different reactions mediated by transglutaminases a cross-linking between the lysine (Lys) residue of the targeted donor protein (purple ellipse) and acceptor glutamine (Gln) residue of another protein (blue rectangle) b incorporation of an amine (H₂N) into the Gln residue c acylation of the Lys residue in donor protein. Additionally to transamidation TGases can catalyse d esteryfication between the Gln residue of protein and a ceramide (HOR") and protein hydrolysis e deamidation, and f isopeptide cleavage. R corresponds to the side chain in a primary amine; R' a Gln containing peptide; R"' and R"'' the side chains in isopeptides.
on the donors’ nature, generate different reaction products (Lorand and Graham, 2003, figure 1).

The substrate specificity related to a specific Tgase activity still remains under the investigation (Kim et al., 1994; Nemes et al., 1999; Taguchi et al., 2000). It is known that only specific glutamine and lysine residues can act in proteins both as acyl donors and acyl acceptors (Facchiano and Facchiano, 2009). Many researchers have been exploring the specificity of these residues. Finally the consensus sequences pQx(P,T,S)1 – (where x, p and 1 suggests any amino acid, polar and aliphatic amino acids, respectively) which includes the reactive glutamines was found (Keresztessy et al., 2006). Meanwhile it was reported that TG2 catalyses the cross-linking reaction within a specific sequence patterns as well QxPhD, QxPh, and QxxhDP (where x can be any amino acid and h can be an hydrophobic amino acid) (Sugimura et al., 2006; Facchiano and Facchiano, 2009). Furthermore another work revealed that substrate specificity is not only restricted by the consensus sequence but is more flexible which explains its ability to be cross-linked by TGases (Fontana et al., 2008).

1.3 Classification of transglutaminases

So far, nine members of the transglutaminase family have been identified at the genomic level in humans (Grenard et al., 2001a). As summarized in Table1.1, the transglutaminase family consist of: the keratinocyte transglutaminase (TG1), the tissue transglutaminase (TG2), the epidermal transglutaminase (TG3), the prostate transglutaminase (TG4), the circulating zymogen FXIII, (the inactive form of the enzyme is converted into the active FXIIIa subunit during thrombin-dependent proteolysis), the recently characterised transglutaminase X, Y, and Z (TG5, TG6, TG7) and erythrocyte band 4.2 (Aeschlimann et al., 1998; Griffin et al., 2002).

Eight of the nine TG genes encode catalytically active enzymes while one encodes a catalytically inactive protein called band 4.2.

Within the transglutamianse family a high level of structural homology has been observed. It is known that all the active members of this family share a four domain tertiary structure with a catalytic triad of Cys-His-Asp or Cys-His-Asn (Ikura et al., 1988; Griffin et al., 2002).
Table 1.1 Classification of TGases, adopted from Lorand and Graham, 2003 and Facchiano and Facchiano, 2009.
Moreover the common feature for all the members is lack of carbohydrate modifications regardless of the presence of N-glycosylation sites, as well as cysteine residue at the active site.

So far, the importance of transglutaminase catalytic activity in physiology, development of many human organs (heart, lung, salivary gland, and nervous system) as well as maintaining tissue stability and integrity has been reported (Lorand and Graham, 2003; Telci and Griffin, 2006). Thus, it is not surprising that alteration of TG expression or activity has been implicated in a wide variety of diseases and pathologies. For example pathological conditions like skin disorders contribute to overexpression of TGases (TG1, TG2) whereas decrease of TG2 expression is involved in tumour growth following disruption of cell-matrix interactions leading to cancer-cell metastasis (Belkin et al., 2001; Lorand and Graham, 2003; Jones et al., 2006).

1.4 Activation of FXIII and role of FXIIIA in mammalian cells

It is known, that the other transglutaminase (FXIIIA) widely employed in maintaining the cell surface homeostasis, appears in a zymogen form requiring proteolytic activation of the pro-enzyme FXIII which occurs by thrombin cleavage (Muszbek et al., 1999; Al-Jallad et al., 2006). FXIII (blood coagulation factor) exists as heterotetramer in plasma or homodimer in cells (Shwartz et al., 1973; Ariëns et al., 2002; Shwartz et al., 2007). Being a tetramer ($A_2B_2$), FXIII is composed of two proenzyme subunits ($A_2$) surrounded by two carrier subunits ($B_2$) while existing as homodimer ($A_2$), FXIII is made exclusively of two subunits A (Shwartz et al., 2007; Shwartz et al., 2009). Subunit A contains the catalytic active site, an activation peptide, a calcium binding site and free sulfhydryl groups, while the carrier subunit B is a glycoprotein that stabilises subunit A, binds it to fibrinogen and influences FXIII activation (Lorand et al., 2001; Ariëns et al., 2002).

The Factor XIII A subunit is synthesised by hematopoetic cells such as megakaryocytes, monocytes and macrophages, while the B subunit is synthesised by liver (Muszbek et al., 1995, 1996). FXIIIA has also been found in the placenta, prostate, uterus and liver (Henriksson et al., 1985; Greenberg et al., 1991; Adany, 1996). The secretion mechanism of FXIII A subunit still remains unknown. Some data indicates that FXIIIA is released as a consequence of cell destruction and is thought to unite with FXIII B.
in the circulation (Kaetsu et al., 1996). However there is some other data suggesting that similarly to other mammalian transglutaminases (TG2 and TG4), FXIII A subunit is secreted through an unconventional pathway. It has been shown that FXIII A can be externalised separately from its carrier subunit FXIII B that undergoes secretion through an ER/Golgi dependent mechanism (Al-Jallad et al., 2006). It was found in monocytes, osteoblasts, and osteocytes and was reported to be secreted from differentiating osteoblasts (Al-Jallad et al., 2006; Nakano et al., 2007).

The gene coding for the FXIII A subunit is mapped to chromosome 6p 24-25 and is composed of 15 exons interrupted by 14 introns encoding a mature protein of 731 amino acids (Board et al., 1988; Hsieh and Nugent, 2008). Following analysis of cDNA sequences of FXIII, it was reported that FXIIIA shares sequence homology with TG2, TG3 and band 4.2 (Ichinose, 1986; Ichinose and Kaetsu 1993). The FXIII B subunit gene is located on chromosome 1q31-32.1 and comprises 12 exons interrupted by 11 introns which encodes a mature protein of 641 amino acids (Webb et al., 1989; Bottenus et al., 1990).

Upon activation by thrombin which is the final step of blood clotting cascade and in the presence of Ca^{2+}, the inactive enzyme (plasma FXIIIA) becomes activated (Fox et al., 1999; Casadio et al., 1999). Studies on FXIIIA structure including x-ray crystallography and computer modeling analysis has additionally shown that the presence of calcium is not only required for activation but also enhances the physical stability of FXIIIA (Fox et al., 1999; Casadio et al., 1999). Triggered by thrombin cleavage of an N-terminal peptide on the A-subunits, dissociation of the A-subunits from the B-subunits exposes the active cysteine binding site of plasma FXIIIA (Ariëns et al., 2002; Lorand and Graham, 2003). The enzymatically active transglutaminase catalyses the cross linking leading to the formation of covalent bonds between proteins (adjacent fibrin monomers) which contain appropriate lysine and glutamine residues (Muszbek et al., 1999) (Figure1.2). Activated FXIII catalyses a transamidation reaction between two different fibrin molecules through an ε-(γ-glutamyl) lysine bonds (Shwartz et al., 1973). This reaction enhances the strength of the blood clot by providing a network for cell migration and new tissue formation (Shainoff et al., 1991; Telci and Griffin 2006). Moreover, the cross-linking reaction catalysed by FXIII influences fibrin breakdown by incorporation of fibrinolysis resistant inhibitors (α2-antiplasmin, PAI-2) (Richie et al., 2000; Ariëns et al., 2002). FXIII forms covalent bonds with α2-antiplasmin, (the main inactivator of plasmin) (Booth, 1999;
Board et al., 1993) and thrombin (activatable fibrinolysis inhibitor) which increases the resistance of the clot to proteolytic degradation by plasmin (Mosesson et al., 2008; Hsieh and Nugent, 2008). Additionally FXIII A can play a wider role by cross-linking several other protein substrates including the blood coagulation factor V (Francis et al., 1986), fibrinogen (Dyr et al., 1989), von Willebrand factor (Hada et al., 1986), type-2 plasminogen activator inhibitor (Richie et al., 2000). Also plasma fibronectin present on the fibroblasts surface as well as fibrinectin synthesised by these cells were suggested to be cross-linked by FXIII A (Barry and Mosher, 1990).

Other studies suggest that in tissues not expressing thrombin the inactive FXIII subunit can still be activated, by other serine proteases including endogenous platelet acid protease and calpain through cleavage of the Arg-37, Gly-38 (Lynch and Pfueller, 1988; Ando et al., 1987). Additionally FXIIIA can also be activated for example in bone by membrane bound protease (MMP-2) or cell surface endopeptidase PHEX (Belkin et al., 2004; Miao et al., 2001). However in the intracellular environment where calcium levels rise in the presence of an appropriate substrate platelet FXIII may undergo a non proteolytic conformation change leading to its activation (Polgar et al., 1990).

Figure 1.2. Activation of FXIII and cross-linking of insoluble fibrin. Adopted from Lorand et al., 2001, and Shwartz et al., 2009.
FXIII was defined in early papers as a fibrin stabilising factor, however recent papers indicate its involvement in many processes which result from its ability to crosslink plasma proteins as well as proteins within the vascular matrix, endothelial cells, maturing monocytes and platelets. So far, it has been reported that FXIIIA plays an important role in many processes including wound healing (Telci and Griffin, 2006; Jones et al., 2005), cell proliferation (Ádany et al., 2001; Schwartz et al. 2009), cell motility (Jayo et al., 2009) osteoblast and chondrocyte differentiation (Al Jallad et al., 2006; Johnson et al., 2008) and bone formation (Nakano et al., 2007), chromatin structure remodeling and even cell death (Ádany et al., 2001; Schwartz et al., 2009). In addition, FXIII has been reported to be involved in angiogenesis through interaction with factors that possess proangiogenic features (fibronectin, thrombospondin1) or interaction with β3 integrins and the VEGF receptor (Dardik et al., 2004; Dardik et al., 2005). Moreover it was suggested that FXIII can play other important roles outside of homeostasis by enhancing the metastasis by a mechanism linked to NK cells (Palumbo et al., 2008).

The deficiency in FXIII activity has been correlated with many diseases mainly affecting homeostasis, normal wound healing as well as healthy pregnancy. It was demonstrated that severe FXIIIA deficiency can lead to bleeding diathesis, delays in wound healing and tissue repair, bleeding during pregnancy which increases the possibility of early miscarriage (Anwar and Miloszewski, 1999; Hsieh and Nugent, 2008).

1.5 Keratinocyte transglutaminase (TG1)

Keratinocyte transglutaminase (transglutaminase type 1) was identified as an intracellular, epidermal specific enzyme (Thacher and Rice, 1985; Philips et al., 1990). TG1 was reported to be primarily expressed in stratified squamous epithelia, however as measured by in situ hybridization, it was noted its expression seemed dependent on keratinocyte proliferation and differentiation (Ta et al., 1990; Michel et al., 1992; Kim et al., 1995). In the skin epidermis it is expressed in the upper spinous and granular layers and is involved in the formation of the outermost skin protection barrier the so called cornified cell envelope (CE) (Thacher and Rice, 1985; Schroeder et al., 1992; Kim et al., 1995). The presence of this insoluble outer cell layer on the cell surface of the epidermis acts as the main barrier against chemical or mechanical injury as well as biological
invasion. Additionally it was reported to be formed of ε-(γ-glutamyl) lysine cross-linked proteins present on the intracellular surface of the plasma membrane due to transamidation activity (Ta et al., 1990). Immunohistochemistry evidence suggests that TG1 appears to be very important in catalysing cross-linking of a range of substrates including involucrin (Simon and Green, 1988), loricrin (Mehrel et al., 1990), cornifins/small proline-rich proteins (Marvin et al., 1992; Gibbs et al., 1993), cystatin-α (Takahashi et al., 1992), keratin intermediate filaments, filaggrin (Steiner and Marekov, 1995), envooplakin (Ruhrberg et al., 1996) and other proteins (Robinson et al., 1997).

Initially keratinocyte transglutaminase from human and rat was shown to be the largest from the whole transglutaminase family with a molecular mass of 90 kDa (Philips et al., 1990). Further studies on proliferating cells revealed the full length of the enzyme to be 106kD (Kim et al., 1994 and 1995). However this discovery was related to the cytoplasmic TG1 which was associated to the inner surface of keratinocyte membrane through mirystyl and palmityl anchors (Philips et al., 1993). Further work showed that keratinocyte differentiation causes increased proteolysis of cytoplasmic TG1 to yield fragments of 10, 33, 67 kDa (Steiner et al., 1996, Kim et al., 1995) which was suggested by in vitro studies to result in increased TG1 activity (Kim et al., 1994, Kim et al., 1995).

The gene coding for human TG1 is located on the 14q11.2 chromosome and encodes a mature protein composed of 816 amino acids (Kim et al., 1992; Yamanishi et al., 1992). Studies on the TGM1 gene have revealed that the TGM1 locus is connected to the autosomal skin disease as Lamellar ichthyosis (Russell et al., 1994). Lamellar ichthyosis is a disorder which affects both skin and hair which results from the mutation in the TG1 gene causing thickening of the epidermis with high risk of dehydration (Huber et al., 1995; Russell et al., 1995; Eckert et al., 2005). This finding indicates that disruptions in TG1 activity are the main source of the disease. Additional research on TG1 deficient mice showed that this enzyme is essential in the development of the stratum corneum and in neonatal adaptation to the environment (Matsuki et al., 1998). Briefly, it was demonstrated that TG1 -/- mice died 4-5 hours after birth due to the abnormal skin keratinisation and an impaired skin barrier (Matsuki et al., 1998).
Chapter I: Introduction

1.6 Epidermal transglutaminase (TG3)

Epidermal transglutaminase was first characterised in 1975 by Buxman and Wuepper as a soluble protein of around 50kDa (Buxman and Wuepper, 1975) and purified from human and bovine skin (Buxman and Wuepper, 1976; Ogawa and Goldsmith, 1976). Further studies revealed that TG3 is synthesised as an inactive zymogen form with a molecular weight of 77 kDa which undergoes limited proteolysis resulting in its activation. The proteolytic activation requires cleavage of the zymogen form into two fragments of 30 kDa and 47 kDa in the region between the catalytic core and the β-barrel 1 and takes place during keratinocyte differentiation (Kim et al., 1990; Kim et al., 1993). Upon proteolysis TG3 like all mammalian TGases requires the presence of calcium ions which influences enzyme activity (Ahvazi et al., 2002; Ahvazi et al., 2003). In addition its activity can also be regulated by binding guanine nucleotides (Ahvazi et al., 2004).

Even though the protease activation of epidermal transglutaminase in vivo still remains elusive, cathepsin-L (Cheng et al., 2006) and the bacterial enzyme dispase (Hitomi et al., 1999) has been reported to proteolyse and activate the enzyme in vitro. Additionally, recent studies showed that the protease inhibitor cystatin M/E can act as an inhibitor of cathepsin-L thus negatively regulate TG3 activation at least during skin morphogenesis in the neonatal phase (Cheng et al., 2006).

Epidermal transglutaminase is expressed mainly as a proenzyme in the brain, testis, small intestine as well as in the skin, generally in its granular and cornified layers (Hitomi et al., 2001; 2003). Additionally it can be localised in the cytoplasm of differentiating keratinocytes as shown by Hitomi et al., (2003). Similar to TG1, TG3 was reported to be involved in the formation of the cornified cell envelope (Buxman and Wuepper, 1975). When activated, TG3 crosslink proteins including loriclin and small proline rich proteins present in the cytoplasm of keratinocytes. In contrary to TG1, TG3 forms mostly intramolecular cross-links between certain lysine and glutamine residues while TG1 favours intermolecular reactions (Candi et al., 1995). Moreover, TG3 cross-links one of the small proline rich proteins in a consecutive manner while TG1 forms large oligomers (Hitomi et al., 2005).

The physiological role of epidermal transglutaminase is still elusive. However it is known to play role in the diagnosis of dermatitis herpetiformis (cutaneous form of celiac
disease). The detection of autoimmune IgA antibodies directed against epidermal transglutaminase was revealed to be a very sensitive test for the detection of dermatitis herpetiformis (Sardy et al., 2002; Rose et al., 2009).

### 1.7 Prostate transglutaminase (TG4)

Prostatic transglutaminase (TG4) is a unique member within the whole transglutaminase family. It was described for the first time in the dorsal prostate and coagulation gland of rats and characterised as a homodimeric protein of 150 kDa composed of two polypeptide chains each with molecular weight of 75 kDa (Seitz et al., 1990; 1991). So far it is known to be found mainly in the prostate gland and on a very low level in other tissues (Gentile et al., 1995; Dubbink et al., 1996).

As shown by in situ hybridization the gene encoding human TG4 is located on the 3p21.33-p22 chromosome (Gentile et al., 1995) and encodes a protein consisting of 684 amino acids with a predicted molecular mass of 77 kDa (Dubbink et al., 1996). In the prostate, the expression of human TG4 is limited to luminal epithelial cells of the gland (Dubbink et al., 1999), however recent studies on a cancerous human cell lines revealed a much broader expression of this enzyme. Colon, lung and prostate cancerous cells showed a wide range of prostate transglutaminase expression level (Davies et al., 2007). Yet very little information has been provided regarding the mechanism of its externalisation. It has been revealed that the 30kDa and 100 kDa GTPase enzymatic activities are linked to the prostatic secretion with the TG4 (Spina et al., 1999). Additional information relates to albumin, which was reported to facilitate TG4 translocation through the membrane (Wiche et al., 2003).

Little is known with regard to the physiological and pathological functions of this enzyme. In rodents, the rat prostate transglutaminase has been demonstrated to be involved in the formation of the copulatory plug (Williams-Ashman, 1984). In addition TG4 has been suggested to play a role in sperm cell motility, immunogenicity and immunoregulation (Ablin and Whyard, 1991). Moreover it has been reported that TG4 may influence the invasiveness of the prostate cancer by being up regulated in some prostate cancer cell lines (Dubbink et al., 1996) and down-regulated in most metastatic prostate cancers (An et al., 1999). Another data base using prostate cancer cells (CAHPV-
10 cells) indicates that when the prostate TG4 is lost from these cells they become less invasive (Davies et al., 2007). Recent studies discovered that over expression of TG4 in prostate cancer cells enhances their capability to adhere to endothelial cells leading to the disturbance of the barrier function of the endothelial cells (Jiang et al., 2009).

1.8 Erythrocyte Band 4.2

The membrane protein, erythrocyte band 4.2, is one of the most abundant proteins within the erythrocyte membrane cytoskeleton, accounting for approximately 5% of the membrane components (Yawata, 2003). Analysis of the genomic organisation of human band 4.2 has revealed that the gene coding for band 4.2 is composed of 13 exons and 12 introns (Korsgen and Cohen, 1991) giving rise to two different isoforms, a minor one with molecular weight of 74 kDa and a major isoform with molecular weight of 72 kDa (Korsgen et al., 1990; Sung et al., 1990; Satchwell et al., 2009). The human band 4.2 protein has been reported to share a sequence homology with other members within the transglutaminase family even though it does not possess the active cysteine site required for the cross-linking activity (Korsgen et al., 1990) thus suggesting that band 4.2 is strictly a structural protein (Cohen et al., 1993).

Studies on the amino acid sequence of the human band 4.2 (Korsgen et al., 1990; Sung et al., 1990) demonstrated that band 4.2 contains an N-terminal glycine residue following the initial methionine which becomes myristylated (Risinger et al., 1992). In addition the human protein was also reported to be palmitoylated on cysteine 203 (Das et al., 1994). Both of these processes have been shown to be required for the direct interaction of band 4.2 with the erythrocyte inner membrane space.

The exact role of protein band 4.2 has not been fully discovered. It is known that band 4.2 associates with the N-terminal cytoplasmic domain of the erythrocyte membrane by binding to the anion exchanger band 3 (Korsgen and Cohen, 1986). In addition it interacts with membrane structural proteins including ankyrin, protein band 4.1 (Korsgen and Cohen, 1988), and spectrin (Mandal et al., 2002) in solution. This interaction influences the structural integrity of the erythrocyte membrane. Moreover band 4.2 has been proposed to be involved in the anchoring of the Rh complex into the red blood cell skeleton by interaction with CD47 which is one of the proteins belonging to the Rh complex (Mouro-Chanteloup et al., 2003; Satchwell et al., 2009) Thus erythrocyte band
4.2 plays an important role in assisting the survival of red blood cells in circulation (Satchwell et al., 2009).

The absence of protein band 4.2, caused by natural mutations in humans or deficiency in its expression by mice can lead to haemolytic anaemia (Bruce et al., 2002; Dahl et al., 2004) for example Hereditary spherocytosis, which is one of the most common anaemic disorders affecting red blood cells in many humans (Satchwell et al., 2009). In addition the deficiency of band 4.2 protein in mouse influences physiological functions of the erythrocytes membrane (Peters et al., 1999) indicating the importance of this protein in maintaining the stability of the red blood cell membrane.

1.9 Transglutaminases 5, 6, and 7 (X, Y, and Z)

Within the transglutaminase family, three transglutaminases including transglutaminase X (TG5), transglutaminase Y (TG6) and transglutaminase Z (TG7) have now been discovered and therefore they are less characterised at the functional level. However, genomic analysis within all the family members revealed a high degree of sequence homology (Aeschlimann et al., 1998; Grenard et al., 2001a).

Native Transglutaminase X, as characterised for the first time by Aeschlimann et al., 1998 is composed of 720 amino acids with a molecular mass of approximately 82kDa. When compared to the other family members, novel transglutaminase showed an overall 35% sequence homology with the conserved domain structure, active site and calcium binding site (Aeschlimann et al., 1998). Further investigation revealed that similar to TG2, and TG3, TG5 is regulated by adenine-guanine nucleotides with a capability of hydrolysing GTP (Candi et al., 2004), however it still remains unknown how its GTP activity influences cell function (Eckert et al., 2005). Recent studies reported that TG5 undergoes proteolysis in mammalian epithelial cells which activates the enzyme itself (Pietroni et al., 2008).

The gene encoding TG5 is located on the 15q15.2 chromosome and consists of 13 exons separated by 12 introns (Grenard et al., 2001a). Originally TG5 was described in the spinous and granular layers of the human epidermis and further analysis revealed its presence in differentiating keratinocytes (Candi et al., 2001; Candi et al., 2002). As shown by in vitro experiments TG5 can cross-link small proline rich proteins, involucrin and loricrin indicating that similar to TG1 and TG3, TG5 can be involved in the formation of
the cornified cell envelope (Candi et al., 2001). The abolishment of the activity of TG5 in the skin caused by mutations in the human TG5 gene has been linked to acral peeling skin syndrome (Cassidy et al., 2005).

Mapping of the chromosomal segment encoding for TG2 and TG3, revealed the presence of another novel gene transglutaminase Y (TG6) located on the 20q11 region of chromosome 20 (Grenard et al., 2001a).

Additionally during extensive investigations another novel transglutaminase has been discovered in a human prostate carcinoma tissue. Transglutaminase Z, (TG7) has been reported to consist of 710 amino acids with a molecular weight of 80 kDa. When compared to the existing mammalian transglutaminases, TG7 has been shown to possess similarities and a high level of conservation for the TGase active and calcium binding sites as well as the core domain containing the catalytic amino acid triad. In addition sequencing analysis revealed genes encoding for TG7 and TG5 to be localised in tandem with the band 4.2 protein on chromosome 15 (Grenard et al., 2001a).

1.10 Tissue transglutaminase (TG2)

To date tissue transglutaminase (TG2) appears to be the most studied and the best characterised enzyme within the whole transglutaminase family. Under normal conditions, inside the cell the enzyme is mainly localised in the cytoplasm. Moreover its presence was also found in the nucleus, nuclear membrane, mitochondria, at the cell surface and in the extracellular matrix (Lesort et al, 1998; Upchurch et al, 1991; Griffin et al., 2002; Piacentini et al., 2005; Collighan and Griffin, 2009).

TG2 is a unique member of the family widely distributed in both the intracellular and extracellular cell environment in tissues and in brain; however its expression levels differ dependent on the cell and tissue type (Thomazy and Fesus, 1989). There are cells that show high expression of TG2 (endothelial, epithelial cells and macrophages), which sometimes remains at a constitutive level (endothelium) or is up- regulated by specific stimuli such as inflammatory cytokines (INF-\(\gamma\) in rat intestinal cells and IL-6 in human Hepatoblastoma cells) as well as steroids, vitamin D, and growth factors including TGF-\(\beta_1\) by the potential TGF-\(\beta_1\)/BMP4 response element in TG2 gene (Suto et al., 1993; Ritter and
Davies, 1998; Kim et al., 2002; reviewed by Zemskov et al., 2006). Additionally tissue specific TG2 expression can be influenced by retinoic acid. Analysis of the TG2 gene revealed the presence of a retinoid response element within the functional promoter of the enzyme (Moor et al., 1984; Gentile et al., 1991). This specific retinoid response element (mTGRRE1) when coupled with short DNA (HR-1) triggers promoter activation by retinoic acid (Nagy et al., 1996). Furthermore, differences in TG2 expression can be caused by variations in methylation of its gene promoter. As shown for Ras-transformed fibroblastic cells, hypermethylation of the promoter region can play an important role in the transcriptional regulation of TG2 gene (Lu and Davies, 1997).

1.10.1 Structure of TG2

Following cloning studies of the TG2 cDNA sequence from guinea pig liver (Ikura et al., 1988), mouse macrophages (Gentile et al., 1991), chicken erythrocytes (Weraarchakul-Boonmark et al., 1992), bovine aorta (Nakanishi et al., 1991), and human endothelial cells (Gentile et al., 1991) the structure of TG2 has been identified. The TG2 is a monomeric protein of 685-691 amino acids with a molecular mass of 76-85 kDa (Aeschlimann and Paulson, 1994) and is allosterically regulated by binding calcium or GTP (Liu et al., 2002; Fesus and Piacentini, 2002; Lorand and Graham, 2003). The gene encoding human TG2 has been mapped to the chromosome 20 and is composed of 13 exons separated by 12 introns (Fraj et al., 1992; Gentile et al., 1994). Exon 13 is made up around 50% of the enzyme cDNA and contains both the C-terminal coding region and the 3' end of the cDNA (Fraj et al., 1992). Analysis of the amino acid sequence of the guinea pig liver has shown a cysteine residue to be present at position 276 (Ikura et al., 1988) while 277 in human TG2 (Gentile et al., 1991). The calcium binding site thought to regulate TG2 transamidation activity has been localised in the core domain around 138 and 185 amino acids (Figure 1.3a) while recent studies has additionally demonstrated the presence of a few negatively charged amino acids localised in TG2 among the whole core domain which can influence TG2 activity by their ability to binding calcium (Király et al., 2009).

Human tissue transglutaminase has been crystallized in two different conformations (Liu et al., 2002; Pinkas et al., 2007). When in a GDP-bound form, TG2 exists in a closed conformation whereas interaction with a pentapeptide inhibitor stabilises its open, extended form. Although the two structures of the enzyme show different movement,
both of them were proposed to be a monomeric protein composed of four distinct domains (Liu et al., 2002; Pinkas et al., 2007). The TG2 structure has been derived from that of FXIII and is composed of an N-terminal β-sandwich (residues 1-138), a large core domain (residues 139-471) and two β-barrels on its C-terminal site (residues 472-687) (Chen and Mehta, 1999; Lorand and Graham, 2003). Also the crystal structure of the red sea bream liver transglutaminase (fish derived TGase) has been shown to be similar to the structure of human FXIIIa, additionally indicating overall similarities between TG2 and FXIII (Noguchi et al., 2001). Although this four-sequential domain organisation, and the relative position of catalytic triad (Cys277, His335, and Asp358) residues is highly conserved among TG isoforms, (Griffin et al., 2002; Lorand and Graham, 2003), the charge distribution varies between different isoenzymes (Chen and Mehta, 1999). This may influence substrate specificity and further functions of each enzyme. The TG2 core domain contains the calcium binding site and the active transamidation regulatory site, while the last two domains have an inhibitory effect on the transamidating activity by impeding the substrate access to the active site (Casadio et al., 1999). The cysteine (Cys277) residue is essential for TG2 transamidation activity (Folk and Cole, 1966; Lee et al., 1993). However studies performed by Ismaa and co-workers (1997) have demonstrated that in addition to the catalytic domain also the flanking domains (N-terminal β sandwich) can also influence the activity of the enzyme. Moreover another residue present in the core domain (tryptophan 241) was also implicated to be involved in TG cross-linking activity (Murthy et al., 2002) while recent studies has additionally demonstrated the presence of a few negatively charged amino acids localised in the TG2 core domain which can influence TG2 activity by their ability to binding calcium (Király et al., 2009). Although the main function of TG2 core domain is its transamidation activity, further analysis revealed its engagement in the hydrolysis of ATP and GTP (Ismaa et al., 1997). Ismaa and co-workers (2000) has localised the GTP binding site to a 15 amino acid segment of the core domain between 159 and 173 residues while further analysis revealed additional GTP binding sites present on a barrel 1 between 476-482 and 580-583 amino acids (Liu et al., 2002; Begg et al., 2006).
Figure 1.3  A Proposed functional and structural domains organisation of tissue transglutaminase (Lesort et al., 2000); B as well as its three-dimensional (closed) structure designed and kindly provided by Dr Russell Collighan from Aston University.
1.10.2 Regulation of activity, expression, and conformation of tissue transglutaminase

Tissue transglutaminase is a bifunctional enzyme that in addition to its calcium-dependent cross-linking activity is capable of catalysing the magnesium dependent reaction by binding and hydrolysis of ATP and GTP (Lee et al., 1989). An understanding of these unique features of tissue transglutaminase has been the main task undertaken by many researchers over numerous years. Early in vitro studies on guinea pig liver transglutaminase have demonstrated that guanine nucleotides, especially GTP, can have an inhibitory effect on the calcium dependent activity of the enzyme (Achyuthan and Greenenberg, 1987).

It is known that TG2 is located in many compartments of the human body, where it is engaged in physiological and pathological processes. Three-dimensional structure of TG2 is allosterically regulated by binding calcium or nucleotides (GTP, ATP) (Begg et al., 2006b). Being mainly a cytosolic protein, where the Ca\(^{2+}\) concentration is low, TG2 transamidating activity remains latent (Monsonego et al., 1998, Liu et al., 2002). The binding of GTP locks TG2 in a closed conformation (Figure 1.4b) thus reducing its affinity for calcium ions and blocking its cross-linking functions (Achyuthan and Greenenberg, 1987; Smethurst and Griffin, 1996; di Venere et al., 2000). In its latent conformation, a specific interaction between the catalytic core and barrel-1 and barrel-2 occurs which hinders accessibility of the TG2 active site to the substrate. The inhibitory effect of GTP is mediated by its binding to amino acids present on the barrel-1 domain (476-482 and 580-583) and two residues present on the TG2 core domain (Ser171 and Lys173) and which stand out on a loop close to barrel 1 leading to its stabilisation and further masking TG2 active site (Liu et al., 2002; Ismaa et al., 2000; Begg et al., 2006a). In addition, Begg and co-workers (2006b) have postulated that GTP when bound to the enzyme, destabilises Arg-579 and facilities the formation of the H bound between Tyr-516 located on the barrel-1 and Cys-277 present on the core domain thus favoring compact, inactive conformation of the enzyme. However the inhibition of cross-linking activity of the enzyme can be partially reversed by addition of calcium ions (Smethurst and Griffin, 1996).
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Figure 1.4 Structural conformation of tissue transglutaminase designed by Dr Russell Collighan from Aston University. The crystal structures of a calcium or an inhibitor-bound, and b GTP/GDP-bound TG2. The structures are shown as ribbons and present the N-terminal β-sandwich domain (in green), the catalytic core (in light blue), the C-terminal β-barrel1 and β-barrel2 (in red and blue) respectively (Pinkas et al., 2007).

When calcium is bound to the enzyme it alters its conformation by displacing the β-barrel TG2 domains further apart from the catalytic core thus opening an access to the active transamidation site (Liu et al., 2002) (Figure 1.4 a).

As described earlier, ATP has also been shown to bind to TG2 and undergo hydrolysis in a magnesium dependent manner (Lee et al., 1993). Studies carried out by Lai and co-workers (1998) demonstrated that both Mg$^{2+}$-GTP and Mg$^{2+}$-ATP complexes are real substrates for TG2 hydrolysis and key regulators of the different enzymatic activities of TG2.
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The binding of Mg\(^{2+}\)-GTP by TG2 inhibits its protein cross-linking activity without affecting ATPase activity (Lai et al., 1998). In contrary, Mg\(^{2+}\)-ATP induces conformational changes by preventing its ability to bind GTP, in addition having no influence on its protein transamidation activity (Lai et al., 1998). Inhibition of the GTPase activity by ATP, if GTP binding is not affected, could increase the TG2 Gh activity (Johnson and Terkeltaub 2005).

Under normal physiological conditions the intracellular level of calcium ions is insufficient to support transamidation by TG2. A membrane lipid sphingosylphosphocholine (Lyso-Sm) has been suggested to modulate TG2 TGase activity by enhancing it at low calcium concentration (10μM Ca\(^{2+}\)) (Lai et al., 1997). During interaction with TG2, Lyso-Sm induces changes in protein conformation that influence its affinity for calcium whilst not affecting substrate binding (Lai et al., 1997). Another molecule suggested to modify TG2 activity is nitric oxide, an inorganic free-radical molecule which targets TG2 via S-nitrosylation of protein thiol groups (TG2 has 18 free cysteine residues including the active site Cys 277) leading to reversible inhibition of its transamidation activity (Bernassola et al., 1999; Lai et. al., 2001) and increased TG2 sensitivity to GTP. Recently, Telci et al. (2009) suggested that nitrosylation of TG2 influences cellular deposition of the enzyme into the ECM thus regulating the function of the enzyme in the extracellular matrix.

Additional studies on TG2 demonstrated its ability to act as a protein disulfide isomerase (PDI) by catalysing the formation of disulphide bonds (Hasegawa et al., 2003). This PDI activity of TG2 was shown to be independent from the presence of calcium ions and contrary to other TG2 activities remained unaffected by nucleotides including GTP (Hasegawa et al., 2003). Furthermore PDI activity was enhanced by the presence of glutathione disulfide, which suggests that TG2 may act as an isomerase in a cytosolic environment (Hasegawa et al., 2003).

In addition, former studies identified TG2 from a human breast cancer cells as a serine/threonine kinase involved in the phosphorylation of the insulin-like growth factor (IGF)-binding protein 3 (IGFBP-3) (Mishra and Murthy, 2004). Recently, the biochemical activities of TG2 was shown to be regulated by protein kinase A which phosphorylate TG2 thus enhancing its kinase activity as well as its transamidation activity (Mishra et al., 2007).
1.10.3 Subcellular localisation of tissue transglutaminase

Multiple factors can regulate the activity of tissue transglutaminase. TG2 is mainly a cytoplasmic protein but interaction with a variety of stimuli can lead TG2 to the nucleus, or to the extracellular matrix and cell surface (Lorand and Graham, 2003; Verderio and Griffin, 2005). A very little amount of enzyme can be found outside the cell however its presence can be detected in the cell membrane fractions (Barnes et al., 1985). Although TG2 does not contain a hydrophobic amino acid leader sequence it has been shown to dynamically translocate through the plasma membrane and into various subcellular compartments (Korner et al., 1989; Peng et al., 1999). Several factors have been suggested to be directly or indirectly involved in TG2 translocation (Gaudry et al., 1999a; Balklava et al., 2002; Akimov et al., 2000; Scarpellini et al., 2009), however the exact mechanism by which the enzyme is actually transported still remains unknown. It is known that when released from cells TG2 accumulates in the extracellular matrix where it interacts with fibronectin, having an influence on its stabilisation, as well as being involved in cell-matrix adhesion and many other processes, including cell migration and signal transduction (Gaudry et al., 1999; Balklava et al., 2002; Akimov et al., 2000; Akimov and Belkin, 2001; Belkin et al., 2001; Kabir-Salmani et al., 2005; Zemskov et al., 2006). The externalisation mechanism and physiological functions of TG2 in the ECM will be described in more detail further on in the thesis. Nevertheless, available evidence indicates the importance of the subcellular location of the enzyme for the regulation of its biochemical activities (Park et al., 2010).

1.10.4 Tissue transglutaminase in the cytoplasm

In the cytoplasmic environment TG2 activity is mainly regulated by low concentrations of calcium ions and high nucleotide concentrations, as described above. A study performed on a human endothelial cells revealed the activity of cytosolic TG2 remains latent under physiological concentration of ATP and GTP, and in the presence of 10µM calcium (Smethurst and Griffin, 1996). Depletion of GTP in situ (Zhang et al., 1998) and many other factors including UV irradiation, and oxidative stress (Shin et al., 2004; Yi et al., 2004; lentile et al., 2007) enhances TG2 activity. In particular, the free radical species
(ROS) are shown to affect enzyme activity. When stress conditions are increased during exposure of the cell to UV light or heat the level of ROS increases. A study using Swiss 3T3 fibroblasts and NIH3T3 cells indicated ROS involvement in stimulating TG2 activity in response to transforming growth factor-β (TGF-β) and arachidonic acids (Lee et al., 2003; Yi et al., 2004).

1.10.5 Effects of tissue transglutaminase on the cell death

Multiple factors can regulate the activity of tissue transglutaminase. Simultaneously the cross-linking activity of TG2 has been implicated in regulating numerous physiological processes including cell growth and differentiation, endocytosis and cell survival. However the most frequent role of cytosolic TG2 related to aberrant transamidation activity of enzyme has been associated with apoptosis, indicating that the extent activity of the enzyme may play crucial role in the dying cell.

So far two major mechanisms of cell death have been described to take place under normal physiological conditions. Apoptosis is one of the two mechanisms which occurs via programmed cell death. It was characterised by changes in the nucleus and cytoplasm including chromatin cleavage and formation of vesicles which further undergo phagocytosis by neighbouring cells and macrophages. Apoptosis has been associated with embryonic morphogenesis, tissue remodeling and tumour growth (Green and Reed, 1998). Initially, the involvement of TGase in programmed cell death was proposed by Fesus et al. (1987). Since then many studies have been undertaken on in vivo and in vitro models regarding TG2 engagement in apoptosis and revealed that when activated TG2 catalyses the formation of insoluble apoptotic bodies (Fesus et al., 1989; Aeschlimann and Paulsson, 1994; Knight et al., 1993). The activation of TG2 is thought to stabilise the apoptotic cells before their clearance by phagocytotic cells thus contributing to the prevention of inflammatory response. Studies in vivo using thymus and liver as model systems revealed that induction of apoptosis in TG2 knock-out mice is possible; nevertheless the clearance of apoptotic cells is delayed suggesting the importance of TG2 for efficient phagocytosis (Szondy et al., 2003). The disturbance of apoptosis resulted from a deficiency in TGF-β activation and was associated with the inflammatory response (Szondy et al., 2003). Further investigation on the inflammatory response in TG2 knockout
mice revealed that TG2 facilitates phagocytosis not only in apoptotic cells but also in macrophages (Falasca et al., 2005). The lack of TG2 expression resulted in an impaired capacity of macrophages for engulfing but not for binding apoptotic cells (Falasca et al., 2005). Thus all the evidence suggests involvement of TG2 in the clearance of apoptotic cells.

Instead of playing an important role during apoptotic cell death TG2 can also affect the decision of the cell whether to undergo death or survive. As shown by Antonyak et al. (2002 and 2003) increased TG2 expression in NIH3T3 cells prevents apoptosis via a GTP-binding mechanism associated with the cell survival. However when TG2 loses its GTP-binding activity, the TG2 protective role can be converted into a cell death stimulating factor (Datta et al, 2007). Additional studies on TG2 in cancerous cell lines has shown that its anti-apoptotic activity can result from activation of the NF-κB signaling pathway (Mann et al., 2006, Cao et al., 2008). In contrast a pro-apoptotic stimulus has been suggested for TG2 in NIH3T3 cells treated with calphositin C. TG2 induces apoptosis by promoting oligomerization of a dual leucine zipper-bearing kinase (DLK) (Robitaille et al., 2008). Moreover TG2 was shown to stimulate apoptotic cell death in a pancreatic cancer cell line Panc-28 cells (Fok and Mehta, 2007). Thus results indicate that these diverse downstream effects of the enzyme in dying cells depend on multiple parameters including cell type as well as apoptotic stimuli (Park et al., 2010).

Necrosis is another mechanism of cell death that differs from apoptosis and occurs as a result of acute physical and chemical cell damage in an uncontrolled destructive manner. Necrotic cells are characterised by disruption of the membrane integrity, cell swelling, and active release of the intracellular macromolecules into the extracellular space leading to an inflammatory response. Necrosis can be closely associated with the elevating concentration of calcium ions which may lead to activation of calcium dependent enzymes (Fukuda et al., 1993). As reported by Zatloukal et al., (1992) enhanced activity of TG2 can lead to cell liver necrosis. Additional studies on mechanically injured fibroblasts, demonstrated that when TG2 is activated, it can cross-link proteins which results in the formation of protective SDS-insoluble shells thus preventing the leakage of harmful contents from death cells (Piredda et al., 1997; Nicholas et al., 2003). Nemes and co-workers, (1997) showed that when the calcium concentration increases in response to calcium ionophore A-23187, the activity of the enzyme increases leading to cross-linking
of cytoskeletal proteins. Such cross-linked protein-rich cells are believed to facilitate a cell death and exclusion of death cells thus contribute to reduced inflammation and apparent tissue damage (Fesus and Szondy, 2005). However the lack of TG2, as demonstrated in MRL1pr/lpr mice was suggested to contribute to the development of autoimmune disorders (Piredda et al., 1997). Using a lung cancer–derived cell line, A549, Kawai and co-workers (2008) showed that the transamidation activity of the enzyme is necessary for recovery of the membrane after mechanical wounding. Thus results may suggest that TG2 activity can stabilise tissue post-trauma and further contribute to the maintenance of membrane integrity.

1.10.6 Tissue transglutaminase on the plasma membrane

Tissue transglutaminase when associated with the membrane acts as a G protein, being involved in the signal transmission from the outer to the inner cellular space. As shown by Nakaoka and co-workers (1994) TG2 from the rat liver membrane was engaged in signal transduction through the α₁-adrenergic receptor whereas Nanda and co-workers (2001) reported the lack of signaling in TG2 knock-out mice. Absence of Gₗₗ coupling to α₁-adrenergic receptor, prevented α₁-adrenergic receptor agonist from stimulating [α-32P]GTP-photolabeling of a 74-KDa protein in liver membrane (Nanda et al., 2001). Agonist activation of TPα thromboxan A receptor stimulated TG2 as a G protein which was further demonstrated to trigger phospholipase C (PLC)–mediated inositol phosphate (IP) production (Vezza et al., 1999). However the interaction between Gh and PLC, seemed to have unclarified influence on the latter. On one hand Feng and co–workers (1996) suggested an inducible effect of Gh on PLC δ1, while Murthy et al. (1999) proposed TG2 to be a suppressor of PLC δ1 activity. In addition when bound to GTP, cytosolic Gh/transglutaminase was shown to release PLC δ1 from the inhibitory complex (Murthy et al., 1999) which on the other hand was demonstrated to positively regulate the GTP:TG2 interaction (Baek et al., 2001).

1.10.7 Tissue transglutaminase in the nucleus

Another cell compartment in which detectable levels of TG2 was found is the nucleus. Studies on a human neuroblastoma SH-SY5Y cells revealed that 7% of the total TG2 was
present in the nucleus, from which 6% was associated with chromatin-associated proteins and the rest which is 1% was found in the nuclear matrix fraction (Lesort et al., 1998). Lesort and co-workers demonstrated that the nuclear activity of TG2 remains at a very low level however it can be elevated by maitotoxin mediated increase of intracellular level of calcium. It is yet unknown what is the translocation mechanism of TG2 to the nucleus. Passive transport of TG2 through the nuclear membrane is probably excluded due to the high molecular weight of the enzyme. Thus active transport of TG2 through the membrane was postulated (Peng et al., 1999). So far histones and retinoblastoma protein (Rb) have been identified as TG2 nuclear substrates (Ballestar et al., 1996; Oliviero, et al., 1997). Additionally importin α-3 has been proposed to be a specific binding partner for TG2 that triggers its translocation to the nucleus (Peng et al., 1999). However importin α-3 remains associated with TG2 only in the cytosol and dissociates from TG2 after crossing the nuclear membrane (Peng et al., 1999). Further studies showed glutamate stimulated translocation of the enzyme to the nucleus in differentiating astrocytes (Campisi et al., 2003). In addition, treatment of pancreatic cancer cells Panc28 with calcium ionophore A-23187 enhanced translocation of the TG2 in a complex with p56 in to the nucleus (Mann et al., 2006).

1.10.8 Effect of tissue transglutaminase on cell growth and differentiation

Tissue transglutaminase multiple functions have been implicated in many processes. Early studies indicated an involvement of TG2 cross-linking activity on cell differentiation and proliferation in various cell types (Birckbichler and Patterson, 1978). In fact authors demonstrated that high levels of enzyme activity inside the cell induced cell differentiation in comparison to cells with low levels of TGase activity. Further investigation using human WI-38 lung fibroblasts showed that cell treatment with the TG2 inhibitor, cystamine stimulated their growth (Brickbichler et al., 1981). However, contradictory results were presented by Johnson and co-workers (1994) who reported that in comparable cells growth was due to over expression of TG2 indicating that the growth inhibition of TG2 might have resulted from non-specific effects of the inhibitor. Other discoveries revealed that TG2 can induce growth by indirect interaction with growth factors including transforming growth inhibitor facto TGFβ and hepatocyte growth factor (Kojima et al., 1993; Katoh et al., 1996). Additionally increase in expression of TG2
has been noticed during maturation of human monocytes to macrophages (Seiving et al., 1991). Another study using neuroblastoma Neuro2a cells reported that increased expression of TG2 leads to NGF-induced neuronal differentiation of these cells (Condello et al., 2008). Moreover increases in TG2 expression was further correlated with chondrocyte differentiation although FXIIIA was also suggested to be involved since it is also a marker of chondrocyte differentiation in the growth plate (Aeschlimann et al., 1993).

Transamidation of proteins present in the extracellular matrix by TG2 was demonstrated to affect cell differentiation and function for example collagen cross-linked by the enzyme was shown to induce differentiation of osteoblasts (Chau et al., 2005). TG2 was suggested to be important in accelerating chondrocyte maturation to hyperthrophy in response to retinoic acid which is a well known inducer of TG2 expression (Johnson et. al., 2003). While other studies revealed that even nanomolar amounts of exogenous TG2 are sufficient to directly stimulate hyperthropic differentiation in chondrocytes (Johnson and Terkeltaub, 2005). Additionally when released from chondrocytes, TG2 was suggested to modulate differentiation of osteoblasts via extracellular TG2-induced PKA signaling (Nurminskaya and Kartinen, 2006). However, as stated by Johson et al., (2008) chondrocyte maturation was accelerated by TG2 and FXIIIA networking without requirement of TG-catalysed transamidation activity.

1.10.9 Protein secretion pathways and externalisation of TG2

Although TG2 has been mainly localised in an intracellular compartment playing intracellular functions, its presence has also been demonstrated on the cell surface as well as in the extracellular compartment (Gaudry et al., 1999a, b). Despite recent data indicating extracellular TG2, very little information has been provided regarding the mechanism of its secretion.

During evolution, many mechanisms of protein translocation across eukaryotic membranes (nucleus, endoplasmic reticulum, mitochondria, peroxisomes, lysosomes etc.) have been developed and later on widely described (Görlich and Kutay, 1999; Shatz, 1996; Agarraberes and Dice, 2001; Brocard et al., 2003). Among proteins, there are messengers, characterised by N-terminal signals, hydrophobic sequences and amino acid regions that allow them to interact with translocation complexes located in the
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intracellular space or on the plasma membrane (Zocchi and Rubartelli, 2001). These peptides lead secretory proteins through the classical, ER/Golgi dependent secretion pathway. Following synthesis in endoplasmic reticulum, proteins are transported further to the Golgi apparatus, from where they are carried by vesicles to their final destination (membrane localisation) (Mellman and Warren, 2000). Additionally, when translocation takes place across the intra and extracellular space of the ER, mitochondria and into lysosomes, targeted proteins needs to be in an unfolded conformation (Rapoport et al., 1996; Shatz, 1996; Salvador et al., 2000). This conformation is not required for the proteins that are imported into peroxisomes as well as between the nucleus and cytosol (Brocard et al., 2003; Görlich and Kutay, 1999; Backhaus et al., 2004). However, in eukaryotic cells there are proteins, which are not subjected to the classical secretion pathway, but are still externalised from the inner to outer membrane space. Those proteins were identified to lack the N-terminal signal peptide, not to be glycosylated (even if they bear glycosylation motifs) thus do not pass into the ER, and become insensitive to the typical inhibitors (brefeldin A, monesin) of proteins that undergo classical secretion (Rubartelli et al., 1992; Tanudji et al., 2003).

The secretion mechanism of proteins that lack signal peptides and are maintained in the cytosol before being released from cells, is known as unconventional/non-classical or leaderless protein secretion (Zocchi and Rubartelli, 2001). Proteins, that are translocated via this pathway include; pro-angiogenic growth factors such as FGF-1 and FGF-2 (Jackson et al., 1995; Engling et al., 2002; Shäfer et al., 2004; Backhouse et al., 2003), inflammatory mediators such as 1β-interleukin (Rubartelli et al., 1990; Andrei et al., 1999), lectins belonging to the β-galactoside family (Cooper and Barondes, 1990; Lindstedt et al., 1993; Cho and Cummings, 1995; Lutomski et al., 1997). In addition the trafficking routes of viral proteins such as HIV-1 tat (Chang et al., 1997), herpes virus VP22 (Elliot and O’Hare, 1997), foam virus beta protein (Lecellier et al., 2002), parasitic surface proteins (Denny et al., 2000) occur via unconventional secretion pathways as well.

Similarly, to other proteins that are secreted via non-classical pathways, TG2 and FXIII A lack typical ER targeting signal sequences but still require the active conformation (Ichinose et al., 1990; Balklava et al., 2002). Many researchers have been interested in revealing the TG2 secretion pathway. So far, it is known that TG2 is released from both healthy and damaged cells but its externalisation increases during cell stress (Upchurch et al., 1991; Haroon et al., 1999; Johnson et al., 1999; Gaudry et al., 1999a, b; Gross et. al.,
Following mutagenesis studies of TG2, researchers suggested that as well as an active conformation, the presence of an intact N-terminal potential FN binding site is necessary for its secretion (Gaudry et al., 1999a). Mutagenesis studies on TG2 also revealed that when TG2 was in an inactive form (C277S) it was neither deposited into the ECM or secreted into medium (Balklava et al., 2002). Similarly the presence of a non-proline cis bond (Y274A) has also been shown to be important suggesting the involvement of TG2 activity and tertiary conformation in its externalisation mechanism (Balklava et al., 2002; Johnson and Terkeltaub 2005). Additionally, it was postulated that TG2-integrin interactions might have an influence on its externalisation (Akimov et al., 2000). Recently Johnson et al (2008) has shown that interaction between FXIIIA and integrin α1β1 is required for TG2 secretion from chondrocytes by using α1β1 blocking antibody which inhibited TG2 externalisation to the cell surface induced by FXIIIA. In addition, studies on a lung cancer-derived cell line, A549 indicated TG2 to be released from damaged cells during a cell repair process and postulated its possible role in modulation of autoimmunity by preventing the release of the cell contents (Kawai et al., 2008). Once externalised, TG2 can be internalised by endocytosis which requires the presence of LRP1 (a member of the LDL, low density lipoprotein receptor superfamily) and further degraded in lysosomes (Zemskov et al., 2007).

1.11 Tissue transglutaminase in the extracellular environment

1.11.1 Localisation of TG2 in the extracellular space

The extracellular matrix is characterised by a high concentration of calcium ions and low concentration of TG2 inhibitory factors including GTP and zinc. Due to this fact, extracellular TG2 was believed to be in its active form (Park et al., 2010). However a statement presented by Go and Jones, (2008) that the oxidizing potential of the ECM is maintained at a stable level, may influence earlier presumptions. Recently, Pinkas et al., (2007) have demonstrated that most of the extracellular TG2 is locked in a closed conformation regardless of high concentrations of calcium ions. Additionally, studies using GTP-binding site mutant (K173L) in CH-8 cells showed a decrease in its secretion into the medium indicating that externalised TG2 is probably in an inactive, GTP-bound form (Johnson and Terkeltaub, 2005). In addition, interaction between TG2 and integrin
receptors present on the cell surface was demonstrated to be independent of its cross-linking activity (Akimov et al., 2001a, b; Stephens et al., 2004). Meanwhile as shown by many researchers under physiological conditions, the cross-linking activity of extracellular TG2 is implicated in stabilising and remodeling of the ECM. Increased TG2 transamidation activity in the matrix was observed by Skill and co-workers (2004) in an opossum kidney proximal tubular epithelial cell line as a response to elevated level of glucose. In addition TG2 when treated with the TG inhibitor KCC009 was shown to reduce remodeling of fibronectin into the extracellular matrix in both in vitro and in vivo studies on glioblastoma cells (Yuan et al., 2007). Thus it still remains unclear when secreted into the extracellular environment whether TG2 is or is not required to function as an adaptor protein and/or cross-linking enzyme.

Interactions of cells with the extracellular matrix is essential for many cellular processes including cell adhesion, migration, differentiation, growth, apoptosis and assembly of proteins into the ECM. TG2 has been implicated in stabilisation and reconstitution of the extracellular matrix. Its role in the stabilisation of the extracellular environment was first proposed by Battaglia and Shapiro in 1988. Since then TG2 has been shown to be involved in binding and assembly of various proteins into the ECM including fibronectin (Tamaki and Aoki, 1981; Mosher et al., 1991), fibrin and fibrinogen (Cottrell et al., 1979; Guadiz et al., 1997) vitronectin (Sane et al., 1991) osteonectin (Aeschlimann et al., 1995) osteopontin (Prince et al., 1991; Sorenson et al., 1994) osteocalcin (Kaartinen et al., 1997) collagens (Mosher, 1984; Kleman et al., 1995; Esterre et al., 1998) laminin-nidogen complex (Aeschlimann et al., 1991) and elafin (Nara et al., 1994). Moreover TG2 contributes to the organisation of the ECM by promoting tissue mineralization, stabilisation of dermo-epidermal junctions as well as modification and activation of different growth factors including transforming growth factor-β (TGF-β) in the ECM (Lorand and Graham, 2003).

The activity of externalised TG2 into the ECM was found to be involved in connective and bone tissue remodeling (Aeschlimann et al., 1996). During endochondral bone formation which involves chondrocytes differentiation, proliferation, hyperthrophy and cartilage calcification, secretion of TG2 takes place. Initially at the proliferation stage TG2 is present in the intracellular space from where during the chondrocyte differentiation process is externalised to the cell surface where it is implicated in cross-linking proteins prior to mineralization (Aeschlimann et al., 1993).
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Another function that tissue transglutaminase seems to have is to stabilise dermo-epidermal junctions (DEJ). This specialized stable structure organised between the epidermis and the dermis is made up of molecules involved in specific protein-protein interactions from the hemidesmosomes at the basal surface of keratinocytes, the anchoring filaments connecting the hemidesmosomes to the basement membrane and the anchoring fibril linking the epithelium and the dermis (Lorand and Graham, 2003). The stability of skin by maintaining the integrity of dermo-epidermal junction is mainly achieved by anchoring fibrils. The major component of anchoring fibrils is collagen VII (Burgeson, 1993). Expression studies of transglutaminase activity in skin regenerating from cultured epithelial autographs proposed collagen VII to be a potential substrate for TG2 cross-linking activity as well revealing the importance of TG2 in the stability of dermo-epidermal junctions (Raghunath et al., 1996). Transglutaminase derived cross-link activity was also demonstrated to take place along the basement membrane where it was shown to interact with different substrates including laminin-nidogen complexes and osteonectin as well as in the elastic fibrillar apparatus (Aeschlimann and Paulsson, 1991; Aeschlimann et al., 1995; Brown-Augsburger et al., 1994; Trask et al., 2001). Elastic fibers consist of two different compartments: amorphous-appearing elastin and fibres (microfibrils). Microfibrils are found in many tissues as well as at dermo-epidermal junctions. The main components of microfibrils have been identified as fibrillin-1, fibrillin-2 and micro-fibril-associated glycoprotein-1 (MAPG-1) (Brown-Augsburger et al., 1994). The fibrilins are characterised as the major components of microfibrils that possess epidermal growth factor-like repeats in their domain structure (Brown-Augsburger et al., 1994). Another protein known to be a microfibrilis component is MAPG-1, a small molecular weight protein associated with the extracellular matrix microfibrils. Studies performed by Qian and Glanville (1997) and Brown-Augsburger and co-workers (1994) revealed that fibrillin-1 and MAPG-1 are both substrates for tissue transglutaminase transamidation activity. Available data demonstrated that tissue transglutaminase activity may be implicated in stabilising the anchoring fibrils and microfibrils to form dermo-epidermal junctions.

In vitro experiments suggest the involvement of TG2 in the activation of the latent form of transforming growth factor-β TGF-β (Lorand and Graham, 2003). Most of the time, TGF-β when secreted from cells remains in a biologically inactive form of a large latent complex consisting of mature TGF-β, the TGF-β propeptide called latency
associated peptide (LAP) and a latent TGF-β binding protein (LTBP). The dissociation of TGF-β from LAP peptide is required for its ability to bind receptors and further involvement in a wide range of cellular processes including cell proliferation, cell differentiation, cell migration, and the extracellular matrix deposition (Massague, 1990; Lyons et al., 1988; Nunes et al., 1997). It is not necessary in the case of LTBP; however it is involved in targeting TGF-β into the ECM (Taipale et al., 1994). Members of LTBP family are characterised by possessing epidermal growth factor-like repeats and eight cysteine repeats in their domain structure similar to microfibrilar proteins which may suggest the involvement of TG2 in regulation of the intracellular TGF-β. In vitro studies revealed that LTB-1 and large latent complex are substrates for transglutaminase and the covalent incorporation of LTB-1 into the extracellular matrix is transglutaminase dependent (Nunes et al., 1997). The immunochemical studies further confirmed by electron microscopy revealed that increased TG2 expression is correlated with an increased rate of LTB-1 deposition into the ECM (Verderio et al., 1999). Additionally LTB-1 was co-localised together with TG2 and FN on the cell surface where TG2 activity is enhanced by the presence of calcium (Verderio et al., 1999). These results suggest that TG2 may contribute to the targeting of the latent TGF-β complex into the ECM by cross-linking the LTBP to matrix proteins. The engagement of TG2 in TGF-β₁ activation was further demonstrated using TG2 -/- mice indicating that the lack of TG2 expression correlates to deficiency in TGF-β₁ activation leading to impaired phagocytosis and prolonged inflammation (Szondy et al., 2003). These findings were further confirmed by Telci and co-workers (2009) who investigated the effect of nitrosylation on TG2 activity. TG2-induced fibroblasts treated with nitric oxide donor S-nitroso-N-acetylpenicillamine was shown to inhibit TG2 cross-linking activity leading to decrease in expression and activity of TGF-β₁ (Telci et al., 2009).

1.11.2 Fibronectin in the extracellular environment

In vitro studies demonstrated that fibronectin is a well known substrate for TG2 and that binding between both of these molecules occurs with high affinity (Kd around 8-10nM) and 2:1 stoichiometry (Lorand et al., 1988; LeMosy et al., 1992) independent of the GTPase and cross-linking activities of TG (Turner and Lorand, 1989). Fibronectin (FN) is a high molecular weight protein (around 440kDa) mainly present in blood, tissues as well as the ECM. Dependent on its localisation, FN can exist in two different forms: a
soluble protomeric form which can be found in blood plasma while an insoluble monomeric form is present in the extracellular environment of tissues (Magnusson and Mosher, 1998). Soluble FN is a dimeric glycoprotein composed of two similar chains linked by disulfide bonds. Each subunit of FN is made of a series of repeating amino acids sequences: Type I, II and III modules (figure 1.5). The structure of FN is composed of 12 type I modules, 2 type II, 15 to 17 type III (depending on splicing) and a variable (V) sequence not homologous to other FN parts (Wierzbicka-Patynowski and Schwarzbauer, 2003). These modules present on FN represent specific binding sites for various proteins as well as cell receptors. The 2 type III modules (undergo alternative splicing) are called ED-A (ED for extradomain) and ED-B. Plasma FN is characterised by lack of these ED domains; however cellular FN (synthesised locally in tissues) contains various amounts of either or both ED-A and ED-B (Magnusson and Mosher, 1998). Moreover, in plasma FN only one of the subunits contains the V region, while almost all cellular FN subunits contain this region (Schwarzbauer et al., 1989). The multiple domain structure of FN is important in maintaining the integrity of the matrix.

Most adherent cell types synthesize FN and deposit it into the extracellular matrix. Assembly of FN into the ECM is a tightly regulated process that occurs in a multistep manner starting from initiation through elongation to stabilisation (Schwarzbauer and Sechler, 1999).

Initially, soluble protomeric FN binds to specific sites present on the cell surface by means of its N-terminal 70 kDa region, in particular within modules I1-I5 (Sottile et al., 1991). In addition FN interaction with adhesive receptors present on the cell surface, mainly through binding various integrin receptors (as shown in Figure 1.5) leads to its activation (Wierzbicka-Patynowski and Schwarzbauer, 2003). Especially α5β1 integrin was shown to be the major FN receptor which similar to a few other integrin receptors requires the Arg-Gly-Asp (RGD) cell–binding sequence within the III9 module along with the PHSRN synergy sequence located in III10 FN module (Hynes, 1992; Schwarzbauer and Sechler, 1999; Humphries et al., 2004). Other adhesive molecules that FN interacts with are fibrin, transmembrane HSPGs, and collagen. Moreover FN possesses a reactive glutamine residue close to the amino-terminal site by which it interacts with activated plasma transglutaminase FXIIIa. FXIIIa covalently cross-links FN into the fibrin clots leading to increased elasticity and thickness in the blood clot (Muszbek, 1999). In the next step cell associated FN is distributed over the cell surface and it is converted into cell-stabilised
Figure 1.5: Domain structure of fibronectin (FN). Localization of the binding sites for various adhesion receptors on the FN molecule (Zemskov et al., 2006)
multimers forming a fibrillar network (Chen and Mosher, 1996; Magnusson and Mosher, 1998). In the final stage, while assembly progresses, FN is regenerated and gradually converted into a detergent-insoluble form as it becomes further integrated in the organisation of the extracellular matrix (Wierzbicka-Patynowski and Schwarzbauer, 2003).

1.11.3 TG2-Fibronectin interaction

Numerous recent studies point to the involvement of TG2 in interaction that take place between cells and the extracellular matrix (Akimov et al., 2000; Akimov et al., 2001; Lorand and Graham 2003). Although TG2 has no leader sequence and does not undergo any posttranslational modifications it can still be found on the cell surface as well as outside the cell where it interacts with many extracellular proteins (Upchurch et al, 1991; Aeschlimann and Paulsson, 1994; Verderio et al., 1999; Balklava et al., 2002; Zemskov et al., 2006).

A series of studies showed that TG2 can bind directly to the major extracellular protein FN with high affinity and this interaction does not require it’s cross-linking or GTPase activities (Lorand et al., 1988; LeMosey et al., 1992). The engagement of cell surface TG2 in cell-matrix adhesion, cell migration, FN assembly and signalling strictly depends on its interaction with fibronectin (Akimov et al., 2000; Belkin et al., 2001; Akimov and Belkin, 2001; Verderio et al., 2003). This interaction was shown to take place through the 42kDa region of fibronectin consisting of modules I6II1,2I7,9 which has a high affinity to TG2 comparable to the whole FN molecule (Radek et al., 1993). Additionally this interaction was reported to be located apart from the integrin binding motifs on FN suggesting a supportive role for TG2 and integrins in mediating cell adhesion (Akimov et al., 2000; reviewed by Zemskov et al., 2006).

Several contradicting reports appeared contributing to the localisation of the FN-binding site on the TG2 sequence. The TG2-fibronectin interaction was suggested to be dependent on the intact NH2-terminal region of TG2 (Jeong et al., 1995; Gaudry et al., 1999a). Further insight into the FN binding motifs present on guinea pig liver TG2 indicated that the FN binding site is placed within a 28 kDa proteolytic fragment of the TG2 structure (Jeong et al., 1995). More recent site-directed mutagenesis studies based on using TG2/FXIIIA chimeric constructs containing swapped regions between TG2 and FXIIIA and TG2 deletion products demonstrated the presence of additional FN recognition
motif on the TG2 molecule (Hang et al., 2005). Moreover, more detailed investigation on a matter, including using synthetic peptides which encompass the first domain of TG2, revealed that peptide $^{88}$WTATVVDDQCDCTLSSLQLTT$^{106}$ inhibited the TG2-FN interaction. The major FN binding site was mapped to the 88-106 amino acids of TG2 within the hairpin structure consisting of anti-parallel β strands 5 and 6 among which Asp$^{94}$ and Asp$^{97}$ were shown to be critical residues involved in TG2-FN interaction (Hang et al., 2005, Figure 7).

### 1.12 Other molecular interaction of cell surface TG2

In addition to the interaction with FN, recent studies revealed a close association of cell surface TG2 with several integrin receptors mainly via binding to the $\beta_1$ and $\beta_3$ subunits present in different cell types (Gaudry et al., 1999 a, b; Akimov et al., 2000; Belkin et al, 2001; Akimov and Belkin, 2001; Tanaka et al., 2007).

Integrins represents the major class of cell surface transmembrane adhesion receptors for ECM proteins as well as mediators in cell-matrix interaction. The interactions between cells and ECM proteins are important to stabilise tissue integrity as well as many other processes including cell proliferation, differentiation, migration, regulation of gene expression, formation of blood clotting, thrombosis and wound healing (Critchley et al., 1999). These processes rely on the linkage of intracellular cytoskeleton via integrin cytoplasmic tails which allows transport of signals across the plasma membrane (Calderwood et al., 2000; Evans and Calderwood, 2007). Integrins are heterodimers containing $\alpha$ and $\beta$ subunits; 8$\beta$ subunits may interact with 18$\alpha$ subunits leading to the formation of 24 distinct integrins, expressed by all cell types excluding erythrocytes (Hynes, 2002). The association of the subunits plays important roles in binding of the ligands (Gailit and Ruoslahti, 1988).

Integrins are not always active, very often they are expressed by cells in an inactive state in which they do not bind ligands and do not signal (Hynes, 2002). It has been shown that RGD peptides and small ligands can interact with not fully activated integrins while larger ligands including fibronectin, fibrinogen cannot (Coller 1986; Hynes, 2002). Activation is regulated by many inside-out signalling pathways inducing conformational changes within integrin subunits increasing their affinity for ligands (Sims et al., 1991; Calderwood 2004). It still remains unclear how this process proceed. However there is evidence suggesting that mainly talin (Ma et al., 2008) and in addition proteins belonging
to the kindlin family (Ma et al., 2008; Moser et al., 2008) are important for integrin activation. When bound to ligands, integrins form connections between the cytoskeleton and the ECM, cluster at focal contacts and then trigger signals through the outside-in signaling pathway (Harburger and Calderwood, 2009).

In addition to integrins, other molecules were proposed to associate with TG2 at the cell surface and further modulate the biological functions of TG2 (reviewed by Verderio et al., 2009). HSPGs (heparan sulfate proteoglycans) are specialized glycoproteins made of a core protein, heparan sulfate (HS) glycosaminoglycan (GAG) chains and uronic acids (Bishop et al., 2007). Within the family, three distinct classes have been characterised: the membrane-spanning proteoglycans (syndecans, betaglycan and CD44v3), the glycophosphatidyl-inositol (GPI)-linked proteoglycans (glypican) and a varied group of secreted proteoglycans found in the ECM e.g. argin, collagen XVIII and perlecain (Bishop et al., 2007; Kirkpatrick and Selleck, 2007). The HS chains when assembled on core proteins by enzymes of the Golgi, undergo a series of reactions which cause their structural heterogenicity including differences in chain length and size, the spacing of tracts, and the extent of sulphation and epimerization within negatively charged groups (Bishop et al., 2007). This structural variety in the HS chains allows HSPGs to bind a wide range of different proteins and affect many physiological processes (Kirkpatrick and Selleck, 2007).

Signorini and co-workers (1988) when purified human erythrocyte TG2 on a Heparin-Sepharose column, revealed that TG2 has a high affinity for heparin which is an analogue of heparan sulfate (HS). Additional studies on transglutaminase and heparin interaction demonstrated that binding to the heparin affects only slightly the catalytic activity of the enzyme but protects it from thermal unfolding as well as proteolytic degradation (Gambetti et al., 2005). Moreover by using surface plasmon resonance Scarpellini and co-workers (2007, 2009) showed high affinity of recombinant human TG2 for heparin. These findings were further validated by solid phase binding assays indicating that interaction between TG2, HS and heparin are of similar strength and additionally comparable to the strength of TG2-FN interactions (Scarpellini et al., 2009). In addition the ECM adhesive glycoproteins including vitronectin and fibronectin which were also shown to be TG2 enzymatic substrates can bind to heparin indicating that heparin may be involved in TG2 immobilization on these substrates (Sane et al., 1990; Verderio et al., 2009).
1.13 Cell surface and extracellular associated TG2 mediated cell adhesion

Interactions of cells with the surrounding extracellular environment are essential for maintaining physiological cellular processes. These interactions have been shown to take place through a major classes of adhesion receptors that are present on a variety of cell types including integrins, selectins, CAMs (cell adhesion molecules of immunoglobulin superfamily) and HSPGs (Zemskov et al., 2006). Membrane proteins (receptors) mediate cell-cell interactions as well as cell-matrix interactions with ECM glycoproteins such as fibronectin (FN), collagens, laminins as well as ECM proteoglycans (Zemskov et al., 2006).

Initial studies suggesting the involvement of TG2 in promoting cell adhesion was published in 1986 by Slife and coauthors as well as by Tyrell and coworkers, who proposed that plasma membrane associated TG2 may form covalently cross-linked proteins matrices which can increase the adhesiveness of the cells. Since then evidence of cell surface TG2 promoting cell adhesion started to accumulate rapidly. Studies on Balb-C 3T3 fibroblasts stably transfected with TG2 revealed alterations in cell morphology reflecting enhanced cell adhesion as well as their increased resistance to detachment with trypsin (Gentile et al., 1992). The cross-linking of fibronectin by cell associated TG2 was shown to firmly anchor the endothelial cells into the basement membrane (Martinez et al., 1994).

Additional investigations undertaken by Jones and coauthors (1997) on a human umbilical epithelial cell line ECV 304 demonstrated that reduced expression of TG2 by antisense technology resulted in diminished adhesion and spreading of endothelial cells and their sensitivity to detachment by proteolytic enzymes.

So far TG2 transamidating activity has been implicated in the cell adhesion processes, however recent findings suggesting that TG2 can promote cell adhesion independent of its crosslinking activity (Gaudry et al., 1999b; Akimov et al., 2000; Takahashi et al., 2000), but is dependent on TG2 interactions with integrin receptors present on the cell surface. Following immunochemistry and electron microscopy studies using cells undergoing attachment and spreading, TG2 was found to localise in focal adhesion sites rich in β1 and β3 integrins where fibronectin fibril assembly progresses (Gaudry et al., 1999b; Akimov and Belkin 2001a,b). TG2 interaction with fibronectin is very important for its adhesion functions. Even more important is that the gelatin binding domain of FN does not
function as both an integrin and other receptors binding site (Zemskov et al., 2006). Thus, TG2 and integrins can play collaborative roles in the process of cell adhesion rather than compete between each other (Zemskov et al., 2006). Using different antibodies directed against TG2, different investigators managed to abolish the interaction between the enzyme and gelatin fragment of FN suggesting cell surface TG2 to be the principal adhesive receptor for this part of FN (Akimov et al., 2000; Belkin et al., 2001; Zemskov et al., 2006). Moreover this interaction was demonstrated to be involved in the formation of focal adhesions at the cell surface and further influencing intracellular signaling pathways by the activation of focal adhesion kinase FAK (Akimov et al., 2000). In addition FN bound extracellular TG2 was shown to be able to bind heparan sulfate chains present on the cell surface independently of the RGD-mediated fibronectin binding (Verderio et al., 2003). Therefore rescuing the inhibitory effect of cell adhesion induced by inhibitory RGD peptides on fibronectin alone (Verderio et al., 2003). This mechanism was shown to require activation of protein kinase (PKCα), further its interaction with integrin β1 leading to enhancement of actin stress fiber organization and FAK, ERK1/2 MAP kinases activation (Telci et al., 2008).

TG2 released during cell damage or tissue injury was proposed to enhance or substitute for integrin mediated cell adhesion and intracellular signaling thus promoting cell survival (Telci et al., 2008; Collighan and Griffin, 2009).

1.14 Tissue transglutaminase - a wound healing enzyme

Injury that can cause internal and external tissue damage activates a complex, repair process, composed of a series of successive phases (inflammation, proliferation, formation and remodeling of extracellular matrix), which leads to reconstitution of wounded areas (Upchurch et al., 1991; Werner and Grose, 2003; Telci and Griffin, 2006). Wound healing is a carefully regulated process, based on complex interactions between the cells and mediators that are released from them during each of the healing phases. Those regulatory factors include various growth factors (PDGF, TGF-β, and VEGF), cytokines, chemokines and proteins delivered from disrupted blood vessels and degranulated platelets (Werner and Grose, 2003; Anitua et al., 2004, Telci and Griffin, 2006). Homeostasis, initiated at the beginning of injury, involves platelet aggregation and formation of a fibrin clot (Anitua et al., 2004). Further immune responses promote
capillary vasodilatation and phagocytosis that is carried out by neutrophils and macrophages (Gillitzer and Goebeler, 2001; Park and Barbul, 2004). Following the inflammatory phase, proliferation begins. At this stage, keratinocytes take part in recreation of the normal pattern of epithelial barrier and new capillaries are formed (angiogenesis) which helps to maintain the nutrition of the wounded area. Stimulated by regulatory factors, fibroblasts differentiate and migrate to the wound where they are engaged in granulation tissue formation and collagen production that is later on deposited into the wound area. Additionally, several matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) have been associated with matrix remodeling by creating a path for migrating cells into the wound area (McCawley and Matrisian, 2001). After pulling together the edges of the wound, the maturation phase takes place. Finally, granulation tissue is transformed into a mature scar whose architecture is re-modeled as random collagen fibrils are cross-linked. The remodeling continues for a prolonged period until deposition of collagen has increased the tensile strength of the scar (Werner and Grose, 2003; Verderio et al., 2004; Midwood et al., 2004; Verderio et al., 2005; Telci and Griffin, 2006).}

So far numerous studies reported the involvement of transglutaminases (with respect to their ability to mediate cross-linking reactions) in inflammation, general maintenance of tissue integrity and the wound healing process. Upon injury the formation of the blood clot takes place to fill in the wounded area. So far activated by thrombin the FXIIIa subunit was shown to catalyse fibrin transamidation which enhances the thickness of the clot (Muszbek et al., 1999). Nevertheless the evidence also suggests that abundant erythrocyte TG2 may also be involved in the blood clot formation and further in its stabilisation (Auld et al., 2001). During wounding cells present in disrupted area synthesise and release components which help to regulate the healing of injured tissue. One of these molecules are ECM proteins including FN to which TG2 has been shown to have a high affinity (Gaudry et al., 1999a). As demonstrated by Upchurch and co-workers (1987 and 1991) after mechanical or chemical injury TG2 becomes released from fibroblasts into the ECM around the wound where it associates with FN. The FN-TG2 interaction has been shown to down-regulate TG2 transamidation activity (LeMosey et al., 1992) whereas the cross-linking function of the enzyme has been implicated in the stabilisation and/or remodeling of the ECM and further tissue repair (Figure 1.6) (Aeschlimann and Thomazy 2000). However as shown by numerous studies redistribution
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of the enzyme in the ECM can take place as a result of its interaction with stress factors released during deterioration of organs such as liver, kidney or lung (Griffin et al., 1979, Johnson et al., 1999; Grenard et al., 2001b). The importance of TG2 activity in the different stages of healing process has been confirmed by the observation of impaired wound healing in TG2-/- mice (Mears et al., 2002). Initial studies undertaken by Griffin et al., (1979) suggest enhanced TG2 activity in herbicide-damaged lung in an experimental model of pulmonary fibrosis. Further examination on wounded rat dorsal skin reported increased activity of the enzyme in all skin layers during the healing process (Bowness et al., 1988).

In addition, monitoring of the healing process on anesthetized rats using histological and immunohistoligical methods demonstrated elevated activity of TG2 in endothelial cells, macrophages and skeletal muscle cells at sites of neovascularization in the provisional fibrin matrix within all stages of the wounding process (Haroon et al., 1999). Moreover the expression of the enzyme has been shown to be regulated by cytokines including TGF-β1, IL-6, and TNF-α present at the inflammatory stage of the healing process which may promote adhesion of white blood cells during inflammation (Haroon et al., 1999; Suto et al., 1993; Kim et al., 2002). Some investigators have connected TG2 with the inflammatory phase by its ability to enhance the activity of secretory phospholipase A₂ enzyme (sPLA₂) (Cordella-Miele et al., 1990). As reported by different studies TG2 can modify of sPLA₂ activity which when stimulated could increase release of arachidonic acid from the membrane leading to reduced biosynthesis of eicosanoids by cyclooxygenase (Verderio et al., 2004; Telci and Griffin, 2006). To reverse that effect, recombinant peptides capable of inhibiting TG2-catalysed post-translational activation of sPLA₂ were generated (Sohn et al., 2003). Moreover the increase in the TG2 expression has been linked with macrophages and promoting their role in phagocytosis (Szondy et al., 2003; Telci et al., 2006). It was demonstrated following examination of TG2-deficient moice which revealed a defect in the clearance of apoptotic cells by macrophages (Szondy et al., 2003). Further investigation on inflammatory response in TG2 knockout mice confirmed the correlation between TG2 levels and macrophage mediated phagocytosis (Falasca et al., 2005). Additional evidence of tissue transglutaminase engagement in the wound healing process came from the analysis of biopsies from human patient’s burn skin autografts. In skin regenerating from keratinocyte autografts TG2-derived cross-links
Figure 1.6 Schematic modified from Verderio et al., 2005, showing wound healing phases with specification of actions that are undertaken by transglutaminases (TG1, TG2 and FXIIIa) during the healing process. A few hours after injury (12-24h), platelets aggregate to form a fibrin clot. Afterwards, inflammation begins (2-5 days), neutrophils and macrophages are directed into the wounded area where they release different factors that trigger matrix synthesis and, later on the fibroblast influx and replacement of the provisional matrix with granulation tissue (5 days-3 weeks). At the end of the wound process, which can last from the 3rd week as long as a few years, fibroblasts are transformed into myofibroblasts, and granulation tissue is replaced with a scar rich in collagen bundles.
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were confirmed at the dermo-epidermal junctions, papillary dermis and surrounding capillaries (Raughunath et al., 1996). In a model of cell wounding, TG2 cross-linking activity was also shown to control the release of the intracellular contents of dying cells into the wound thus contributing to the maintenance of tissue integrity and reduced inflammatory response which results from the loss of cellular Ca\textsuperscript{2+} homeostasis (Nicholas et al., 2003).

The involvement of TG2 in inflammatory phase of wound healing was also shown by various investigators in inflammatory diseases which correlated with abnormal activities of TG2 (Esposito and Caputo, 2005).

1.15 Tissue transglutamianse and matrix associated pathologies

1.15.1 Abnormal wound healing - fibrosis and scarring

Successful wound healing results in reconstitution of the damaged area; however, some internal tissues and organs can encounter wound aberrations consequently leading to a variety of disorders. The difficulty occurs when injury becomes chronic resulting in over expression of the wound healing response that finally leads to scarring and fibrosis as well as cell deletion and damage of the tissue (Kim et al., 2002, Verderio et al., 2005). Numerous studies demonstrated the involvement of TG2 in maintaining the repair mechanism; however it has also been reported that TG2 may play pathological role when cell trauma or tissue insult occurs leading to increased inflammation, tissue fibrosis and scarring (Verderio et al., 2004). The first evidence of TG2 involvement in fibrotic disease came from Griffin et al., (1979) who observed alterations in TG2 activity in an experimental model of pulmonary fibrosis induced by paraquat (Griffin et al., 1979). Further investigations using animal models as well as human kidney biopsy material have shown correlation between elevated levels of TG2 expression and progressive renal scarring (Johnson et al., 1997; Johnson et al., 2003). Subsequently it was confirmed by Skill et al. (2004) who investigated the influence of increased levels of glucose on the tubular epithelial cells and its consequences on TG2 secretion into the ECM (Skill et al., 2004). As further documented increased TG2 expression was shown to contribute to progression of interstitial fibrosis and scarring in diabetic nephropathy (Skill et al., 2004).
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Additionally extracellular TG2 has also been reported to be involved in the severe chronic inflammatory states such as liver conditions (cirrhosis and fibrosis, alcoholic hepatopathy and hepatitis C) (Mirza et al., 1997; Grenard et al., 2001) and in renal and lung fibrosis (Griffin et al., 1979; Richards et al., 1991) as well as in heart where cardiac overexpression of TG2 leads to interstitial fibrosis in transgenic mice (Small et al., 1999). The major function of TG2 in many of these conditions has been linked to its ability to activate pro-inflammatory cytokines such as TGF-β1 (which is a multi-step process ending with release of the active TGF-β1 subunit), leading to increased deposition of ECM (Verderio et al., 1999; Griffin et al., 2002). The lack of TG2 expression in TG-deficient mice showed reduced activation of TGF-β1 resulting in decreased interstitial inflammation and protection against the development of renal fibrosis (Schweke et al., 2008).

Impairment of homeostasis in ECM turnover is likely to be involved in the development of the atherosclerotic plaque (Ientile et al., 2007). One of the factors that has been found to stabilise the atherosclerotic plaque by preventing it from rupture is TG2 (Haroon et al., 2001). Recent studies on the glaucomatous human trabecular meshwork suggested that elevated levels of TG2 and its tight TG2-FN interaction in the ECM may be associated with the pathologies of glaucoma (Tovar-Vidales et al., 2008).

So far increased expression of TG2 has been highlighted by many researchers as one of the agents causing fibrosis and scarring. Therefore investigations have been undertaken to generate TG2 inhibitors. Two inhibitors including the membrane soluble irreversible inhibitor R283 or (1,3-dimethyl2[(oxopropyl)thio]-imidazolium ) (Freund et al., 1994) and a membrane-impermeable irreversible inhibitor R281, or (N-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulphonium-5-oxo-L-norleucin) (Griffin et al., 2008) have been shown to target extracellular TG2 transamidating activity and reduce interstitial scarring in a rat subtotal nephrectomy model of chronic renal fibrosis (Johnson et al., 2007; Collighan and Griffin, 2009). Thus the use of inhibitors looks like a very promising tool for the treatment of fibrotic conditions correlated with overexpression of TG2.
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1.15.2 Cancer

Numerous reports suggest the involvement of TG2 in the development of different cancer types. Alterations in the transamidation activity as well as GTP-binding properties of the enzyme could also affect tumour progression as well as the malignant phenotype. Early studies on the relationship between transglutaminase activity in malignant hepatoma, transformed human and hamster cells as well as mouse cells revealed a reduction in TG2 cross-linking activity in neoplastic cells in comparison to their normal counterparts (Birckbichler and Patterson, 1980). As further demonstrated by immunofluorescence the reduction of TG activity was correlated with the reduced amounts of TG2 antigen present in the particulate-fractions of transformed cells. Subsequently, induction of hepatocellular carcinomas in rats treated with diethylnitrosamine or 6-p-dimethylaminophenylazobenzothiazole lead to the reduction of enzyme activity and its redistribution to the particulate fraction of the cell (Barnes et al., 1985). Following the subject, many other researchers observed the inverse correlation of TG2 activity and the susceptibility of a tumour to metastasis as well as redistribution of the enzyme to the particulate fractions (Hand et al., 1990; Knight et al., 1991; Beninati et al., 1993). In order to differentiate TG2 transamidation activity from its GTP-binding function, Mian et al. (1995) used transfected malignant hamster fibrosarcoma (MetB) cells with the wild type TG2 and (C277S) inactive mutant form. When synchronized into the S-phase of the cell cycle, both cells with overexpressed TG2 and inactive mutant in respect to the controls showed delayed progression from S-phase to G2/M phase (Mian et al., 1995) which remains in agreement with previous finding that reduced activity of the enzyme may favour tumour cell growth and invasion (Hand et al., 1990; Knight et al., 1991).

As a consequence of these findings, Jones et al., (2006) performed in vitro angiogenesis assays and further in vivo studies on two tumour models using exogenously applied TG2. Angiogenesis assays showed that application of active TG2 resulted in suppression of angiogenesis without causing cell death. Moreover in vivo experiments revealed that when injected into the CT26 colon carcinoma tumourus in mice, TG2 caused delays in tumour growth by inducing changes in the stability of the ECM while similar treatment on TG2 deficient mice resulted in increased tumour growth leading to increased death (Jones et al., 2006). Additionally recent investigations on tumour
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suppressing proteins indicated TG2 to be a binding partner for GRP56 (down-regulated protein in metastatic malignant melanoma cells) and through this interaction to play a role in suppression of metastases and tumour growth (Xu et al., 2006).

Multiple reports however documented overexpression of TG2 in several cancerous tissues including pancreatic ductal adenocarcinoma (PDAC) (Verma et al., 2006); breast carcinoma (Mehta et al., 2004; Mangala et al., 2007), malignant melanoma (Fok et al., 2006) ovarian carcinoma (Satpathy et al., 2007; Hwang et al., 2009) and glioblastoma (Yuan et al., 2006). High expression of TG2 was shown to contribute to the development of chemoresistance as well as survival and metastasis of cancerous cells (Mehta et al., 2004; Verma et al., 2006; Fok et al., 2006; Mangala et al., 2007; Hwang et al., 2008). For example in breast cancer cells TG2 was demonstrated to promote cell survival by activating integrin-mediated signaling pathways. Additionally in these cells, downregulation of endogenous TG2 by siRNA inhibited fibronectin mediated cell attachment and cell survival, while overexpression of the enzyme enhanced invasion of breast cancer cells and attachment to fibronectin (Herman et al., 2006; Mangala et al., 2007).

Moreover there are additional reports suggesting that TG2 when up-regulated in certain types of cancerous cells may play an anti-apoptotic function whereas siRNA down-regulation of TG2 by treatment of these cells with inhibitors sensitizes them to apoptosis (Mehta, 1994; Antonyak et al., 2001; Herman et al., 2006; Kim et al., 2006; Mehta et al., 2009).

1.16 Tissue transglutaminase association with diseases

Tissue transglutamianse is known to play pleiotropic functions in multiple cellular processes which help to maintain the homeostasis. However alterations in TG2 expression have been suggested to contribute to the development of several pathologies including autoimmune celiac disease and variety of neurodegenerative diseases (Huntington, Alzheimer and Parkinson disease).
1.16.1 Tissue transglutaminase in celiac disease

Numerous studies have been performed within the last few years on the engagement of TG2 activity in the pathogenesis of celiac disease. Celiac disease (CD) also termed celiac sprue and gluten-sensitive enteropathy is an inflammatory disorder characterised by damage of the intestinal epithelium resulting from abnormal intestinal immune response to dietary gluten proteins in the diet (Trier J.S. 1998; Jabri and Sollid 2009). In 1985, Bruce and co-workers described that tissue transglutaminase is expressed at low levels in a healthy small intestine, in contrary elevated levels of the enzyme were noticed in jejunal biopsies from the CD patients suggesting involvement of TG2 in the pathogenesis of the disease (Bruce et al., 1985). Further investigations identified TG2 as the autoantigen of the endomysium specific autoantibodies that are characteristic for celiac disorder (Dieterich et al., 1997). As a result of these findings other researchers used IgA TG2 antibodies as a tool to develop screening tests for the presence of celiac disease (Gillett and Freeman, 2000; Fabiani et al., 2001).

In addition to the role of TG2 in the humoral response, the activity of the enzyme was also shown to be associated with generating gluten peptides that trigger a T-cell mediated reaction in the small intestine of CD individuals (Moelberg et al., 1998; van de Wal et al., 1998; Vader et al., 2002). Genetic predisposition to develop celiac disorder has been linked with MHC class II genes that encode HLA-DQ2 and HLA-DQ8 molecules (Lundin et al., 1994). HLA-DQ2 and HLA-DQ8 display a high affinity for negatively charged amino acids (van de Wal et al., 1996; Godkin et al., 1997). However native gluten possesses only a few negatively charged residues for binding to DQ2 and DQ8. Increase in the number of these negatively charged amino-acids is triggered by post-translational modifications. Conversion of glutamine residues in gluten derived peptides into glutamic acid has been shown to be catalysed by tissue transglutaminase (Moelberg et al., 1998; van der Wal et al., 1998). However TG2 deamidates only specific glutamine residues bearing Gln-X-Pro motifs (where X represents any amino acid residues) which are often present in gluten proteins (Vader et al., 2002; Jabri et al., 2009). The deamidation can take place in the acidic environment of the stomach or in the one that is deprived of amine donors. Therefore TG2 mediated reactions targeted against specific substrates stimulates T-cell epitopes by generating negatively charged peptides which binds the HLA-DQ2 and HLA-DQ8 molecules with high affinity (Vader et al., 2002; Dorum et al., 2009).
Chapter I: Introduction

To sum up, tissue transglutaminase was characterised as the humoral auto-antigen in celiac disease but it still remains poorly understood whether the enzyme can function as T cell auto-antigen. However it was indicated by recent in vitro experiments performed by Ciccocioppo et al., (2010) on CD patients who revealed a role of TG2-specific T-cells mediated immune response in the pathogenesis of the disease (Ciccocioppo et al., 2010).

1.16.2 Tissue transglutaminase in neurodegeneration

Knowing that tissue transglutaminase is ubiquitously distributed throughout the human body, it is not surprising that its expression was also found in the brain (Kim et al., 1999). Although the enzyme expression was detected in the central nervous system (CNS) mainly in neurons it has also been observed in glial cells (Kim et al., 1999; Lesort et al., 1999). Within the central nervous system (CNS) TG2 has been postulated to play physiological and pathological functions. So far it has been shown to be involved in neuronal differentiation (Tucholski et al., 2001), neuronal apoptosis (Melino et al., 1994) and in maturation of the brain (Bailey and Johnson 2004). However TG2 has also been implicated in the development of the pathologies in the CNS. For example, mRNA, full length TG2 (Tolentino et al., 2002) and its short splice variants encoding a truncated form of enzyme missing the GTP-binding site (Citron et al., 2001) are elevated following the injury in the CNS. The increased level of TG2 and transcription of short splice variants facilitates uncontrolled cross-linking activity of the enzyme resulting in neuronal dysfunction and death (Citron et al., 2002). Given the findings, it has been hypothesized that TG2 may contribute to development of neurodengenerative diseases. Neurodegeneration is characterised by elevated levels of TG2 enzymatic activity include Huntington’s disease (Karpuj et al., 1999; Lesort et al., 1999), Alzheimer’s disease (Johnson et al., 1997; Citron et al., 2001; Kim et al., 1999), Parkinson’s disease (Citron et al., 2002) and progressive supranuclear palsy (Zemaitatis et al., 2003).

The most common form of neurodegenerative disorder is Alzheimer’s disease (AD). It is caused by the formation of senile plaques and neurofibrillary tangles consisting of amyloid-β protein or neurotoxin protein aggregates consisting of hyperphosphorylated tau which accumulation leads to neuronal death (Wilhelmus et al., 2008). There is a strong evidence suggesting the TG2 plays role in the pathology of AD since the enzyme was found to co-localise with plaques (Zhang et al., 1998) and neurofibrillar tangles
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(Citron et al., 2002) where additionally its elevated enzymatic activity was identified (Singer et al., 2002). Moreover β-amyloid which is present in fibrillar in plaques (Rasmussen et al., 1994) and the microtubule-binding protein tau that is a component of neurofibrillar tangles (Murthy et al., 1998) was demonstrated to act as substrates for TG2 which was further shown to facilitate the formation of amyloid-β oligomers and insoluble tau aggregates or tau filamentous. Additionally TG2 was reported to mediate polyamination of tau protein leading to formation of non-degradable tau in neurons (Tucholski et al., 1999). The available data suggest that regulation of TG2 activity during aggregates formation may be used as a therapeutic target for Alzheimer’s diseases (Wilhelmus et al., 2008).

Parkinson’s disease (PD) is a progressive movement disorder that is characterised by the loss of dopaminergic neurons in the substantia nigra. Pathologically this neurodegenration is manifested by the presence of intraneuronal inclusions known as Lewy bodies consisting of the α-synuclein protein (α-syn) (Spillantini et al., 1997). So far elevated expression of TG2 has been observed in substantia nigra of PD brain as well in Lewy bodies (Adringa et al., 2004; Junn et al., 2003). The first evidence of TG2 possible involvement in the aggregation of α-synuclein was documented by Jensen et al. (1995). Further investigations within that field using in vitro and cell models confirmed that TG2 interacts with α-synuclein resulting in increased formation of intramolecular α-synuclein aggregates (Junn et al., 2003). However contradictory findings suggested that even though the enzyme catalyses cross-linking of intramolecular α-synuclein monomers it inhibits its fibrillization in vitro (Konno et al., 2005). The basis of these interactions remains poorly understood nevertheless recent studies indentified two lysine residues (Gln\textsuperscript{79} and Gln\textsuperscript{109}) as the main α-synuclein substrate for the cross-linking activity of the enzyme (Schmid et al., 2009).

An additional disorder linked with elevated levels of TG2 activity is Huntington’s disease (HD). Huntington’s disease is a progressive autosomal-dominant neurodegenerative disorder that is caused by polyglutamine tract expansion in the gene encoding for huntingtin protein (Lesort et al., 2000). Huntingtin is present in the aggregates that are specific for HD (Becher et al., 1998) and has been demonstrated to act as a substrate for TG2 crosslinking activity in vitro (Kahlem et al., 1996; Karpuj et al., 1999). TG2 cross-links were detected in neurons in specific areas of Huntington’s brain where it co-localises with huntingtin protein in intracellular inclusions (Zainelli et al., 2002).
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Thus the enzyme was suggested to mediate the formation of huntingtin protein aggregates and in this manner to be involved in the pathogenesis of HD (Wilhelmus et al., 2008).

Based upon many available findings, elevated TG2 activity has been suggested to mediate the formation of neurotoxic aggregates in neurodegenerative disorders including Huntington’s disease, Alzheimer’s disease, and Parkinson’s disease. Therefore inhibition of the enzymes transamidating activity might be a therapeutic strategy in prevention of development of these diseases (Wilhelmus et al., 2008).

1.17 Aims

Numerous studies have reported the versatility of TG2, revealing information about its involvement in physiological and pathological processes that take place in eukaryotic cells in vivo. Many of these pathologies are instigated by TG2 in the extracellular environment but the mechanism of secretion of the enzyme is still not known. Evidence indicates that the enzyme does not undergo a conventional ER/Golgi dependent secretion pathway. Data suggest that for TG2 to be secreted, an intact N-terminal FN binding site (for which it has high affinity) is required however in addition interaction of TG2 with its high affinity binding partners present both in the intracellular and extracellular space as well as with specific cell surface receptors may be involved in that process.

Although there are likely to be many factors involved in externalisation of TG2 its translocation to the cell surface and its conformational state when secreted still remain unknown.

Thus, the initial objective of this project was to develop suitable cell models to investigate the externalisation of TG2 and its translocation into the ECM. The second aim was focused on studying specific amino acid sequences or TG2 regions which can be engaged in this process. To further identify whether the enzyme interacts with any so far known proteins, mutagenesis studies based on generating TG2 point or single mutations as well as deletion products were undertaken. Knowing that GTP (one of the intracellular binding partner of the enzyme), FN (its extracellular partner) and cell surface receptors including heparan sulfate proteoglycans and integrins may play a role in its translocation to the ECM, the specific characterised or uncharacterised TG2 binding sites for each of its high affinity binding partners were targeted for mutagenesis.
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Therefore, depending on the results obtained in the initial investigations, the influence of mutations on the conformational state of the enzyme, its final localisation as well as its role in extracellular environment with a more in depth view on its involvement in mediating cell-adhesion will be further explored in this thesis.
Chapter II: Materials and Methods
Chapter II: Materials and Methods

MATERIALS

2.1.1 General chemicals:

All sterile solutions, reagents and tips were prepared by autoclaving at 121 °C; otherwise solutions were filtered through a 0.22 µm Whatman sterile filter. Most of the general chemicals were purchased from Sigma-Aldrich as stated below:

Sigma – Aldrich Company Ltd, Dorset, UK:

Serum free medium (DMEM), Magnesium chloride, sodium chloride, sulphuric acid, glycine, Tris base, Paraformaldehyde, Dimethyl Sulphoxide (DMSO), D- (+)-Glucose, Ethylenediaminetetraacetic acid (EDTA), Magnesium sulfate, Casein enzymatic hydrolase, and SigmaFast OPD, May Grunwald and Giemsa stains

Additionally, other reagents and chemicals were supplied from:

Bachem, Merseyside, UK:
The synthetic peptides: H-Gly-Arg-Gly-Asp-Thr-Pro-OH (GRGDTP) and H-Gly-Arg-Ala-Asp-Ser-Pro-OH (GRADSP)

Peptide Synthetics, Fareham, UK:

Sigma Genosys, UK:
Oligonuclotide primers:
Forward primers:
5’ GGACAGCACCACGTGGTAGCCACGACGACGCTGCACCCCTCTCGC 3’
5’ GTTTTATCTACAGGGCGAGGCAGGCTTATCAAGCCTG 3’
5’ TACCAAGGGCTCGGCCTTCTCTCAAGAACGATAAC 3’
5’ GGGGAGGCATACAGCGGCGGCGACGTGGTTGGGCTTCAGGAAGGT 3’
5’ CAACCCGAGTCTGGCCGAGGCTGGACGACTGCTCCCCG 3’
5’ GGACGGTACCAGGACCATGACGCTGGCTCTCCT 3’
5’ GGACGGTACCAGGACCATGACGCTGGCTCTCCT 3’
Reverse primers:
5’ GCCAGAGGGTAAAGGCTGGCGTGCTACACGTTTGGCTATGC 3’
5’ CTTGATGAA ACTTGCCTGGCTGAGGGATAAACACG 3’
5’ GTATGTTTCTGGTAGAAGAGGGCGAGGCCTCCCCATGTA 3’
5’ GACACCCAGCACCCAGCGGCGGCGCTGGCTGCTGCTCCC 3’
5’ CAGGAGCAGTGCGCCGAGGCGTCTCAGGAGGGTTG 3’
5’ GGACGGCAGGAGGCTGGGGAAGGCAATGATGAC 3’
5’ GGACGCGGCCGCTCACCGGATCTTGATTTCTGG 3’

Sequencing primers:
5’ GGTTCTTCCAGCGCGCA 3’
5’ GGGTAAGCTTTCCGTATGTAGC 3’
5’ TAATACGACTCTATAGGG 3’
5’ GGGTAAGCTTTCCGTATGTAGC 3’

Molecular Probes, Eugene (OR), USA:
Biotin-X-cadaverine

Melford, Suffolk, UK:
Tris hydrochloride, Dithiothreitol (DTT), Carbenicillin disodium salt

Merck Chemical Limited, Nottingham, UK:
Albumin Bovine Serum, glacial acetic acid and Casein N,N-Dimethyl-Bovine

Thermo Scientific, Northumberland, UK:
EZ-link Sulf-NHS-LC-Biotin, High Capacity NeutrAvidin Agarose

2.1.2 Bacterial lines and transformation reagents:

Invitrogen, Ltd., Paisley, UK:
DH5α strain of Escherichia coli

Merck Chemical Limited, Nottingham, UK:
NovaBlue Singles TM Competent cells, strain of Escherichia coli

Oxoid Limited, Hampshire, UK:
Yeast extract, Trypton T, and Agar bacteriological

Sigma – Aldrich Company Ltd, Dorset, UK:
Ampicilin

2.1.3 Immunochemicals:

Lab Vision Products, Thermo Fisher Scientific, UK:
Transglutaminase II Ab-1 monoclonal antibody (CUB 7402)

Sigma – Aldrich Company Ltd, Dorset, UK:
Goat anti-mouse IgG-HRP conjugate, anti-mouse IgG α-tubulin

2.1.4 Protein reagents:

Sigma – Aldrich Company Ltd, Dorset, UK:
Fibronectin from human plasma (FN), and extravidin- peroxidise
70 kDa fibronectin from human plasma, 30kDa fibronectin from human plasma
**Chapter II: Materials and Methods**

**Zedira GmBh, Darmstadt, Germany:**
Guinea pig liver tissue transglutaminase
Human tissue transglutaminase recombinant in *E.coli*

**Domestic supplier:**
Marvel skimmed dried milk powder

**2.1.5 Electrophoresis and Western blotting reagents:**

**Santa Cruz Biotechnology Inc., Calne, UK:**
RIPA cell lysis buffer

**Sigma – Aldrich Company Ltd, Dorset, UK:**
Acrylamide, Ammonium persulphate (AP), protease inhibitor cocktail for mammalian cells, Laemmli buffer (2x concentrated), GBX Developer/Replenisher, GBX fixer/Repelnisher, Kodak Biomax XAR detection Film

**Melford, Suffolk, UK:**
Tris-Glycine-SDS buffer, pH 8.5, sodium dodecyl sulphate (SDS)

**Bio-Rad Laboratories Ltd., Hertfordshire, UK:**
Gel loading tips

**Fisher Scientific UK Ltd., Leicestershire, UK:**
Western blotting filter papers

**GE Water & Process Technologies, UK:**
Nitrocellulose membrane

**Amersham Pharmacia Biotech. UK Ltd, Little Chalfont, UK:**
ECL development kit and the full range rainbow molecular marker

**2.1.6 Expression and cloning vectors:**

**Invitrogen Ltd., Paisley, UK:**
pcDNA3.1/CT-GFP expression vector

**Merck Chemical Limited, Nottingham, UK:**
psTBlue-1 Perfectly blunt cloning kit

**2.1.7 Molecular biology kits, chemicals and reagents:**

**UK New England Biolabs, Knowl Piece, UK:**
DNA marker - 2-log DNA ladder (0.1-10kb), restriction enzymes - Not I, Kpn I, Ear I, Eag I, Bst XI, T4 DNA ligase
**Chapter II: Materials and Methods**

**Fermentas, York, UK:**
Restriction enzyme – PpiI

**Promega, Southampton, UK:**
Pfu DNA Polymerise, plasmid DNA purification kits: Pure Yield Plasmid Miniprep system, Wizard plus SV Minipreps DNA Purification system, Wizard SV gel and PCR Clean-up system,

**Qiagen, Crawley, UK:**
Plasmid DNA purification kit: Endotoxin free plasmid maxi

**Merck Chemical Limited, Nottingham, UK:**
KOD HOT Start DNA Polymerase

**Stratagene, Cheshire, UK:**
Quick change II site directed mutagenesis kit

**Melford, Suffolk, UK:**
Molecular grade water, agarose, Low melting temperature agarose, Tris-Acetate-EDTA buffer pH 8.0, Isopropyl-β-D-1-thio-galactopyranoside IPTG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside X-gal

**2.1.8 Cell transfection reagents:**

**Sigma – Aldrich Company Ltd, Dorset, UK:**
HEPES buffered saline, Calcium chloride solution

**Lonza Wokingham LTD., Berkshire, UK:**
Nukleofactor kit R

**2.1.9 Every day use consumables:**

**Appleton Woods Ltd., Birmingham, UK:**
Tissue culture plates (TCP) (6- and 96-well plates), Petri dishes, wide range of flasks (T25, T75, T100), 2 ml microcentrifuge tubes, 15ml and 50ml sterile centrifuge tubes, 5ml, 10ml, 25ml sterile pipettes, 10µl, 100µl and 1ml pipette tips

**2.1.10 Other consumables:**

**Sterilin Ltd., Bargoed, UK:**
Microtiter 96-well plates

**Nunc™, Thermo Fisher Scientific, Roskilde, Denmark:**
Cryotube vials, high binding 96-well plates

**Laboratory supplier:**
the irreversible peptidic inhibitor R281
2.1.11 Equipment:

Laboratory instruments listed below were purchased from the following distributors:

**Appleton Woods Ltd., Birmingham, UK**
Spectrafuge 24D, Mini gyro-rocker (Stuart), Orbital shaker

**Beckam Coulter Ltd., High Wycombe, UK:**
Avanti J-E centrifuge, Allegra X-15R centrifuge, Microfuge® 22R centrifuge

**Sanyo Ltd., Watford, UK**
Orbi-safe shaker, Sanyo Tissue culture CO₂ incubator

**Bio-Rad Laboratories Ltd., Watford, UK:**
DNA electrophoresis system - Protean III minigel system, power supply – Power Pac basic

**Bibby Scientific Limited, Stone, UK:**
Jenway Spectrophotometer Genowa

**Amaxa, GmbX, Germany:**
Nukleofaktor system

**Amersham Biosciences, Sweden:**
Heparin Sepharose, HiTrap™ Heparin HP (1ml, 5ml)

**Sigma – Aldrich Company Ltd, Dorset, UK:**
GTP-Agarose column (2ml)

**Cleaver Scientific, Rugby, UK:**
Agarose electrophoresis equipment (tanks and power supply)

**Biotek, Potton, UK:**
Absorbance microplate reader ELx808™

**Amersham Biosciences, Sweden:**
ÄKTA purifier, FPLC system

Fluorescent microscope, Ziess Axiovert 200
Chapter II: Materials and Methods

METHODS

2.2 Methods

2.2.1 Generation of the wild type TG2

2.2.1.1 Expression and purification of wtTG2

The cDNA encoding human endothelial TG2 was cloned into the pcDNA3.1/Myc-HisA/Neo vector and then into the HindIII and Pmel restriction sites of the pGene/V5-His vector for mifepristone inducible expression in mammalian cells (Hang et al., 2005). These clones were kindly provided by Dr A.M. Belkin (University Maryland, USA). Obtained plasmids were transformed into NovaBlue Singles Competent cells (E.coli) (Novagen), and plated directly on ampicillin containing agar plates for selection and incubated overnight at 37 °C. Single colonies were grown in LB medium (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0 plus ampicillin 100μg/ml) for 18 hours at 37 °C. After centrifugation plasmid DNA was purified using Wizard Plus SV Miniprep (Promega) according to the manufacture’s protocol. The purity of plasmids was confirmed by using 0.8% (w/v) agarose gel electrophoresis in TAE buffer (Tris-acetate-EDTA, pH 8.3).

2.2.1.2 DNA analysis on agarose gel electrophoresis

Agarose gels were composed of agarose (w/v depending on gel percentage) by melting it in 1xTris-acetate-EDTA buffer, pH 8.3 (TAE, 40mM Tris, 0.114% glacial acetic acid(v/v) and 1mM EDTA) in a microwave oven. After cooling the solution to 55-60 °C, ethidium bromide (10mg/ml) was added to a final concentration of 0.5μg/ml, the gel was poured on a casting plate in a Bio-Rad DNA electrophoresis tray and allowed to solidify for about 1hour at RT or +4 °C (low melting point agarose gel). Earlier prepared DNA samples were diluted 1/10 with 10x DNA loading buffer (100mM EDTA, 1% SDS(w/v), 0.25% bromophenol blue(w/v) and 0.25% xylene cyanol(w/v)) and loaded into wells. As a marker
2.2.1.3 Primer design and DNA amplification

The purified inserts were amplified with primers (forward and reverse primers especially designed for TG2 using PrimerSelect software, Lasergene), which introduced a KpnI site before the TG2 initiation codons and a NotI site after the stop codons (Table I). PCR reactions were assembled with the following components:

### Table I

**A list of primers for TG2 amplification**

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG2 FP</td>
<td>5’ GGTACCATGGCCAGGAGCTGGTC 3’</td>
</tr>
<tr>
<td>TG2 RP</td>
<td>5’ GCCGGCCGCTTAGGCGGGCAATGATGAC 3’</td>
</tr>
</tbody>
</table>

The reactions were carried out in the presence of Pfu DNA Polymerase (Promega) and cycled in a Primus thermal cycler using the following conditions (Table III):

### Table II

**A list of components used for TG2 amplification**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfu DNA Polymerase 10x buffer with MgSO₄</td>
<td>5μl  1x</td>
</tr>
<tr>
<td>2mM dNTPs mix</td>
<td>5μl  200μM each</td>
</tr>
<tr>
<td>Forward primer (TG2)</td>
<td>0.5μl  TG2 0.372μg/0.5μl</td>
</tr>
<tr>
<td>Reverse primer (TG2)</td>
<td>0.5μl  TG2 0.449μg/0.5μl</td>
</tr>
<tr>
<td>DNA template</td>
<td>1μl  0.5μg/50μl, 1000x</td>
</tr>
<tr>
<td>Pfu DNA Polymerase</td>
<td>1μl  1.25u/50μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>37μl</td>
</tr>
</tbody>
</table>

The reactions were carried out in the presence of Pfu DNA Polymerase (Promega) and cycled in a Primus thermal cycler using the following conditions (Table III):
Chapter II: Materials and Methods

Table III

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>2 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>42-65 °C</td>
<td>1 minute</td>
<td>25-35 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72-74 °C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>74 °C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>Indefinite</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

2.2.1.4 Subcloning of amplified cDNAs

The PCR products were separated on 0.8% agarose gels and purified using Wizard SV gel plus a PCR clean up system and then cloned into psTBlue-1 vector using the Perfectly Blunt Cloning kit from Novagen. The cultures from E.coli, transformed with the ligation products, were grown overnight at 37 °C, on agar plates with 100µg/ml ampicillin (Sigma-Aldrich) and 50µg/ml carbenicillin (Melford laboratories), which were additionally supplemented with 35ul of 50mg/ml X-gal (Melford laboratories) and 20ul of 100mM IPTG (Melford laboratories) to allow blue/white screening. The blue colonies, assumed to contain religated vector, were not taken for further examination. The white colonies, were spread again onto ampicillin agar plates and grown overnight at 37 °C. The size of the cDNA was rapidly estimated following the cracking procedure described in “Promega-protocols guide book”. Briefly, each picked colony was added into an eppendorf tube that contained 50µl of 10mM EDTA pH 8.0, then suspended in 50ul 2xcracking buffer (0.2M NaOH, 0.5% SDS, 20% sucrose) and incubated at 70 °C for 5 min. After cooling to RT, 1.5µl of 4M KCL plus 0.5µl of 0.4% bromophenol blue was transferred into each tube which were placed on ice for 5 min. Obtained suspensions were centrifuged at 13 000rpm for 3 min at 4 °C and loaded on 0.8% agarose gels. After performing electrophoresis, samples which contained cDNA constructs that were significantly bigger from the rest were chosen for miniprep purification following the instructions of Wizard Plus SV Miniprep DNA Purification system (Promega). To separate inserts from the vectors, first they were
digested with KpnI and NotI restriction enzymes and run for 1 hour on 1.5% low melting point agarose gels with a constant power of 100V. Cleaved inserts were cut from the gel, once more purified using Promega clean up system and subcloned into the pcDNA3.1/CT-GFP expression vector. To isolate endotoxin-free DNAs for transfection, obtained plasmids were purified using Endotoxin free plasmid maxi kit (Qiagen, UK) according to the manufacturer’s protocol. Plasmid DNA quality was estimated by agarose gel electrophoresis and the concentration was calculated by the absorbance at 260nm, by the ratio of $A_{260} \text{ nm}$ to $A_{280} \text{ nm}$ absorbance values.

2.2.2 Mutagenesis studies

2.2.2.1 Primer design and DNA amplification

2.2.2.1 A TG2 point mutations

Endotoxin free plasmid DNA containing wild type TG2 was used as a DNA template to generate a set of different TG2 mutants. The Quick change site-directed mutagenesis kit (Stratagene) was used to create single point mutations (GTP1 and GTP2 mutants) and multiple point mutations (HS1, HS2 and FN1 mutants) as shown in Table IV. All the reactions were performed according to the manufacturer’s protocol.

<table>
<thead>
<tr>
<th>Construct</th>
<th>TG2 Point Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN1</td>
<td>(D94A, D97A)</td>
</tr>
<tr>
<td>GTP1</td>
<td>(S171E)</td>
</tr>
<tr>
<td>GTP2</td>
<td>(K173L)</td>
</tr>
<tr>
<td>HS1</td>
<td>(K600A, R601A, K602A)</td>
</tr>
<tr>
<td>HS2</td>
<td>(K205A, R209A)</td>
</tr>
</tbody>
</table>
This method requires designing two oligonucleotide primers, each complementary to the opposite strands of the vector containing the insert of interest and containing the required mutation(s) (Table V). Specific oligonucleotide primers are extended during temperature cycling by Pfu DNA Polymerase which leads to generation of the mutated plasmids.

Table V

A list of primers and their sequences

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primers</strong></td>
<td></td>
</tr>
<tr>
<td>FN1</td>
<td>5’ GGACAGCCACCCTGGTAGACCAGCAAGCCTGCACCCTCTTCGC 3’</td>
</tr>
<tr>
<td>GTP1</td>
<td>5’ GCTTTATCTACGAGGCAGGGCAGGCAAGTTTCATCAAG 3’</td>
</tr>
<tr>
<td>GTP2</td>
<td>5’ TACCAGGGCTCGGCCTCTTCATCAAGAAGATAAC 3’</td>
</tr>
<tr>
<td>HS1</td>
<td>5’ GGGGAGCGAAGCGGCCGCTGGTGCTGGGTAGGCTGTC 3’</td>
</tr>
<tr>
<td>HS2</td>
<td>5’ CAACCCCAAGTTTCTGGCGAAGCGCGCTGACTGCTCCCG 3’</td>
</tr>
<tr>
<td><strong>Reverse primers</strong></td>
<td></td>
</tr>
<tr>
<td>FN1</td>
<td>5’ GCGAGAGGTGAAGGCTTGGCTACCACCGTGCTGTC 3’</td>
</tr>
<tr>
<td>GTP1</td>
<td>5’ CTTGATGAACCTTGGGTGCTGTCCTGTAAGAAGC 3’</td>
</tr>
<tr>
<td>GTP2</td>
<td>5’ GTATGTTCCTGATGAAGGCGAGCCGCTGGTA 3’</td>
</tr>
<tr>
<td>HS1</td>
<td>5’ GACACCTCAGCCACCGCGGCGGCGCTGCTGGGTAGG 3’</td>
</tr>
<tr>
<td>HS2</td>
<td>5’ CGGGAGCGAGTCAGCGCGGCGGCTGGACCTTGGGTG 3’</td>
</tr>
</tbody>
</table>

As shown in Table VI, the obtained plasmids were digested with specifically chosen restriction enzymes (since the mutation creates new or removes existing restriction sites) and run on a 0.8% agarose gel with constant voltage 100V to verify whether the obtained constructs contained the desired mutation.
Table VI

A list of restriction enzymes used to digest desired TG2 mutation

<table>
<thead>
<tr>
<th>Construct</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN1</td>
<td>BstXI</td>
</tr>
<tr>
<td>GTP1</td>
<td>PpiI</td>
</tr>
<tr>
<td>GTP2</td>
<td>EarI</td>
</tr>
<tr>
<td>HS1</td>
<td>NotI</td>
</tr>
<tr>
<td>HS2</td>
<td>Eagl</td>
</tr>
</tbody>
</table>

To further confirm the correct sequences of the TG2 mutants each plasmid DNA was sequenced. Again for that purpose a set of primers (either forward and reverse or only reverse in case of the HS1 mutant) were designed using PrimerSelect software, Lasergene.

Table VII

A list of sequencing primers

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN1</td>
<td>5' GGGTAAGCTTTCCGTATGTAGC 3'</td>
</tr>
<tr>
<td>GTP1</td>
<td>5' GACCTGTGCCGGAGAAGC 3'</td>
</tr>
<tr>
<td>GTP2</td>
<td>5' GGTTCTTCCAGCGCCGCA 3'</td>
</tr>
<tr>
<td>HS2</td>
<td>5' GGGTAAGCTTTCCGTATGTAGC 3'</td>
</tr>
</tbody>
</table>

Forward primers

Reverse primers
To isolate endotoxin-free DNAs for transfection, obtained plasmids were purified using Endotoxin free plasmid maxi kit (Qiagen, UK) according to the manufacturer’s protocol. Plasmid DNA quality was estimated by agarose gel electrophoresis and the concentration was calculated by the absorbance at 260nm, by the ratio of $A_{260}$ nm to $A_{280}$ nm.

2.2.2.1 B TG2 deletion products

A set of TG2 deletion mutants (deletion of the first 15 amino acids of the N-terminal site and deletion of the whole C-terminal domain of the wild type TG2) was generated based on the TG2 insert expressed in the pcDNA3.1/CT-GFP vector.

<table>
<thead>
<tr>
<th>Construct</th>
<th>TG2 deletion products</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-</td>
<td>TG2 (del 1-15 amino acids)</td>
</tr>
<tr>
<td>C-</td>
<td>TG2 (del 594-687 amino acids)</td>
</tr>
</tbody>
</table>

Both deletion mutants were created by PCR amplification using the set of oligonucleotide primers as shown in Table IX which introduces mutations between KpnI and NotI restriction sites on the DNA template.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primers</strong></td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td>5’ GGACGTTACCATGACCAATGGCCGAGACCACCAC 3’</td>
</tr>
<tr>
<td>C-</td>
<td>5’ GGACGTTACCATGGCCGAGGAGCTGGTCTTTAGAG 3’</td>
</tr>
<tr>
<td><strong>Reverse primers</strong></td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td>5’ GGACCGGGAAGCTTAGGCGGGGCCAATGATGAC 3’</td>
</tr>
<tr>
<td>C-</td>
<td>5’ GGACCGGCGCTCACCAGCTTCTAGTTCGCGC 3’</td>
</tr>
</tbody>
</table>
PCR reactions were assembled with the following components:

**Table X**

*A list of components used for PCR reaction*

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOD Hot Start DNA Polymerase 10x buffer</td>
<td>5µl</td>
<td>1x</td>
</tr>
<tr>
<td>2mM dNTPs mix</td>
<td>5µl</td>
<td>200µM each</td>
</tr>
<tr>
<td>25mM MgSO₄</td>
<td>3µl</td>
<td>1.5mM MgSO₄</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5µl</td>
<td>N- (0.52µg/0.5µl)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.5µl</td>
<td>C- (0.53µg/0.5µl)</td>
</tr>
<tr>
<td>DNA template (TG2)</td>
<td>1µl</td>
<td>0.5µg/50µl, 1000x</td>
</tr>
<tr>
<td>KOD Hot Start DNA Polymerase (1U/µl)</td>
<td>1µl</td>
<td>0.02U/50µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>34µl</td>
<td></td>
</tr>
</tbody>
</table>

The reactions were carried out in the presence of KOD Hot Start DNA Polymerase (Novagen) and cycled in a Primus thermal cycler using the following conditions:

**Table XI**

*Condition of PCR reaction*

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>2 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>0.5 minute</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>0.5 minute</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72-74°C</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>74°C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

Obtained PCR products and an empty expression vector pcDNA3.1/CT-GFP were digested with Kpnl and Notl restriction enzymes. To eliminate any remaining buffers all
constructs were purified using Wizard SV gel plus a PCR clean up system and visualised on a 0.8% agarose gel with a constant power of 100V. Purified inserts were then subcloned into the pcDNA3.1/CT-GFP expression vector. To isolate endotoxin-free DNAs for transfection, obtained plasmids were purified using Endotoxin free plasmid maxi kit (Qiagen, UK) according to the manufacturer’s protocol. Plasmid DNAs quality was estimated by agarose gel electrophoresis and the concentration was calculated by the absorbance at 260nm, by the ratio of $A_{260}$ nm to $A_{280}$ nm absorbance values.

Again the identity and proper arrangement of the TG2 deletion mutants was analysed by DNA sequencing by means of specially designed sequencing primers as shown in Table XII.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequencing primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primers</strong></td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td>$5'$ TAATACGACTCACTATAGGG 3'</td>
</tr>
<tr>
<td>C-</td>
<td></td>
</tr>
<tr>
<td><strong>Reverse primers</strong></td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td>$5'$ GGGTAAGCTTCCGTATGAGC 3'</td>
</tr>
<tr>
<td>C-</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.3 Cell culture

#### 2.2.3.1 Cell lines

Cell lines used in this project including human kidney epithelial cells HEK 293/T17 and mouse embryo fibroblasts NIH3T3 were obtained from American Type Culture Collection (ATCC, USA). HEK293 is a cell line that is generated by the transformation of normal human kidney embryonic cells with adenovirus type 5 DNA (Graham et al., 1977). The type of kidney cell that the HEK293 cells are derived from is unknown. It has been speculated that the cells may be neuronal in origin. However it is more plausible that cells
derived from an embryonic kidney would rather be epithelial, endothelial or fibroblasts. Nevertheless the reason for using those cells results from their ability to efficiently express wide range of recombinant proteins that are subcloned into the expression vector, which carries SV40 as origin of replication to allow its amplification in the host cell (van Craenenbroeck et al., 2000; Durocher et al., 2002). Additionally human dermal osteoblasts (HOB) were kindly provided by Dr S. Anderson (University of Nottingham, UK). This cell line was obtained from femoral heads of trabecular bone and spontaneously immortalised.

Each cell type was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, nonessential amino acids, 100 U/ml of penicillin and 100µg/ml of streptomycin followed by incubation in a humidified atmosphere at 37°C, 5%(v/v) CO₂, 95%(v/v) air to maintain their optimal growth conditions.

The human kidney epithelial cells HEK293/T17 expressing wt TG2 as well as TGase mutants were grown in a supplemented growth medium (DMEM) excluding any penicillin/streptomycin supplement since the presence of antibiotic in the growing medium could affect the efficiency of transfection.

The NIH3T3 fibroblasts expressing wt TG2 as well as TGase mutants were supplemented with antibiotics in complete DMEM.

2.2.3.2 Cell passaging and counting

Cells were passaged at approximately 90% confluence. The cell monolayer was rinsed with PBS pH 7.4 and treated with 0.25% (w/v) trypsin, 2 mM EDTA solution in PBS, pH 7.4 at 37 °C prior to detachment of the cells. To neutralize the trypsin, cells were collected in the growth medium and centrifuged at 250xg for 5 minutes and then reseeded in the tissue culture flask until they reached the required confluence. To determine the concentration of cells in a suspension a hemacytometer was used. Approximately 10ul of cell suspension was added into each hemacytometer chamber and then cells were counted 4 times using phase/contrast microscopy. The cell number was calculated as follows: ‘established number of cells x dilution factor/mm² of counted area x chamber depth’.
2.2.3.3 Cryopreservation of cells

Cells were trypsinised, and treated as described before (2.2.3.2). Centrifuged again to discard remaining medium and resuspended in a cryopreservative solution containing heat inactivated FCS containing 10 % (v/v) DMSO. Cells were then transferred into cryogenic vials and frozen in – 70°C overnight before long-term storage in liquid nitrogen.

2.2.3.4 Thawing frozen cells

Cryogenic vials containing frozen cells were removed from liquid nitrogen and thawed in a water bath at 37 °C. The cell suspension was placed into a falcon tube and mixed very gently with 5ml of supplemented growth medium. The whole suspension was then centrifuged at 250xg to eliminate any traces of DMSO. The remaining pellet was resuspended in the growth medium and transferred into the T25 tissue culture flask which was placed in the incubator at 37 °C.

2.2.3.5 Transient transfection of cells with the wild type TG2 and TG2 constructs

The human kidney epithelial cells were used for experimental procedures between 5 to 30 passages. After reaching 80%-90% confluence, cells were passaged using supplemented growth medium without antibiotic and required amount of cells was calculated in a hemacytometer (2.2.3.2). For transient transfection, 6.5 x 10^5 cells were seeded into each well of a 6-well tissue culture plate (TCP). To optimize the experimental conditions, first HEK 293/T17 cells were transfected with 2µg of empty expression vector (pcDNA3.1/CT-GFP) using 250mM CaCl_2 as a tranfection reagent. Then cells were transfected with 2µg of wild type and mutant (FN1, GTP1, GTP2, HS1, HS2, N-, C-) TGase cDNA, respectively. Transfection was performed following the manufacturer’s protocol. After 24 and 48 hours incubation at 37°C the efficiency of transfection was estimated using fluorescence microscopy.

Similarly the NIH3T3 fibroblasts were used for experimental procedures between 5 to 30 passages. After reaching 80%-90% confluence, cells were passaged using supplemented growth medium with antibiotic and the required amount of cells was
calculated in a haemocytometer (2.2.3.2). To transiently transfect these cells, a kit from Lonza (Amaza cell line nukleofaktor kit R) was used following the manufacturer’s instructions. To verify the efficiency of transfection, 1 x 10^6 cells were transfected with 4μg of empty expression vector (pcDNA3.1/CT-GFP). For transfection of wild type and mutant (FN1, GTP1, GTP2, HS1, HS2, N-, C-) TGase cDNA, 4ug of DNA was used. After 24 and 48 hours incubation at 37°C the strength of signal was estimated using fluorescence microscopy.

2.2.3.6 Preparation of total cell lysate

After 48 hours incubation at 37°C, the adherent cells were washed twice with ice cold PBS, pH 7.4 and resuspended in 60 µl of ice-cold buffer made after mixing 1ml of RIPA lysis buffer (Santa Cruz Biotechnology) plus 10μl protease inhibitor cocktail (Sigma) and 20µg/ml PMSF. After 20 minutes incubation on ice the cells lysate was scraped from the bottom of wells. Cell extracts were clarified by centrifugation at 300g for 5 minutes at 4°C. Clear supernatants were collected into eppendorf tubes and stored at -70°C for further analysis by electrophoresis.

2.2.3.7 Determination of protein’s concentration

The protein content in cells lysates was determined by the Lowry method (Lowry et al., 1951) using a commercially available kit from Bio-Rad, following the manufacturer’s instructions. Briefly, 5μl of BSA solution ranging from 0.1-1.5 mg/ml was used as a standard and added into the 96 wells plate in triplicate. A further 1μl of cells lysate dissolved in 4μl of dH_2O was added into the microtitre plate in triplicate respectively. 25 μl of Reagent A and 200 μl of reagent B were then added into each well and incubated at room temperature for 15 minutes. Absorbance values were read at 750nm using an Absorbance microplate reader ELx808™ to produce the calibration graph.
2.2.4 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE electrophoresis)

2.2.4.1 Sample preparation

The protein extracts (total cell lysates) collected from untransfected as well as transfected cells were resolved by SDS-PAGE electrophoresis using a Protean III minigel system. All cell lysates were equally (1:1 ratio) dissolved in 2x Laemmli buffer (125mM Tris-Cl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-β-mercaptoethanol and 0.004% bromophenol blue) and denatured in boiling water for 5 min. After cooling, a maximum of 50ug of protein was loaded on the gel. To visualize protein separation during electrophoresis a full range of rainbow molecular markers were used (Amersham Biosciences).

2.2.4.2 SDS-PAGE electrophoresis

Separation of protein extracts by SDS-PAGE electrophoresis was based on the modification of the method described by Laemmli et al., 1970 using a Protean III minigel system (BioRad, UK). Both, resolving and stacking gels were made in the Protean III minigel system from 3% (w/v) polyacrylamide, and Tris-Cl/SDS buffer solution. The running gel was composed of 0.75M Tris, 0.2% (w/v) SDS pH 8.8 while the stacking gel was made of 0.25M Tris, 0.2% (w/v) SDS pH 6.8. Gel polymerization was initiated using 10% (w/v) Ammonium persulphate and the required volume of N,N,N’,N’-Tetramethylethylenediamine (TEMED). The exact recipe for preparing resolving and stacking gels is shown in Tables XIII, and XIV.
Table XIII

A list of Resolving gel components

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (w/v) acrylamide/bis-acrylamide</td>
<td>8% 10%</td>
</tr>
<tr>
<td>Tris-SDS pH 8.8</td>
<td>3.75 ml 3.75 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>7.25 ml 6.25 ml</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulphate</td>
<td>50 µl 50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl 10 µl</td>
</tr>
</tbody>
</table>

Table XIV

A list of Stacking gel components

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (w/v) acrylamide/bis-acrylamide</td>
<td>650 µl</td>
</tr>
<tr>
<td>Tris-SDS pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulphate</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

The resolving gel was pipetted into the sandwich and then covered by water saturated butan-2-ol to flatten the gel’s surface and prevent air bubbles from getting inside the gel. The prepared gel was left for 1 hour at room temperature to polymerize. Afterwards, the butan 2-ol was removed and washed with distilled water and the wet surface of the gel was dried using a filter paper. Meanwhile the 0.75mm stacking gel was prepared and poured on the top of the resolving gel and allowed to polymerize (Laemmli et al., 1970). A 10-well or 15-well comb was placed into the stacking gel. The gel was allowed to polymerize at room temperature for another 45 minutes. Then the combs were removed.
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and gel placed in a Protean III mini gel tank which contained Tris-glycine electrode running buffer, pH 8.5 (25mM Tris-Base, 192mM glycine and 0.1% (w/v) SDS). Electrophoresis was performed at the constant voltage of 100V through the stacking gel and 120V through the resolving gel. When the bromophenol blue dye reached the bottom of the gels, the gels were taken out from the chambers and processed further.

2.2.5 Western blotting of separated by electrophoresis proteins

Gels containing separated proteins were transferred to a nitrocellulose membrane by a Bio-Rad wet blot system following the protocol of Towbin et al., 1979. The fiber pads, filter paper and nitrocellulose membrane were pre soaked in an ice cold transfer buffer (48 mM Tris-Cl, 39 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol). The protein gels were removed from the chambers and soaked in a transfer buffer. Afterwards the sandwich was formed; first one fibre pad was placed on the black side of the gel holder cassette, then on its top a filter paper was laid onto it. Another layer was made by the protein gel which was covered by a nitrocellulose membrane. Next a filter paper was placed on the top of the nitrocellulose membrane and covered with a second fibre pad. When the sandwich was formed, the gel holder cassette was finally closed and the blotting cassette inserted into the blotting apparatus filled and with ice cold transfer buffer. The cassette was inserted in a blotting apparatus so that the membrane faced the anode electrode. The transfer was carried out for 90 minutes at 150mA and the blotted membrane was washed once with PBS, pH 7.4 then further processed to blocking procedure.

2.2.5.1 Detection of TG2 antigen by Western blotting

The blotted membranes were rinsed in deionised water and shaken 1.5 hour with gentle agitation in blocking solution (5% dried milk dissolved in TBS-0.05%Tween 20(v/v), pH 7.4) to eliminate any no specific binding which could occur during the transfer. The blots were incubated overnight at 4 °C with the required antibody diluted in blocking solution. Following probing with primary antibody, the blots were washed three times.
with TBS-0.05%Tween (v/v), pH 7.4 and additionally with blocking solution. After washing
the membranes were incubated 2 hours at room temperature with Horseradish
peroxidase conjugated antibody diluted 1:2000 in blocking solution. Afterwards, the
secondary antibody was decanted; the membranes were washed once again, rinsed and
soaked with PBS pH 7.4. TG2 bands were detected using the Amersham ECL
Chemiluminescence system kit (Amersham Biosciences) following the manufacture’s
instruction to allow visualisation of the proteins on the developed X-ray film (Kodak
Biomax XAR film, Sigma). Detection was carried out in a dark room, for 5 minutes to 1
hour depending on the intensity of the signal. The film was developed using GBX
developer/replenisher (Sigma-Aldrich, UK), rinsed with water and then fixed in GBX
fixer/replenisher (Sigma-Aldrich, UK).

To control the equal loading of proteins, obtained blots were first stripped and then
reprobed with a monoclonal antibody directed against tubulin (internal marker for equal
loading). Following the 30 minutes stripping in the stripping buffer (100mM 2-
mercaptoethanol, 2% SDS and 62.5mM Tris-HCl, pH 6.7), the membranes were twice
washed with TBS-0.05%Tween(v/v), pH 7.4 and blocked at RT for 1 hour in blocking buffer
(3% dried milk dissolved in TBS-0.05%Tween 20(v/v), pH 7.4). The chemiluminescence
signal was detected on X-ray film after reprobing the membranes with control mouse
monoclonal anti-tubulin antibody (Sigma-Aldrich, UK) diluted 1:1000 in a blocking buffer,
followed by peroxidase conjugate monoclonal anti-mouse IgG (Sigma-Aldrich, UK).

To quantitate the obtained results the densitometry analysis was performed using
ImageJ software. The net values obtained from TG2 bands were normalised against
values from the corresponding tubulin bands.

2.2.6 Characterisation of the mutants

2.2.6.1 Affinity chromatography

2.2.6.1 A Preparation of cleared cell lysates for the affinity chromatography

HEK cells were transiently transfected with wild type TG2, and TG2 mutants as
described in section (2.2.3.5). After 48 hours incubation at 37°C cells were washed twice
with ice cold PBS, pH 7.4 and lysed by addition of non-denaturating buffer composed of
(20mM Tris-Cl pH 7.4, 10mM EGTA, 2mM EDTA, 1mM NaF and 1mM Na$_3$VO$_4$). Lysed cells were clarified by centrifugation for 5 minutes at 300g at 4°C. Cleared supernatants were collected into eppendorf tubes and stored at – 70 °C until required.

2.2.6.1 B Heparin Sepharose affinity column

The resulting supernatants obtained from HEK293/T17 cells transfected with wtTG2, and TG2 mutants, as described before (section 2.2.3.5) were pelleted to discard cellular debris and membranes and applied to a 5ml bed volume Heparin Sepharose column (Amersham Biosciences, Sweden) equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer. Each sample was applied manually to the column in equilibration buffer at an approximate flow rate at 1ml/min at room temperature. After washing the column with 15ml of equilibration buffer bound proteins were eluted with 1M NaCl in 50mM Tris-Cl, 1mM EDTA, and 1mM DTT, pH 7.5. Both flow-through and eluted volumes were fractionated into 300μl aliquots of each well of a microtiter plate and assayed for TG2 activity. The resulting fractions were verified by SDS-PAGE electrophoresis and Western blotting using anti-TG2 monoclonal antibody for detection of TG2 bands.

2.2.6.2 Solid phase binding assay (ELISA)

The assay is a modification of the original method established by Achyuthan et al. (1995). The solid phase binding assay used to measure the binding affinity of TGase for fibronectin was performed on 96 well TCP plates (Corning Costar, UK) coated with 5µg/ml of plasma fibronectin (Sigma-Aldrich, UK) diluted in a 50mM Tris-Cl, pH 7.4. The coating procedure was carried out at 4°C whereas most steps were performed at room temperature. To eliminate any possibility of non specific binding the wells were blocked for 30 min with 5% (w/v) fat-free milk in PBS, pH 7.4. After washing twice with PBS-Tween and once with PBS pH 7.4, cell homogenates (50µl per well) were added to the coated TCP wells and incubated for 1 hour at 37°C. After washing as before, 50µl of the anti-TG2 antibody (CUB7402) diluted 1:1000 in a blocking solution was added to the wells and left for 2 hours and washed again. Followed by 2 hours incubation with 50µl of goat anti-mouse IgG HRP conjugate antibody similarly diluted in a blocking buffer bound
horseradish peroxidase activity was measured by addition of Sigma Fast OPD substrate and the colorimetric reaction was stopped with 2.5M $\text{H}_2\text{SO}_4$ and further assayed spectrophotometrically at 490nm using an Absorbance microplate reader (Biotek ELx808™).

2.2.7 TG2 activity assays

2.2.7.1 Biotin X-cadaverine incorporation into N,N'-dimethylcasein

This assay is based on measuring TG2-activity by a modification of the technique originally described by Slaughter and co-workers (Slaughter et al., 1992). Briefly, each well of a 96-well plate was coated with 100µl of 10mg/ml N,N'-dimethylcasein in 50mM Tris-Cl, pH 8.0, and incubated overnight at 4°C. After one wash with TBS-0.05%Tween 20(v/v), pH 7.6 and TBS, pH 7.6, 50µl of column flow-through as well as each eluted fraction collected from the Heparin Sepharose column, was added into the coated wells of the 96-well plate. Additionally 50µl of 50mM Tris-Cl pH 8.0, 0.25mM biotin X-cadaverine, 10mM DTT, 20mM CaCl$_2$ was added into each well. The reaction was allowed to proceed for 1 hour at 37°C. The plate was then washed once with TBS-0.05%Tween 20(v/v), pH 7.6 and TBS, pH 7.6 before being blocked with 100µl of 3% (w/v) BSA in TBS, pH 7.6 for 30 minutes at 37°C. After another wash, biotin X-cadaverine incorporation into N,N'-dimethylcasein was detected by incubation for 1 hour at 37°C with 100µl extravidin-peroxidase (Sigma-Aldrich, UK) diluted 1:2000 in 3% (w/v) BSA in TBS, pH 7.6. After another set of washes, TG2 activity was measured using Sigma Fast OPD, tablets dissolved in 20ml of distilled H$_2$O. The colour was developed by adding 2.5M $\text{H}_2\text{SO}_4$ and the absorbance at 490nm measured using a microplate reader ELx808™.

2.2.7.2 Measurement of TG2 activity by Biotin X-cadaverine incorporation into fibronectin

This TG2-activity assay is based on measuring the incorporation of biotin X-cadaverine into fibronectin as described by Jones and his collaborators (Jones et. al., 1997). Briefly, wells of a 96-well TCP plate were coated with 5 µg/ml of human plasma fibronectin and
incubated overnight at 4°C. After 30 min blocking with 3% BSA (w/v) in Tris-Cl, pH 7.4, the total volume of 100 µl of each TG2 construct resuspended in the Tris-based buffer containing 5mM CaCl$_2$, 10mM DTT, 0.132mM biotin-x-cadaverine was added to the plate and TG2 immobilisation was allowed to proceed for 2 hours at 37°C. To monitor the reaction, two different controls containing guinea pig liver TG2 used at 10ng/well were used in the assay. To confirm the influence of calcium on the activity of the enzyme, 5mM CaCl$_2$ was replaced with 5mM EDTA. In order to stop the reaction, the wells were washed three times with 2mM EDTA in PBS pH 7.4. 0.1% (w/v) sodium deoxycholate in PBS pH 7.4 containing 2mM EDTA was then added to the plates which were then gently agitated for 10 minutes at room temperature. Following three washes with 50mM Tris-Cl, pH 7.4 the incorporated biotin X-cadaverine was detected by incubation for 1 hour at 37°C with 100µl extravidin-peroxidase (Sigma-Aldrich, UK) diluted 1:5000 in 3% (w/v) BSA in TBS, pH 7.4. After another set of washes, the TG2 activity was analysed using Sigma Fast OPD, tablets dissolved in 20ml of distilled H$_2$0. The colour development was stopped by addition of 2.5M H$_2$SO$_4$ and the absorbance was measured at 490nm using a microplate reader ELx808™.

2.2.8 Peptide synthesis

2.2.8.1 Peptides synthesis and characterisation

The possible heparin sulfate and FN binding site peptides corresponding to human TG2 were synthesised by Peptide Synthetics, UK. The purity (>95%) and composition of the peptides were confirmed by high performance liquid chromatography. The peptides included possible heparin binding site: NPKFLNAGRDCSRRSS (P1); NPKFLKNA (P2); GRDCSRRSS (P3) and scrambled peptide FNRADLKPRCGSSKNKSR (P1s). The possible FN binding site on the N-terminal end of TG2: AEELVLERCDLELE (P4) and scrambled peptide EECRLAELLEDVL (P4s). Additionally as a positive control for P4 peptide WTATVVDQDCTLSSLQLT (P5) peptide was synthesized as stated above and used as described by Hang et al., (2005).
2.2.9 Cell adhesion assay:

2.2.9.1 Fibronectin (FN) coating

The tissue culture plates (TCP) were coated with 50µl of 5µg/ml fibronectin (FN) diluted in 50mM Tris-Cl, pH 7.4. Coated plates were then incubated overnight at 4°C.

2.2.9.2 Immobilisation of TG2 on FN matrices

The stock of purified guinea pig liver TG2 (Zedira, Germany) was prepared at concentrations of 1mg/ml. Following one wash with 50mM Tris-Cl, pH 7.4 the enzyme was immobilised on the FN coated plates. The 20µg/ml of guinea pig liver TG2 was prepared by diluting it in PBS containing 2mM EDTA, pH 7.4, to suppress the Ca²⁺-dependent cross-linking activity of TG2. The immobilisation of TG2 proceeded for 1 hour at 37°C.

2.2.9.3 RGD-mediated cell adhesion

Exponentially growing cells in a supplemented growth medium (DMEM), were trypsinised. After centrifugation at 300g for 5 minutes, the supernatant was discarded and the obtained pellet was suspended in a serum free medium in order to remove any remaining serum proteins. Following two washes, 2.5x10⁵ cells/ml cell suspensions were incubated for 20-25 minutes at 37°C in a 5% CO₂, 95% (v/v) air atmosphere, with two synthetic peptides, named as follow: GRGDTP and GRADSP (Bachem, UK). The concentration of peptides was prepared at: 50µg/ml (~75µM), 100µg/ml (~150µM), 150µg/ml (~225µM), 200µg/ml (~300µM) and 250µg/ml (~375µM). Meanwhile the FN coated plate, with and without immobilized guinea pig TG2, was washed once with Tris-Cl, pH 7.4 and serum free medium. A further 100µl of cells were seeded into each well of the TCP plate and allowed to attach in the presence of peptides during incubation at 37°C. The cell adhesion assay was limited to 20-50 minutes to minimize the secretion of any endogenous proteins.
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2.2.9.4 Cell fixation and permeabilisation

Following cell attachment, medium was removed from the TCP wells and plates were washed once with PBS, pH 7.4. The remaining cells were fixed by using 100µl/well of 3.7% (w/v) paraformaldehyde prepared in PBS, pH 7.4 for 15 minutes at room temperature. To remove the fixative, wells of the plate were washed twice with PBS, pH 7.4 and then incubated with 100µl of 0.1% (v/v) Triton in PBS, pH 7.4 for 15 minutes at room temperature. The wells were then washed once with PBS, pH 7.4.

2.2.9.5 May - Grunwald -Giemsa staining

To assign the amount of attached cells, a two-step staining method was used. Briefly, this method is based on using two different dyes, one May Grunwald dye (Sigma-Aldrich, UK) stains cytoplasm while the other one Giemsa (Sigma-Aldrich, UK) stains nucleus. Following fixation and permeabilisation, 100µl of May - Grunwald stain was added into each well of TCP plate and left for 15 minutes at room temperature. When the cytoplasm was stained, the dye was removed and the plate was once washed with PBS, pH 7.4. Then the TCP wells were incubated with 100µl of 5% (v/v) in dH₂O Giemsa stain for 20 minutes at room temperature. Afterwards the stain was removed and the plate was washed once with dH₂O and left to dry.

2.2.9.6 Quantitative determination of adherent cells

To quantify the amount of attached versus spread cells on a fibronectin coated tissue culture plate, the sum of three images of non overlapping fields from the centre of the well was calculated. Each image of cells was captured by a digital camera (Nikon) at 20x magnification and further analysed using Scion Image program written at the National Institutes of Health. The amount of cells from the centre of the well per image was assessed through threshold and particle analysis settings. Spread cells were characterised by a round and flattened shape, which results from the rearrangement in the actin skeleton. Spread cells were distinguished from attached (non-spread) cells by their two-colour staining (pink cytoplasm, purple nucleus). They were quantified by density slicing,
particle analysis settings and highlighted by adopting the right Lut intensity. In contrary the amount of attached cells was determined by summing up three images from three non overlapping fields. As a control, cells not-treated with any peptide were used. Similarly, the average mean of those attached cells was calculated using non overlapping images from three different wells and used as the 100% attached cells. The mean number of attached cells (cell attachment) was determined as the percentage of cell attachment on FN. The mean percentage of spread cells (cell spreading) on FN was examined separately and expressed as 100%. The mean percentage of cell attachment and cell spreading on a FN coated plate containing immobilised guinea pig liver TG2 was then normalised against and compared to the mean percentage of cell attachment and spreading on a control FN coated plated.

2.2.10 Localisation of mutants and wild type TG2 at the cell surface and their deposition into the ECM

2.2.10.1 Biotinylation of cell surface proteins

After 48 hours post-transfection of HEK293/T17 and NIH3T3 cells, cell monolayers were rinsed three times with ice-cold PBS pH 8.0 and labelled 20 minutes on ice with 0.8mM sulfo-NHS-LC-biotin (Sigma-Aldrich, UK) dissolved in PBS pH 8.0. To stop the reaction cells were washed with 50mM Tris-HCl, pH8.0 and then lysed for 30 minutes on ice with 1% SDS (w/v) in PBS pH 8.0. Following protein determination lysates were denaturated in boiling water and clarified by 20 min centrifugation at 14 000xg at room temperature. Afterwards cleared supernatants containing 200μg protein aliquots were incubated overnight at 4°C with NeutrAvidin-Agarose resins (Thermo-Fisher Scientific, UK). The next day proteins bound to NeutrAvidin-agarose were washed three times with PBS pH 8.0 and further dissolved in 2x Laemmli loading buffer (Sigma-Aldrich, UK). Following denaturation proteins were separated by 8% (w/v) gel electrophoresis and analyzed by Western blot analysis by using anti-TG2 monoclonal antibody.
2.2.10.2 Changes in ECM-deposited TG2 by Western blotting analysis

To determine the distribution of wild type TG2 and TGase mutants (FN, GTP1, GTP2, HS1, HS2, N-, C-) in the extracellular matrix (ECM), NIH 3T3 cells were transiently transfected as described in section (2.2.3.6) using 6 well plates. For extracellular matrix deposition by NIH 3T3 cells transfected with wild type TG2 as well as TGase constructs, the incubation time for cell growth was prolonged for 72 hours. Additionally to slow down the speed of cell proliferation after 24 hours post-transfection, 10% (v/v) foetal bovine serum present in a supplemented culture medium was replaced with 1% (v/v) foetal bovine serum. After 72 hours incubation at 37°C in a 5% CO₂, 95% (v/v) air atmosphere, culture medium was removed and attached cells were washed three times with ice cold PBS, pH 7.4. In order to detach cells, the bottom of each well was covered with 2mMEDTA in PBS, pH 7.4 and the incubation was allowed to proceed for 30 minutes at 37°C. Detached cells were collected into eppendorf tubes leaving ECM laid down by transfected cells on the bottom of the wells. Following two washes with 2mMEDTA in PBS, pH 7.4, any remaining soluble fractions present on the ECM, were dissolved using 0.1% (w/v) deoxycholate in PBS, pH 7.4 for 5 minutes at room temperature. The wells of the plate were washed with 0.1% (w/v) deoxycholate in PBS pH 7.4 followed by PBS, pH 7.4. Afterwards insoluble ECM fractions were dissolved in 2x Laemmli loading buffer (125mM Tris-Cl, pH 6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 10% (v/v) 2-β-mercaptoethanol and 0.004% bromophenol blue), collected into eppendorf tubes and denatured in boiling water for 5 min. The samples containing the ECM insoluble proteins were then resolved by SDS-Page electrophoresis and transferred onto the nitrocellulose membrane by Western blotting (section 2.2.5). Following overnight incubation at 4°C with TG2 monoclonal antibody (CUB 7402) diluted 1:1000 in a blocking solution the membrane was treated with peroxidase conjugate monoclonal anti-mouse IgG antibody and the presence of extracellular matrix bound TG2 detected by enhanced chemiluminescence system as described in section (2.2.5.1).

2.2.11 Statistical analysis

Results shown are the mean +/- SD. The statistically significant differences between obtained data sets in cell adhesion assays were determined by Mann-Whitney test at 95%
level of significance. When \( p < 0.05 \), the differences between data points were considered to be statistically significant and presented with the *, and ◊ symbols. The statistically significant absorbance and net area values were analysed by Student’s t test and presented as significant when the \( p < 0.05 \) (*, ◊, ◎), and \( p < 0.001 \) (•) using the Excel statistical analysis programme.
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3.1 INTRODUCTION

To date, tissue transglutaminase (TG2) is the most studied and the best characterised enzyme within the whole transglutaminase family. The enzyme is expressed by a variety of cells and tissues and plays a crucial role in a variety of intra as well as extracellular biological phenomena that take place in mammalian organisms. Apart from its transglutaminase activity, TG2 also has GTPase, ATPase, protein disulphide isomerase and serine/threonine kinase activities (Griffin et al., 2002; Lee et al., 1989; Hasegawa et al., 2003; Mishra and Murthy, 2004) although not all of these have been fully characterised. In relation to subcellular localisation, TG2 is found mainly in the cytosol and also in the nucleus and mitochondria. Additionally, a moderate but significant amount of the enzyme is associated with the cell surface from where it is deposited into the extracellular matrix. Being mainly a cytosolic protein, where the Ca$^{2+}$ concentration is low and GTP concentration high, TG2 transamidating activity remains latent (Monsonego et al., 1998; Liu et al., 2002). Analysis of the structure of TG2 shows that it consists of 4 domains (Lesort et al., 2000). Domain 2 contains the active site which consists of the catalytic triad Cys277, His335 and Asp358. The active site is inaccessible to substrate in the GTP-bound conformation by the juxtaposition of domains 3 and 4. In the presence of calcium, conformational changes within TG2 cause domains 3 and 4 to fold away from domain 2, exposing the active site (Griffin et al., 2002; Bergamini, 2007). When GTP is bound, TG2 is locked in a closed conformation, reducing its affinity for calcium ions and blocking its cross-linking activity (Achyutan and Greenenberg, 1987; Smethurst and Griffin, 1996; di Venere et al., 2000). There have been many attempts to localise the GTP binding site of TG2. Initial studies by Takeuchi et al., (1992) proposed the existence of three possible GTP-nucleotide binding sites located at amino acid residues 46-69, 345-367, and 520-544 of guinea pig liver transglutaminase. Further investigations using rabbit liver transglutaminase demonstrated much higher affinity of a 36 kDa fragment for [alpha-32P] GTP compared to the 80 kDa fragment, suggesting the importance of the N-terminal domain of TG2 in nucleotide binding (Singh et al., 1995). Also the N-terminus of recombinant human TG2, in particular amino acid residues 1-185 were later reported to be important for GTP hydrolysis (Lai et al., 1996). Molecular modeling studies based on a
sequence homology with FXIIIA revealed, however, that neither the N-terminal nor C-terminal domains of TG2 contain nucleotide hydrolysis sites, but are rather localised within the catalytic core domain (amino acid reissued 139-185) distinct from the catalytic triad (Ismaa et al., 1997). Despite those suggestions, one of the C-terminal domains, barrel 1 with 517-523 residues was found to be further implicated in stabilisation of GTP binding (Monsonego et al., 1997). These findings were later confirmed by Ismaa et al., (2000) using TG2/FXIIIA chimeras and TG2 single point mutants all localised within the catalytic core and further mapped to amino residues 159-173, with peptide YVLTTQGFIYQGSVK reported to be important for GTP binding to TG2. In addition, x-ray crystallography of human TG2 revealed that the GTP-binding site is placed in a hydrophobic pocket between domain 2 and domain 3, and is composed of mostly domain 3 amino acid residues 476-482, 580-583 as well as Phe174 which is located on domain 2 (Liu et al., 2002). Other studies have shown that TG2 is the only exception within the transglutaminase family that can bind guanine nucleotides to control its cross-linking activity (Liu et al., 2002, Figure 4). This is in agreement with additional investigators who confirmed the involvement of GTP in regulating the activity of the enzyme inside the cell (Begg et al., 2006a).

Multiple factors can regulate the activity of tissue transglutaminase leading it to the extracellular matrix and cell surface (Lorand and Graham, 2003; Verderio and Griffin, 2005). Several factors have been suggested to be directly or indirectly involved in TG2 translocation (Gaudry et al., 1999; Balklava et al., 2002; Akimov et al., 2000; Scarpellini et al., 2009). However, the exact mechanism by which the enzyme is actually secreted remains unclear. So far, the diversity of proteins translocation mechanisms across plasma membranes have been investigated and reported (Zocchi and Rubartelli, 2001; Nickel et al., 2005; Nickel, 2007). However, there are limited data regarding the mechanism of TG2 externalisation, the subsequent fate of its externalisation as well as possible involvement of any of TG2 high affinity binding partners in that process.

It is known that released TG2 accumulates in the extracellular matrix where it interacts with fibronectin (Gaudry et al., 1999a). Following immunofluorescence experiments on WI-38 human lung fibroblasts, TG2 was shown to co-localise with FN in the pericellular matrix. This binding was found to be inhibited after cell treatment with anti-fibronectin.
antibodies (Upchurch et al., 1987). As suggested by Lorand et al., (1988) FN might work as a specific carrier for TG2, possibly also playing a role in preventing unnecessary cross-linking of plasma membrane proteins by the enzyme. Moreover, TG2 was found to bind plasma FN independently of the presence of calcium indicating the involvement of a domain other than the catalytic core in its complexation with FN (Turner and Lorand, 1989). Affinity binding experiments based on using three different proteolytic fragments of FN (21 kDa, 30 kDa, and 42 kDa) revealed the 42 kDa fragment to contain the TG2 binding site (Radek et al., 1993). The interaction of FN with erythrocyte and liver TG2 was also examined by electron microscopy and identified the binding site for TG2 within 5-10 nm of the N-terminal site of FN (LeMosey et al., 1992). The TG2-fibronectin interaction was further suggested to be dependent on the intact NH₂-terminal region of TG2 (Jeong et al., 1995; Gaudry et al., 1999a). Deeper insight into the FN binding motifs indicated that the FN binding site is contained within a 28 kDa proteolytic fragment of TG2, with the first 7 amino acids considered to be of great importance for this interaction (Jeong et al., 1995). In addition, as shown by Gaudry et al., (1999a) the first 7 amino acids are also important for the localisation of the enzyme at the cell surface of transiently-transfected COS-27 cells which takes place in association with FN. More recently, site-directed mutagenesis studies based on using TG2/FXIia chimeric constructs and TG2 deletion products demonstrated the presence of an additional FN recognition motif on a TG2 molecule (Hang et al., 2005). This novel FN binding site was localised to amino acid residues 88-106, which is mainly hairpin structure consisting of anti-parallel β strands 5 and 6 among which Asp⁹⁴ and Asp⁹⁷ were shown to be critical for TG2-FN interaction (Hang et al., 2005).

The interaction of TG2 with fibronectin was suggested to play an important role in the localisation of the enzyme at the cell surface and also be involved in its translocation. In addition, it was also postulated to be very important for its adhesive functions (Akimov et al., 2000; Belkin et al., 2001; Zemskov et al., 2006). Additionally, integrins were suggested to play a collaborative role to TG2 in the process of cell adhesion rather than compete with enzyme (reviewed by Zemskov et al., 2006). Furthermore a TG2-integrin interaction was postulated to have an influence on externalisation of the enzyme (Akimov et al.,
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2000; Johnson et al., 2008). As stated by Akimov et al., (2000) the C-terminal domain of the enzyme was suggested to bear the integrin binding motifs.

In addition to integrins, other molecules were proposed to associate with TG2 at the cell surface and further be involved in its translocation across the cell membrane (Verderio et al., 2009). Heparan sulfate proteoglycans (HSPGs) are known to function as cell surface co-receptors for ECM and growth factors and can display ligands which attract their high affinity partners. These interactions are mainly mediated through their glycosaminoglycan chains (Bernfield et al., 1999). Over the past years, the influence of both glycosaminoglycans heparan sulfate (HS) and heparin on cell behaviour has been demonstrated (reviewed by Bernfield et al., 1999). Additionally heparin has been considered to be synonymous with HS since both molecules contain negatively charged linear polysaccharides. Furthermore they undergo similar biosynthetic routes which however results in their sequence diversity (reviewed by Esko and Lindahl, 2001). Additionally when it comes to the localisation, heparin can be found in vivo in the granules of mast cells while HS is present mainly on the cell surface where it covalently binds different core proteins which form HSPGs (Bernfield et al., 1999). Even thought they have been shown to differ in the degree of sulfation, higher sulfation of liver HS (Lyon et al., 1994) the isolation of HS resembling heparin from ologodendrocyte type-2 progenitor cells indicates that those two molecules might be related in this respect (Stringer et al., 1999; Powell et al., 2004).

The heparin-TG2 interaction has been suggested to be high affinity; however the localisation of heparan sulfation binding motifs on TG2 still remains unexplored. The known heparin-binding sites of other proteins seem to share certain common features that have been used to identify the heparin-binding sites of uncharacterised proteins. For instance, many linear HSPG-binding motifs are composed of a particular organisation of basic amino acid residues, such as XBBXBX or XBBBBXXBX, where B is a basic amino acid whose side chain is exposed on the protein surface and X is a neutral or hydrophobic amino acid whose side chain is directed towards the protein interior (Cardin and Weintraub, 1989). Other motifs are composed of a 3D organisation of basic amino acid residues that are not necessarily adjacent to each other in the primary sequence. Consequently, a combination
of both sequence and structure analysis is required for the identification of novel HSPG binding motifs.

In order to understand whether any TG2 high affinity binding partners influence TG2 localisation at the cell surface or into the extracellular matrix, mutagenesis studies were undertaken. The main regions of TG2 targeted for mutagenesis included the suggested FN binding sites, the GTP binding site, as well as proposed integrin binding motifs. Additionally it was of most importance to identify the HSPG-binding site on TG2 using a combination of molecular modelling and site-directed mutagenesis.
3.2 METHODS

3.2.1 Analysis of transfection efficiency by fluorescence microscopy

To determine the transfection efficiency, HEK 293/T17 and NIH 3T3 cells were transiently transfected with 2μg/ml and 4μg/ml of empty vector pcDNA3.1/CT-GFP, respectively. 48 hours after transfection, images were obtained using a Zeiss Axiovert 200M fluorescence microscope equipped with laser adjusted at 507 nm. Transfection efficiency was evaluated using the Scion image analysis program (www.scioncorp.com) which was developed at the National Institute of Health (Washington DC, USA).

3.2.2 Insertion of wt TG2 cDNA into pcDNA3.1/CT-GFP vector

Plasmid vector pGene/V5-His, containing the TG2 cDNA (kind gift from Alexey Belkin, University of Maryland, US), was used as the source of TG2 cDNA. The TG2 coding region was amplified by PCR (Materials and Methods 2.2.1.3) using primers 5’GGTACCATGGCCGAGGAGCTGGTC3’ and 5’GCGGCCGCTTAGGCGGGAAATGATGAC3’ which were designed with KpnI and NotI restriction sites to facilitate directional cloning into plasmid pcDNA3.1/CT-GFP. The PCR product was cloned using Perfectly Blunt Cloning kit (Novagen) according to the manufacturer’s protocol. Plasmid pSTBlue1 containing the cloned PCR product was digested (see 2.2.1.4) with KpnI and NotI enzymes to liberate the TG2 cDNA, which was purified and subcloned into KpnI and NotI digested plasmid pcDNA3.1/CT-GFP. Recombinant clones were identified on the basis of their increased size, confirmed by digestion with KpnI and NotI, and their inserts nucleotide sequenced. pcDNA3.1/CT-GFP:TG2 plasmid DNA was then purified with Endotoxin free plasmid maxi kit (Qiagen, UK) to isolate DNA suitable for transfection.
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3.2.3 Generation of TG2 mutants

QuickChange site directed mutagenesis kit (Stratagene, UK) was used to create point mutations in TG2. Briefly, complementary sense and antisense oligonucleotide primers were designed for each mutation. Using plasmid pcDNA3.1/CT-GFP: TG2 as template, PCR was performed according to the manufacturer’s protocol to generate mutant plasmid DNA. The methylation dependent endonuclease DpnI was used to selectively digest parent plasmid DNA and mutated plasmid DNA transformed into XL1 supercompetent cells. Plasmid DNA was purified from colonies and analysed by restriction digestion analysis where appropriate (Materials and Methods 2.2.2.1 A). Additionally, nucleotide sequencing analysis was performed on all mutated sequences of the enzyme.

3.2.4 Generation of TG2 deletion products

Both TG2 deletion mutants (Δ1-15TG2 and Δ594-687) were generated by PCR amplification using appropriate primers (N-terminal mutant - 5’ GGACGTTACCATGA CCAATGCGCGAGACCACCAC 3’ and 5’ GGACGCGGAAGCTTAGGCGGG CCAATGATGAC 3’; C-terminal mutant - 5’ GGACGTTACCATGCCGCGAGGAGCTGGTCTTAGAG 3’ and 5’ GGACGCCGCTCACCAGCATTGCTTCTTGG 3’). As detailed in Materials and Methods (2.2.2.1 B) when amplified, obtained plasmids DNA were transformed into the DHα5 competent cells (Invitrogen, UK) and further purified, and digested with NotI and KpnI enzymes in order to confirm desired deletion. Additionally each single deletion mutant was sent for sequencing to verify its DNA sequence.
3.2.5 Characterisation of the mutants

3.2.5.1 Analysis of wild type TG2 and TG2 mutant’s expression

To verify the expression level of recombinant TG2, plasmid DNA was transiently transfected into the human embryonic kidney cells (HEK 293/T17) as well as mouse NIH 3T3 fibroblasts. Cell extracts (lysed as in Materials and Methods 2.2.3.6) were analysed by SDS-PAGE electrophoresis and verified for the presence of TG2 antigen by Western blotting as described in Materials and Methods (2.2.5).

3.2.5.2 Solid phase fibronectin binding assay (ELISA)

For the determination of TG2 binding to immobilised fibronectin, a modification of the method of Achyuthan et al. (1995) was used as described in Methods section (2.2.6.2). Bound TG2 protein was expressed as absorbance at 490 nm per mg of total protein measured by Lowry method (Lowry et al., 1951).

3.2.5.3 Guanosine 5’-triphosphate-Agarose (GTP-Agarose) affinity chromatography

All chromatographic steps were performed using an Äkta Purifier (GE Healthcare) Cell lysates (150 µl) were mixed with 450µl of 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 10mM MgCl₂, pH 7.5 and the mixture was then applied to a 2ml bed volume GTP-Agarose column (Sigma-Aldrich, UK) equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 2mM MgCl₂, pH 7.5 at a flow rate of 1 ml/min at room temperature. After washing with 20ml of 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 10mM MgCl₂, pH 7.5, bound protein was eluted from the column with the same buffer containing 5mM GTP, collecting 0.3ml fractions into a microtitre plate using a Frac-950 fraction collector (GE Healthcare). Fractions were
analysed by SDS-PAGE and Western blotting using CUB 7402 as detailed in Materials and Methods (2.2.4 and 2.2.5).

3.2.5.4 Heparin Sepharose affinity chromatography

All chromatographic steps were performed using an Äkta Purifier (GE Healthcare) Cell lysates (150 µl) were mixed with 450 µl of 50mM Tris-Cl, 1mM EDTA, 1mM DTT, pH 7.5 and applied to a 5ml bed volume Heparin Sepharose column (HiTrap™ Heparin HP, Amersham Biosciences, Sweden) equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer at a flow rate of 1 ml/min at room temperature. The column was then washed with 25ml of the same buffer before elution with a linear gradient of 0-1M NaCl in 50mM Tris-Cl, 1mM EDTA, and 1mM DTT, pH 7.5, collecting 0.3 ml fractions into a microtitre plate using a Frac-950 fraction collector (GE Healthcare). Fractions were analysed for TG2 activity (as described in Materials and Methods Section 2.2.7.1) and also by SDS-PAGE and Western blotting using CUB 7402.

3.2.6 Influence of GTP and TG2 inhibitor on TG2-Heparin interaction

Cell lysates were incubated for 1 hour at room temperature in 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 0.5mM GTP, 2mM MgCl₂ pH 7.5 to ensure that all TG2 was present in the GTP-bound closed conformation. Alternatively, cell lysates were incubated with 0.5mM TG2 inhibitor in 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer for 30 minutes on ice to ensure that all TG2 was converted to the inactive inhibitor-reacted open form. Cell lysates were then subjected to heparin affinity chromatography as described in 3.3.14.
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3.2.7 Measurement of TG2 activity

3.2.7.1 Biotin X-cadaverine incorporation into N,N'-dimethylcasein

The transamidating activity of the enzyme was verified by Biotin X-cadaverine incorporation into N,N'-dimethylcasein as described in Methods section (2.2.7.1).
3.3 RESULTS

3.3.1 Mutagenesis studies

TG2 cDNA was amplified by PCR using primers which contained flanking \textit{KpnI} and \textit{NotI} sites to facilitate directional cloning, and was cloned into pSTBlue-1 vector (Merck Chemical Limited, UK). After confirmation of successful cloning, the TG2 cDNA was excised from pSTBlue-1 by \textit{KpnI} and \textit{NotI} digestion and subcloned between the \textit{KpnI} and \textit{NotI} sites of pcDNA3.1/CT-GFP vector (Fig 3.3.1). An additional advantage of using the pcDNA3.1/CT-GFP vector is that GFP expression will allow simple detection of transfected cells by fluorescent microscopy.

3.3.2 Subcloning of TG2 cDNA into pSTBlue-1 and pcDNA3.1/CT-GFP

Plasmid DNA was digested with \textit{KpnI} and \textit{NotI} to ensure that the TG2 insert was the correct size. As can be seen in Fig 3.3.2A, digestion of plasmid pSTBlue-1:TG2 liberated a fragment of 2.3 kb in agreement with that calculated from the nucleotide sequence. The 2.3 kb insert was further subcloned into pcDNA3.1/CT-GFP (Fig 3.3.2 B).

3.3.3 Expression of wild type TG2 in human embryonic HEK293/T17 cells

To examine the expression level of TG2 from pcDNA3.1/CT-GFP: TG2, plasmid DNA was transiently transfected into the human embryonic kidney cells (HEK293T). These cells were chosen because they express negligible levels of endogenous TG2. To obtain a high efficiency of transfection, the plasmid DNA was purified using endotoxin free GenElute Endotoxin-Free Maxiprep kit according to the manufacturer’s protocol.
Figure 3.3.1 Schematic showing the map of pcDNA3.1/CT-GFP expression vector (Invitrogen, UK).
Figure 3.3.2 A, B Electrophoretic analysis of psTBlue-1:TG2 and pcDNA3.1/CT-GFP:TG2 digested with NotI and KpnI. A The upper band represents psTBlue-1 vector (3.95kbp) while the lower band corresponds to inserts size (around 2.3kbp). B The upper band represents pcDNA3.1 vector (6.1kbp) and lower correspond to inserts size (around 2.3kbp).
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The plasmid quality was verified on agarose gel, and the concentration was calculated using Genowa spectrophotometer. HEK cells were transiently transfected by a calcium phosphate-based method on cells grown to 80-90% confluence. The use of GFP as a marker of transfection allowed direct observation of transfection efficiency which was estimated at around 60-70% after 48 hours post-transfection (Fig. 3.3.3B). After transient transfection of HEK293/T17 cells with 2 μg/ml of plasmid DNA encoding wt TG2 and 48 hours incubation at 37°C the expression of the protein was assessed using SDS-PAGE followed by Western blot analysis (Materials and Methods 2.2.4 and 2.2.5). As shown in Fig. 3.3.3C, the wild type TG2 showed band of the expected molecular weight (78kDa) when compared to negative controls including empty vector transfected and untransfected cells.

3.3.4 Expression of wild type TG2 in NIH3T3 mouse fibroblasts

Similarly to HEK293/T17 cells, NIH3T3 cells were characterised by undetectable levels of endogenous TG2 expression, thus being a good model for investigating the expression of the enzyme. NIH3T3 cells were transiently transfected by electroporation using Nucleofector® (Lonza) kit R as described in Materials and Methods (2.2.3.5). To optimize the transfection efficiency, cells were initially transfected with pcDNA3.1/GFP only using a range of amounts of plasmid DNA (2-5μg) and transfected cells counted by fluorescent microscopy (results not shown). As shown in Fig. 3.3.4B, optimum transfection efficiency was achieved using 4μg of plasmid after 48 hours post-transfection. Using these conditions, TG2 plasmids were transfected into NIH3T3 cells. After 48 hours incubation at 37°C, protein expression was analysed by SDS-PAGE and TG2 antigen determined by Western blot analysis using monoclonal anti-TG2 antibody (CUB 7402) as described in Materials and Methods section (2.2.4 and 2.2.5). All transfected plasmids resulted in an expressed TG2 band at the expected size. Cells transfected with empty vector and untransfected cell controls showed no detectable TG2 (Fig. 3.3.4C)
Figure 3.3.3 A, B  Fluorescent microscopy analysis of expression efficiency of HEK 293/T17 cells after transient transfection with pcDNA3.1/GFP vector. Transiently transfected cells were analysed by fluorescent microscopy using Zeiss Axiovert 200M microscope equipped with laser adjusts at 507 nm. A Untransfected cells showed no signal strength while B transfected with empty vector cells showed around 60% transfection efficiency.
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Figure 3.3.3 C Western blotting to show expression level of wild type TG2 in HEK293/T17 cells 48-hours post-transfection. HEK293/T17 cells were transiently transfected with 2μg of plasmid DNA encoding wild type TG2. Transient transfection was allowed to proceed for 48 hours. Afterwards cells were lysed and the protein content was determined by Lowry method (Lowry et.al., 1951). As a result 50μg of the protein extracts (whole cell lysates) collected from both untransfected, empty vector transfected, and wild-type transfected cells were analysed by SDS-PAGE as described in Materials and Method section (2.2.4). The presence of TG2 antigen was detected using anti-TG2 (CUB7402) antibody followed by peroxidase-conjugated secondary antibody as described in Materials and Methods (2.2.5).
Figure 3.3.4 A, B Analysis of transfection efficiency of NIH3T3 cells transfected with pcDNA3.1/CT-GFP by fluorescent microscopy. A Untransfected cells revealed no signal in comparison to B transfected cells where transfection efficiency was estimated at around 50%.
Figure 3.3.4 C Western blotting showing the expression of wild type TG2 in NIH3T3 cells 48 hours post-transfection. NIH3T3 cells were transiently transfected with 4μg of plasmid DNA encoding wild type TG2 using Nucleofector kit R (Lonza). 48 hours after transfection, cells were lysed and the protein content was determined by Lowry method (Lowry et.al., 1951). 50μg of the protein extracts (whole cell lysates) collected from untransfected cells, pcDNA3.1/CT-GFP transfected control and pcDNA3.1/CT-GFP: TG2 transfected cells were resolved by SDS-PAGE as described in Materials and Methods section (2.2.4). The presence of TG2 antigen was detected by Western blotting using anti-TG2 (CUB7402) antibody followed by goat anti-mouse IgG HRP-conjugated secondary antibody as described in Materials and Methods (2.2.5). The membrane was stripped and further reprobed with anti-mouse α-tubulin antibody to normalise protein loading.
3.3.5 Identification of the sites on TG2 responsible for binding high affinity partners

In order to find out whether any of the TG2 binding partners affected its intra or extracellular localisation, the goal was to generate mutants which were show abolished or at least reduced binding strength to specific TG2 partners. As shown in Fig. 3.3.5I and Fig. 3.3.5II, targeted TG2 amino acid sequences included: Asp\(^94\) and Asp\(^97\) (residues claimed by Hang et al., 2005 to be crucial for TG2-FN interaction), N-terminal β-sandwich TG2 domain (which was indicated by Gaudry et al., (1999) to as well play an important role in TG2-FN interaction), possible heparan sulfate binding site residues (assuming that TG2 can interact with HSPGs receptors when released from cells to the cell surface as stated by Verderio et al., 2009) as well as Ser\(^171\) and Lys\(^173\), the main residues responsible for TG2-GTP interaction when present in an intracellular environment (Ismaa et al., 2000). Additionally a TG2 region that was suggested to be involved in TG2-integrin interaction (Akimov et al., 2000) and might contain the possible heparan sulfate binding motifs, the C-terminal domain, was targeted for deletion.

3.3.6 Identification of putative heparin-binding motifs in TG2

Examination of the primary amino acid sequence of TG2 for the linear consensus GAG binding motifs XBBXBX and XBBBXXBX, where B is a basic amino acid whose side chain is exposed on the protein surface and X is a neutral or hydrophobic amino acid whose side chain is directed towards the protein interior (Cardin & Weintraub, 1989), reveals one such sequence \(^{261}LRRWKN\) close to the active site of TG2. This sequence has already been suggested as a possible HSPG binding site for TG2 (Verderio et al. 2009). However, examination of the crystal structure of TG2 (1KV3) shows that \(^{261}LRRWKN\) is part of an alpha helix, whereas the XBBXBX consensus must be in a β-sheet in order for the basic residues to face the same direction. A common structural theme of linear GAG binding motifs is that there are two basic residues approximately 20Å apart to accommodate a pentasaccharide, facing in opposite directions on an alpha helix (Margalit et al., 1993).
Figure 3.3.5I Schematic showing organisation of structural domains of tissue transglutaminase with indication of amino acid sequences or regions that were targeted for mutagenesis to generate different TG2 mutants. Illustrated mutations include: deletion of the first 15 amino acids from the first NH$_2$-terminal domain of TG2 (Δ1-15TG2), FN1 mutant (D94A, D97A), GTP1 mutant (S171E), GTP2 mutant (K173L), HS2 (K205A, R209A) and HS1 (K600A, R601A, K602A) mutants and deletion of the last C-terminal domain of TG2 (Δ594-687TG2).
Figure 3.3.5II A

Figure 3.3.5II Illustration of all the TG2 mutants generated for the purpose of investigation the interaction of the enzyme with his high affinity binding partners including fibronectin, HSPGs, integrins as well as GTP. A FN1 mutant (D94A, D97A), B deletion of the first 15 amino acids from the first NH$_2$-terminal domain of TG2 (Δ1-15TG2) C HS1 (K600A, R601A, K602A) and HS2 (K205A, R209A) mutants D GTP1 (S171E) and GTP2 (K173L) mutants E deletion of the last C-terminal domain of TG2 (Δ594-687TG2).
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Figure 3.3.5II B, C
Figure 3.3.5ii D, E

D

GTP1 (S171E)

GTP2 (K173L)

E

C-terminal deletion
Since $^{261}$LRRWKN$^{266}$ is too short to satisfy this requirement either, it is unlikely to be able to bind GAGs and so was not investigated. It has been suggested that the three dimensional arrangement of basic amino acid residues is more important than linear clustering, such that many GAG binding motifs can also be comprised of sequence-distant basic amino acid residues (Hileman et al., 1998). Examination of the crystal structure of TG2 revealed two likely candidate motifs, $^{590}$KIRILGEPQKRK$^{602}$ (HS1) which is located at the tip of C-terminal beta barrel 2 and another comprised of $^{202}$KFLKNAGRDCRRSPVYVGR$^{222}$, with K387 (HS2), forming a shallow pocket lined with basic residues (Fig. 3.3.6).

### 3.3.7 Generation of TG2 point mutants

**Plasmid pcDNA3.1/CT-GFP**: TG2 vector was used as a template to generate the TG2 mutants. All the point mutations were generated using Quickchange mutagenesis kit (Stratagene) as described in section (2.2.2). Primers were designed to also include silent mutations to either introduce or delete a restriction site, allowing simple assessment of successful mutagenesis by restriction analysis. The resulting plasmids were digested with the appropriate restriction enzymes and compared to the wild type. Possible heparan sulfate binding site mutant HS1 showed two linear bands (~ 8.9kbp, ~ 6.8kbp) in comparison to the wild type digested with the same (NotI) enzyme (Fig., 3.3.7A). In addition HS2 mutant showed three different bands (~ 4.2kbp, ~ 2.1kbp, ~ 1.9kbp) when digested with Eagl enzyme in comparison to the control showing four linear bands on the gel. FN1 mutant when digested with BstXI enzyme showed two linear bands as well (~ 8.3kbp, ~ 4.9kbp) when compared to the wild type. After digestion of plasmid DNA carrying GTP1 mutation with Ppil, four different linear bands (~ 2.9kbp, ~ 2.7kbp, ~ 1.3kbp, and 870bp) were seen on an agarose gel which differed when compared to plasmid carrying wt TG2 digested with the same restriction enzyme. Similarly, GTP2 mutant showed 10 linear bands on agarose gel (~ 2.8kbp, ~ 1.8kbp, ~ 1.5kbp, ~ 680bp, ~ 550bp, ~ 450bp, ~ 530bp, ~ 210bp, ~ 75bp, ~ 34bp) in comparison to 8 bands which appeared on a gel after digesting the wild type control with Earl (Fig 3.3.7B).
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Figure 3.3.6 A, B Illustration of 3D structure analysis of possible heparan sulfate binding site motifs present on TG2 molecule.
Figure 3.3.7 A Electrophoretic analysis of restriction digested wt TG2, HS1 mutant (K600A, R601A, K602A), FN mutant (D94A, D97A) and HS2 mutant (K205A, R209A) plasmids. Each of the mutants was generated using Quick change site directed mutagenesis kit from Stratagene by means of specially designed primers (section 2.2.2.1 A) using pcDNA3.1/CT-GFP: TG2 vector as a DNA template. Obtained plasmids DNA were run on 0.8% agarose gel using DNA log ladder (1-10kbp) (Lane 1 and Lane 7).
Figure 3.3.7 B Electrophoretic analysis of restriction digested wt TG2, GTP1 mutant (S171E) and GTP2 mutant (K173L). Each of the mutants was generated using Quick change site directed mutagenesis kit from Stratagene by means of specially designed primers (section 2.2.2.1 A) using pcDNA3.1/CT-GFP: TG2 vector as a DNA template. Obtained plasmids DNA were run on 0.8% agarose gel using DNA log ladder (1-10kbp) or DNA log ladder (0.1bp-1.5kbp).
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All mutant plasmids were subjected to nucleotide sequencing to confirm mutations and integrity of the TG2 sequence.

3.3.8 Generation of TG2 deletion mutants

Both deletion mutants including deletion of the first 15 amino acids from the NH$_2$-β sandwich TG2 domain (Δ1-15TG2) and deletion of the last domain of the enzyme (Δ594-687TG2) were generated by PCR amplification using pcDNA3.1/CT-GFP: TG2 as template and oligonucleotide primers with flanking KpnI and NotI restriction sites to allow directional cloning of products into pcDNA3.1/CT-GFP. Resulting plasmids were purified and digested with NotI and KpnI enzymes and compared to the wild type (Fig. 3.3.8). As expected there was a noticeable difference in the size of the bands corresponding to the TG2 insert when compared to the wild type. For Δ1-15 TG2 this was 2.2kbp and for Δ394-687 it was 2kbp. Plasmid DNA was verified by nucleotide sequence analysis.

3.3.9 Expression of wild type TG2 and TG2 mutants in HEK293/T17 cells

Mutant TG2 enzymes for binding of fibronectin FN1 (D94A, D97A) and N-terminal deletion (Δ1-15TG2), HSPG binding mutants HS1 (K600A, R601A, K602A) and HS2 (K205A, R209A), GTP binding mutants GTP1 (S171E) and GTP2 (K173L) as well as C-terminal deletion (Δ594-687TG2) were transfected into HEK293T cells to assess expression levels. All mutants showed bands of the expected molecular weight (Fig. 3.3.9A) however differed in expression levels (Fig. 3.3.9B). GTP-defective binding site mutant (GTP1) and N-terminal deletion were shown to be expressed less in comparison to the wild type protein, while HSPGs mutants as well as FN1, GTP2 and C-terminal deletion mutant were expressed at a much higher or similar level by cells, respectively.
Figure 3.3.8 Electrophoretic analysis of *Not*I and *Kpn*I digested wt TG2 and TG2 deletion products (Δ1-15 TG2 and Δ594-687 TG2). Each of these mutants was generated by PCR by means of specially designed primers (section 2.2.2.1 B) using pcDNA3.1/CT-GFP: TG2 as template. Inserts were subcloned into pcDNA3.1/CT-GFP and resulting plasmid were digested with *Kpn*I and *Not*I and run on 0.8% agarose gel using DNA log ladder (1-10kbp).
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3.3.10 Expression of wild type TG2 and TG2 mutants in NIH3T3 cells

Mutant plasmids were transfected into NIH3T3 cells to assess the expression level. 48 hours after transfection, cells were lysed and 50 μg of protein from each cell extract were analysed by SDS-PAGE and Western blotting. All mutants showed expressed TG2 proteins of the expected molecular weight (Fig. 3.3.10A) with untransfected and empty vector controls showing no bands. Some of the mutants showed additional bands at 50kDa, possibly due to proteolytic degradation. Differences in expression level were revealed after densitometry analysis (Fig. 3.3.10B). GTP1 mutant (S171E), and TG2 deletion mutant (Δ 1-15 TG2) were shown to be expressed less in comparison to wild type TG2, whereas HS1 (K600A, R601A, K602A), HS2 (K205A, R209A), FN1 (D94A, D97A), GTP2 (K173L) mutants and C-terminal deletion (Δ594-687) were expressed at a higher or similar level.

3.3.11 GTP-agarose affinity chromatography of GTP1 and GTP2 mutants

The TG2 constructs containing GTP-defective binding site mutants were transiently transfected into the HEK293/T17 cells as detailed in Materials and Methods (2.2.3.5). In order to assess impairment in GTP binding by these mutants (Ismaa et al., 2000), cell extracts were subjected to GTP-agarose affinity chromatography (Methods 3.2.5.3). As a positive control, cell lysate containing the wild type protein was also analysed. Fractions collected from the column were subjected to SDS-PAGE and Western blotted with anti-TG2 antibody (CUB7402) for the presence of TG2 antigen. As shown in Fig. 3.3.11, both GTP1 and GTP2 mutants were found in a flow-through after failing to bind to the GTP-agarose whereas wt TG2 bound to the column and was only eluted in the presence of competing GTP (5mM).
Figure 3.3.9 Expression level of wt TG2 and TG2 mutants in HEK293/T17 cells. A HEK293/T17 cells were transiently transfected with 2μg of plasmid DNA encoding the wild type enzyme and TG2 mutants and incubated for 48 hours at 37°C. Transfected cells were lysed, protein content was determined using Lowry method (section 2.2.3.7) and 50μg of protein was resolved by SDS-PAGE and the presence of TG2 antigen determined by Western blotting as described in Materials and Methods (2.2.5). B Relative expression levels of TG2 proteins normalised to tubulin after densitometric analysis using ImageJ software.
Figure 3.3.10 Expression level of wt TG2 and TG2 mutants in NIH3T3 cells. A NIH3T3 cells were transiently transfected with 4μg of plasmid DNA encoding the wild type enzyme and TG2 mutants and incubated for 48 hours at 37°C. Transfected cells were lysed, protein content was determined using Lowry method (section 2.2.3.7) and 50μg of protein was resolved by SDS-PAGE and the presence of TG2 antigen determined by Western blotting as described in Materials and Methods (2.2.5). B Relative expression levels of TG2 proteins normalised to tubulin after densitometric analysis using ImageJ software.
Figure 3.3.11 Western blot analysis of GTP-agarose affinity chromatography fractions for GTP1, GTP2 and TG2. HEK293/T17 cells were transiently transfected using 2μg of each plasmid DNA. After 48 hours, transfected cells were lysed using a non-denaturating method as described in Materials and Methods (2.2.6.1 A). The resulting cell extracts were mixed with 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 10mM MgCl2, pH 7.5 and then to the column equilibrated in the same buffer. After washing with buffer, proteins were eluted with the same buffer containing 5mM GTP. The flow-through (1-8), wash (9-12) and eluted fractions (13-25) were analysed by SDS-PAGE electrophoresis followed by Western blotting as described under Materials and Methods (2.2.4 and 2.2.5).
3.3.12 The NH₂-β sandwich of the TG2 first domain is crucial for binding to fibronectin

The TG2-FN interaction has been stated to be important in regulating many processes that take place in mammals. TG2 binds FN via its 28kDa proteolytic fragment which contains the first and a big part of the second TG2 domain (Jeong et al., 1995). Moreover the N-terminal domain and residues Asp⁹⁴ and Asp⁹⁷ were suggested to be crucial (Gaudry et al., 1999; Hang et al., 2005). Early studies revealed that a mutant deficient in the first seven N-terminal amino acid residues abolished binding to fibronectin. However, this mutant was also shown to be unstable (Jeong et al., 1995). Therefore to verify whether the first domain plays an important role in TG2-FN interaction, we produced a TG2 deletion mutant deficient in the first 15 amino acids (Δ 1-15TG2), which has been demonstrated to be expressed and stable (Hang et al., 2005). In addition, knowing that Asp⁹⁴ and Asp⁹⁷ are also essential for FN binding, we have mutated them by substituting alanine in a place of aspartic acid to generate the FN1 mutant (D94A, D97A). The NΔ1-15 and FN1 mutants were assessed for their ability to bind to fibronectin in a solid phase binding assay utilising purified FN as stated in Methods (2.2.6.2) and then normalised to the relative expression level of mutant TG2 in the cell lysates as described under Materials and Methods (2.2.5.1). Both of the mutants showed a significant loss of binding ability to the FN when compared to the wild type (Fig. 3.3.12). However the binding strength differed depending on the mutation. The D94A, D97A mutant described previously (Hang et al., 2005) demonstrated approximately 45% binding compared to wild type TG2, whereas the Δ1-15 deletion mutant had approximately 30% binding compared to wild type.

3.3.13 The high affinity heparin binding site of TG2 is located in the catalytic core domain

It is known that TG2 binds to heparin (an analogue of heparan sulfate) with high affinity, a property that has been exploited for its purification (Signorini et al., 1988).
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Figure 3.3.12 Binding of mutant FN1 (D94A and D97A) and N-terminal deletion mutant (Δ 1-15 TG2) to fibronectin. A Fibronectin binding was assayed by ELISA using microtitre plates coated with 5 μg/ml fibronectin. 50-60 μg of proteins from cell extracts were added to each well and bound TG2 was determined using monoclonal anti-TG2 antibody (CUB7402) followed by HRP-conjugated secondary antibody (2.2.5). The reaction was determined spectrophotometrically at 490nm wavelength. B The expression of total TG2 in cell lysates was evaluated by Western blotting and compared to the equal loading marker α-tubulin by densitometry analysis. The binding strength to FN of wild type and mutant TG2 was then normalised to the relative expression levels of wild type and mutant TG2 in the cell lysates, respectively after densitometric analysis of blots.
To study the interaction between TG2 and HSPGs and to test whether any of the identified possible heparan sulfate binding sites shows impairment in the binding affinity to the heparan sulfate, the wild type as well as mutants HS1 (K600A, R601A, K602A) and HS2 (K205A, R209A) were tested for their ability to bind to a heparin Sepharose column. All constructs were transfected into HEK293/T17 cells and when ready were lysed using a non-denaturating lysis buffer to avoid denaturation (Materials and Methods 2.2.6.1 A). The binding strength of TG2 and mutants HS1 and HS2 to heparin Sepharose was determined by elution with an increasing salt gradient (Figure 3.3.13 A). All fractions (flow-through, wash and elution) that were collected from the column were verified for the presence of TG2 antigen by immunoblotting with anti-TG2 antibody as detailed in Materials and Methods (2.2.4 and 2.2.5). As shown in Fig. 3.3.13 B, TG2 was eluted at two distinct salt concentrations 100 mM and 330 mM, suggesting that two different populations of enzyme were present. To exclude the possibility that the observed low affinity binding was due to denatured TG2, the transglutaminase activity of eluted fractions was determined and this showed that specific activity did not differ between the high and low affinity binding fractions (Fig. 3.3.13 C).

Mutant HS1 bound heparin identically to TG2, with low and high affinity, whereas HS2 lost the capability to bind with high affinity to heparin (Fig. 3.3.13 B). However, HS2 retained its low affinity binding to heparin (60mM), suggesting that residues K205 and R209 contributed to high affinity binding to heparin. Since both mutants HS1 and HS2 resulted in the same net charge reduction, this alteration in HS2 binding was not simply due to electrostatic interaction.

HS1 mutant showed high affinity binding to heparin so it was not necessary to determine its activity. As shown in Fig. 3.3.13 D, HS2 fractions eluted with low salt concentration showed activity suggesting that the loss of binding was not due to denaturation or a gross alteration in structure. Also, activity was also noticed in flow-through fractions indicating that some of the mutant remained unbound.
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Figure 3.3.13 A Graph showing TG2-Heparin interaction. Green line is % of elution buffer (1M NaCl in 50mMTris-Cl, 1mMEDTA, 1mMDTT, pH 7.5), brown line is % conductivity, blue line is absorbance at 280nm.
Figure 3.3.13 B Analysis of possible heparan sulfate binding site mutants, HS1 (K600A, R6001A, K602A) and HS2 (K205A, R209A) binding ability to heparin. Cell extracts obtained after non-denaturating lysis of transiently transfected HEK293/T17 cells, were diluted with 50mM Tris-Cl, 1mM EDTA, 1mM DTT, pH 7.5 (Methods 3.2.5.4) and applied to the heparin Sepharose column. Proteins were eluted with a linear gradient of 0-1M NaCl in 50mM Tris-Cl, 1mM EDTA, 1mM DTT, pH 7.5. Both flow-through and eluted fractions (1-25 as shown in a results) were analysed by SDS-PAGE and Western blotting using anti-TG2 monoclonal antibody (CUB 7402) for the presence of TG2 antigen.
Figure 3.3.13 C, D Measurement of transglutaminase activity in eluted fractions from the heparin Sepharose column. Fractions were mixed with 0.25mM Biotin X-cadaverine, 10mMDTT, 20mM CaCl₂ in 50mM Tris-Cl pH 8.0 and then incubated on a N,N’-dimethylcasein coated TCP plate. After washing with TBS-0.05%Tween 20(v/v), pH 7.6 and TBS, pH 7.6, plates were blocked with 3% (w/v) BSA in TBS, pH 7.6 and then Biotin X-cadaverine incorporation was measured with extravidin-peroxidase and analysed spectrophotometrically at 490nm (Materials and Methods 2.2.7.1).
3.3.14 High affinity heparin binding is dependent on TG2 conformation

Since TG2 can adopt two extremes of conformation in the presence or absence of GTP, the effect of GTP binding on the association of TG2 with heparin was investigated (Fig. 3.3.14B). In order to do so, cell extracts obtained from transfected cells were pre-incubated either with 0.5mM TG2 inhibitor and then loaded on the heparin Sepharose column and compared to the wild type enzyme. Purified fractions were later collected and the amount of bound TG2 was estimated by Western blot analysis as described in Materials and Method (2.2.4 and 2.2.5). Eluted wt TG2 was again showed to bind to the column with two different affinities (Fig. 3.3.14 A). In the presence of GTP, which results in a compact globular conformation, all of the TG2 bound with high affinity to heparin (Fig. 3.3.14 B), whereas after reaction with the irreversible peptidic inhibitor R281 (Griffin et al., 2009), which restricts the conformation to an extended form, the TG2 bound to heparin with an intermediate affinity (Fig. 3.3.14 C). Since the GTP-bound globular form of TG2 bound to heparin with high affinity whilst the extended R281-bound form still retained an affinity higher than that of HS2, this suggests that the loss of high affinity binding of HS2 is not solely due to an altered conformation. Therefore, residues K205 and R209 in TG2 are very likely to be directly involved in high affinity heparin binding. As shown in Fig. 3.3.14 D, E, F, the activity of TG2 (and similarly HS2) is inhibited by GTP.
Figure 3.3.14 Effect of TG conformation on binding to heparin Sepharose. A Cell extracts (~ 150µl) from HEK293/T17 cells transfected with wtTG2 were loaded onto a 5ml heparin Sepharose column, equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mM DTT pH 7.5 buffer at room temperature. B Cell extracts were pre-incubated with 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 0.5mM GTP, 2mM MgCl₂ pH 7.5 or C with 50mM Tris-Cl, 1mM EDTA, 1mM DTT, 0.5mM TG2 inhibitor R281, pH 7.5. Both flow-through and resulting fractions (1-25 as shown in a results) were assayed for TG2 activity and analysed by SDS-PAGE and Western blotting using anti-TG2 monoclonal antibody (CUB 7402) for the presence of TG2 antigen as stated in Materials and Methods (2.2.4 and 2.2.5).
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A

TG2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

wt TG2

B

TG2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

wt TG2 + GTP

C

TG2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

wt TG2 + R281

Flow-Through    Salt    Eluted fractions with salt concentration
**Figure 3.3.14 D, E, F Measurement of TG2 activity in fractions eluted from the heparin Sepharose column.** Collected fractions were mixed with 50mM Tris-Cl pH 8.0, 0.25mM Biotin X-cadaverine, 10mMDTT, 20mMCaCl₂ and then incubated on a fibronectin coated plate as described in Experimental procedure (Materials and Methods 2.2.7.1). After washing with TBS-0.05% (v/v) Tween 20, pH 7.6 and TBS, pH 7.6, plates were blocked with 3% (w/v) BSA in TBS, pH 7.6 and then biotin X-cadaverine incorporation was measured with extravidin-peroxidase and analysed spectrophotometrically at 490nm.
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![Graph D](image)

TG2 – activity of fractions collected from the Heparin Sepharose column

![Graph E](image)

TG2+GTP – activity of fractions collected from the Heparin Sepharose column

![Graph F](image)

TG2 +R281 – activity of fractions collected from the Heparin Sepharose column
3.3.15 Affinity to heparin differs depending on TG2 mutation

It was also decided to investigate how other TG2 mutant enzymes, including TG2 active site mutant (C277S), FN1 (D94A, D97A), N- and C-terminal deletion mutants (Δ1-15 TG2, Δ594-687 TG2, respectively) affected binding to heparin. The active site mutant (C277S) is known to lack TG2 transamidating activity as well as force the enzyme into its extended conformation (Lee et al., 1993; Begg et al., 2006b). As stated before, the irreversible peptidic inhibitor R281 (Griffin et al., 2009) also stabilizes the enzyme in an extended form. As shown in Fig. 3.3.15 B, C277S mutant bound to heparin with low affinity in a similar manner to TG2 treated with R281 inhibitor (Fig. 3.3.14 C) suggesting that the TG2-heparin interaction is dependent on the enzyme conformation. Additionally, N-terminal mutant Δ1-15 was shown to bind to the heparin with low affinity (Fig. 3.3.15 D), similarly to C277S. The FN1 mutant, however, bound to heparin in a manner comparable to the wild type enzyme. The C-terminal deletion mutant was eluted in a continuous pattern indicating some heparin binding was still present but much weaker than that of the wild type enzyme (Fig. 3.3.15 C and E).

3.3.16 Binding of GTP mutants to heparin

To investigate how GTP binding to the enzyme affects its interaction with heparin, two GTP-defective binding site mutants GTP1 (S171E) and GTP2 (K173L) were applied on the Heparin column. It is known that when GTP binds to the enzyme it blocks it from binding Ca$^{2+}$ and alters the enzyme conformation into the closed form. The mutant S171E was reported (Begg et al., 2006a) to be locked into the open conformation whilst the influence of mutation K173L on TG2 conformation is unknown. To test interaction between TG2 construct and Heparin, cell extracts obtained after transfection of HEK293/T17 cells were applied on the Heparin column as detailed in Materials and Method (2.2.6.1.B).
Figure 3.3.15 Analysis of wt TG2 and TG2 mutants FN1 (D94A, D97A), N - terminal deletion (Δ1-15TG2) and C – terminal deletion (Δ594-687TG2) binding ability to heparin.

Transiently transfected HEK 293/T17 cells were lysed in a non-denaturating lysis buffer and dissolved in an equilibrating buffer (Materials and Methods 2.2.6.1.A) before being applied on the Heparin Sepharose column. Desired proteins were eluted with 1mM NaCl in 50mM Tris-Cl, 1mM EDTA, and 1mM DTT, pH 7.5. Both flow-through and resulting fractions (1-25 as shown in a results) were verified by SDS-PAGE electrophoresis and western blotting using anti-TG2 monoclonal antibody (CUB 7402) for the presence of TG2 antigen.
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A

TG2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

B

C277S 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

C

FN1 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

D

N 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

E

C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Flow-Through Salt Eluted fractions with ↑ salt concentration
Further purified fractions (1-25 fractions) were resolved by SDS-PAGE electrophoresis and the presence of TG2 antigen was determined using anti-TG2 antibody (CUB7402) by Western blotting (2.2.4 and 2.2.5). As shown in Fig. 3.3.16 A, the wild type enzyme revealed the same binding pattern as previously described (Fig. 3.3.15A). Similarly GTP2 mutant was eluted with lower and higher salt concentration suggesting that it binds Heparin with two different affinities (Fig. 3.3.16 C). GTP1 mutant (S171E) binds to the column with low and high affinity, but when eluted retains continuous pattern indicating the weaker affinity to Heparin in comparison to the wild type TG2 (Fig. 3.3.16 B). However this binding pattern show differences when compared to the active site mutant which similarly to GTP1 alters TG2 conformation into an open form.
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Figure 3.3.16 A, B, C Analysis of wt TG2 and GTP-binding defective site mutants, GTP1 (S171E) and GTP2 (K173L) binding ability to heparin. Transiently transfected HEK 293/T17 cells were lysed in a non-denaturating lysis buffer and dissolved in the equilibrating buffer (Methods 3.2.5.4) before being applied on the Heparin Sepharose column. Desired proteins were eluted with 1mM NaCl in 50mM Tris-Cl, 1mM EDTA, and 1mM DTT, pH 7.5. Both flow-through and resulting fractions (1-25 as shown in a results) were verified by SDS-PAGE electrophoresis and Western blotting using anti-TG2 monoclonal antibody (CUB 7402) for the presence of TG2 antigen.
3.4 DISCUSSION

The importance of maintaining homeostasis in mammalian organisms is crucial for their proper functioning. There is strong evidence suggesting that one of the enzymes that belong to the transglutaminase family (TG2) is essential for efficient homeostasis during wound repair processes (Telci and Griffin, 2006; Verderio et al., 2005). Although TG2 has been characterised as a wound healing mediator and its engagement in the healing process and many other processes including cell-cell and cell-matrix interactions was widely described, its secretion mechanism remains unknown. Available evidence suggests that TG2 secretion increases when cells and tissues are exposed to stress and proceeds through an unconventional pathway (Upchurch et al., 1991; Haroon et al., 1999; Johnson et al., 1999; Gaudry et al., 1999; Balklava et al., 2002; reviewed by Zemskov et al., 2006). Nevertheless, the exact fate of TG2 after secretion, involvement of its high affinity binding partners and effect of its conformational state when secreted is still not fully understood.

It is known that TG2 is widely distributed in a variety of mammalian cells as well as tissues that differ in its expression levels (Thomazy and Fesus, 1989; Zemskov et al., 2006). There are cells that show high expression of TG2 (endothelial cells and macrophages), which sometimes remains on a constitutive level (endothelium) or is up-regulated by specific stimuli such as inflammatory cytokines (INF-γ in rat intestinal cells and IL-6 in human Hepatoblastoma cells) as well as steroids, vitamin D, and growth factors including TGF-β1 by possibly TGF-β1/BMP4 response element in TG2 gene (Ritter and Davbies, 1998; Kim et al., 2002; Verderio et al., 2005;Zemskov et al., 2006, ). To determine the expression levels of TG2 and the mutants and their effects on cellular localisation, two different cell lines were used as a model. Human kidney embryonic (HEK293/T17) were chosen as the initial cell model. The motivation for this approach stems from the fact that these cells are known to contain undetectable levels of TG2 therefore being a sensitive model for detection of transfected protein and are easily transfected. However in order to extend these investigations, a cell model using mouse fibroblast line (NIH3T3 cells) was applied. The reason for using this particular cell line arises from the fact that similarly to HEK293/T17 cells, these cells are characterised by negligible amounts of endogenous TG2.
To be able to understand how TG2 is externalised from different cells, and which factors lead to its localisation at the plasma membrane, a series of TG2 mutants were prepared. TG2 lacks a typical signal peptide thus being secreted through non-classical pathway. So far it is known that TG2 when externalised accumulates into the ECM where it interacts with FN. However, there is still some uncertainty about which amino acid sequence(s) is/are involved in that interaction. Hang et al., (2005) suggest that the first domain of the enzyme, in particular the sequence (WTATVVDQDCTLSLQLTT) is directly involved in FN binding. It has also been suggested that the N-terminus is required for FN binding (Gaudry et al., 1999a). Therefore these residues were targeted for mutagenesis. Additionally it was of interest to investigate how GTP can affect TG2 localisation and conformation. Thus two main GTP-binding amino acids, S171 and K173, were substituted (Ismaa et al., 2000). Also, the influence of interaction of TG2 with either integrins or HSPGs present at the cell surface is unknown. Consequently possible integrin and HSPG binding sites on TG2 were targeted for mutagenesis. The entire set of mutants was initially transient transfected into HEK293T/T17 cells (human embryonic cells) to establish an experimental system that allows the exploration of the trafficking route as well as the final localisation of the enzyme.

For transfection of HEK 293/T17 the calcium phosphate method was used, whereas for transfection of NIH3T3 cells the Nukleofactor kit R system was applied. Both of these methods proved to be efficient enough without exposing the cells to any significant toxicity. Measurement of TG2 protein in the transfected cells showed that both wild type and mutant TG2 proteins were efficiently expressed. However densitometric analysis of the expression levels showed differences between the different mutants within the same cell line, and also between the two cell lines. It is possible that the mutations affected protein expression or stability, especially in the case of the N-terminal deletion mutant which when transfected caused minor changes in the morphology of both cell types affecting their expression and appeared to be degraded.

Characterisation of the phenotypes of all the mutants was performed to ensure that the mutations had the desired effect. In agreement with previous findings (Hang et al., 2005; Gaudry et al., 1999), we have confirmed that both D94 and D97 and an intact N-terminus are important for TG2 binding to FN. However, we have shown that the N-
terminal 15 amino acids of the enzyme play even more important role in binding (Fig 3.3.12). This suggests that both of these distinct sites are important for TG2 to be able to fully interact with its high binding partner FN. Analysis of the two GTP-binding mutants GTP1 (S171E) and GTP2 (K173L) (Ismaa et al., 2000) confirmed that they lost the ability to bind GTP. After analysis of the TG2 crystal structures and electrostatic surface charges of the wild type protein and based on the available literature two distinct sites (the core domain and C-terminal domain) were chosen for mutagenesis. Thus we generated site-directed mutations in human TG2 of Lys-600, Arg-601 and Lys-602 to Ala (HS1) and Lys-205 and Arg-209 to Ala (HS2) and analyse their capability to bind to heparin. The HS2 mutant was shown to bind to the column with very low affinity compared to either the HS1 mutant or the wild type protein. It was also confirmed that the HS2 mutant was still catalytically active, indicating that its loss of heparin binding was not due to either misfolding or protein denaturation. This result shows that the region around K205 and R209 comprise the major heparan sulfate binding site of TG2.

One of the goals of our investigation was to explore whether conformational changes of TG2 can influence its interaction with heparin. In order to do that, the wild type TG2 was pre-incubated with GTP, which as shown earlier (Smethurst and Griffin, 1996; di Venere et al., 2000) enforces the closed conformation. In addition, TG2 was also reacted with the irreversible TG2 inhibitor R281 (Griffin et al., 2009) which enforces the open conformational state. Incubation with GTP reinforced high affinity binding to heparin whereas incubation with R281 inhibitor abolished strong interaction between TG2 and heparin, with only low affinity binding observed. Since TG activity assays were used to confirm that bound enzyme retained activity, this suggested that the TG2-heparin interaction is conformation dependent, with the closed conformation (GTP-bound) showing the strongest interaction.

With the binding and conformational requirements for the TG2-heparin interaction established, it was important to assess the behaviour of the other TG2 mutants. It is known that the TG2 active site mutant (C277S) not only abolishes activity but also stabilises TG2 in an extended conformation (Lee et. al., 1993, Begg et. al., 2006b). Therefore it was of interest to determine how this mutant bound to heparin. As expected, C277S did not show high affinity binding to heparin, instead binding with an affinity
similar to that of R281 inhibitor-reacted TG2 (also open conformation). N-terminal deletion mutant (Δ1-15TG2) bound to heparin with low affinity, indicating that this particular deletion may also have altered conformation. The FN1 (D94A and D97A) mutant was observed to bind to heparin in a comparable manner to the wild type. Deletion of the last TG2 domain (Δ594-687) resulted in intermediate binding strength to heparin, which suggests that this mutation may result in a conformation somewhere between the open and closed states.

The GTP2 (K173L) GTP-binding mutant bound to heparin similarly to the wild type. However, GTP1 mutant (S171E), which is known to be in an open conformation (Begg et al., 2006a), had intermediate affinity to heparin. This suggests that other local effects may be important as well as gross conformation. As shown by Begg et al., (2006 a, b), either mutation of GTP-binding or Cys277 residues can influence the conformation of the enzyme. S171E, when mutated loses its ability to bind GTP due to a conformational effect on the positioning of residues Lys$^{173}$ and Phe$^{174}$, which, similarly to Ser$^{171}$, are located on the same β-strand (Begg et al., 2006a). Likewise, C277S showed reduced affinity for GTP, by affecting Tyr$^{516}$ (the residue of β-barrel 1’ domain that contains the main GTP binding site) H bond interaction and greatly decreasing the compact form of the enzyme (Begg et al., 2006b). Therefore any disruptions in that interaction could induce the protein transition into the open form in a different manner than the one induced by the GTP1 mutant which might have caused differences in the binding pattern to heparin.
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM
In mammals nine different transglutaminases have been described at the genomic level (Grenard et al., 2001). Although TGase isoenzymes are characterised by the structural homology conserved within four distinct domains when first crystallized they were trapped in a conformational state that obscured the active site (Grenard et al., 2001; Yee et al., 1994; Ahvazi et al., 2004, Liu et al., 2002). TG2 however has now been fully characterised in two distinct (compact and extended) conformations (Liu et al, 2002; Pinkas et al., 2007). These conformational changes of the enzyme often dependent on its intra or extracellular localisation which may be important for its interactions with its binding partners present within the cell compartments or at the cell surface and ECM. When intracellular, TG2 binds GTP which locks it in a compact conformation. In the extracellular environment where the calcium concentration is high TG2 has been believed to be in an open active state. However this may not be the case, because of variations in the extracellular redox potential (Go and Jones 2008). In addition as shown by Verderio et al., (2003) TG2 added exogenously to the standard culture required the presence of DTT for its activation. Further studies also indicated independently that despite the relatively high concentration of calcium in the ECM, TG2 still could be in its closed, GTP-bound form (Pinkas et al., 2007; Johnson and Terkeltaub 2005).

Predominantly TG2 has been considered to be an intracellular protein, however further studies revealed that it can be also found on the plasma membrane and in the ECM (Gaudry et al., 1999a, b; Akimov and Belkin 2001a, b). Despite available data indicating externalisation of TG2 to be independent of the classical, reticulum/Golgi pathway, the mechanism of its secretion still remains unclear. TG2 does not have a signal peptide thus it is very unlikely for it to undergo ER/Golgi dependent N-glycosylation despite bearing glycosylation sites (Folk and Finlayson 1977, Gentile et al., 1991). Therefore TG2 must be translocated through the membrane via a non-classical pathway (Gaudry et al., 1999a, b; Akimov and Belkin 2001 a, b; Verderio et al., 2009; Collighan and Griffin, 2009). Recent studies suggest that TG2 is mainly released from damaged cells, and contributes to the repair of the damaged cell membrane by cross-linking the proteins.
involved in membrane fusion (Kawai et al., 2008). When externalised, TG2 does not simply leak out the cell which was demonstrated using different TG2 mutants (Cys-277S and Tyr-274) that impair distribution of the enzyme into the ECM (Balklava et al., 2002).

Once exported, TG2 was shown to associate with the plasma membrane and to co-localise with ECM proteins which may play an important role in the externalisation of the enzyme. Initial studies showed that for the enzyme to be secreted it requires an intact N-terminal binding site which also appears to be one of the FN binding site motifs on the TG2 molecule (Gaudry et al., 1999a, b). Whereas further investigation also indicated the involvement of β1 integrin in this process for which TG2 has a high affinity (Akimov et al., 2000). Although the integrin binding sites has never been localised within the TG2 domain structure, at first it was suggested to be present at the C-termini (Akimov et al., 2000) and further at the N-terminal site of the enzyme (Hang et al., 2005). In addition the presence of the non-proline cis bond on Tyr-274 was shown to be important for both the activity of the enzyme and its secretion, while the presence of the active Cys-277 site was demonstrated to play a role in the localisation of the enzyme into the matrix (Balklava et al., 2002; Johnson and Terkeltaub 2005). These data strongly suggest that TG2 externalisation is mediated via conformational changes which may be moderated by Tyr-274 and Cys-277 residues (Johnson and Terkeltaub, 2005; Collighan and Griffin, 2009).

When released from cells, TG2 has been shown to be localised in the ECM where it actively contributes to reconstitution and/or stabilisation of ECM structures. The extracellular matrix is a very complex mesh, which surrounds and supports cells by providing them with a scaffold for adhesion, growth, migration, differentiation and many other aspects that are critical for cell functions. The most prominent components of the ECM are collagen and elastin which provide strength and flexibility to the matrix, fibronectin which plays mainly an adhesive or integral function within the matrix, numerous proteoglycans and heparan sulfates which are important for stabilising its structure. Additionally ECM consists of different bound molecules including growth factors and cytokines, metalloproteinases (MMPs), and enzymes of which TG2 is one of special interest. Initial studies by Upchurch et al., (1991) on TG2 localisation within the ECM revealed that TG2 can be found in the ECM within the wound area where it extensively cross-links the surrounding environment. Its cross-linking activity has been
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further implicated in the matrix resistance to proteolytic degradation (Johnson et al., 1999). There are many extracellular proteins that TG2 has affinity for; however one of them has been shown to be particularly attractive for the enzyme. The tight interaction within TG2 and FN has been reported by many (Gaudry et al., 1999a, b; Hang et al., 2005) who have made attempts to characterise the FN binding site on TG2. This site has been mapped within the N-terminal TG2 domain, with peptide $^{88}$WTATVVDQQDCTL$^{106}$ reported to directly mimic this strong TG2-FN interaction (Hang et al., 2005). The close association between cell surface TG2 and FN has been shown to be important for the assembly of FN into the ECM, which however cannot take place without the presence of $\alpha 5\beta 1$ integrin (Akimov et al., 2001a, and b). The assembly of FN must be properly controlled and the activity of the enzyme was shown to be unnecessary for this process. However once externalised TG2 was suggested to be associated with the assembly of fibronectin fibrils (Akimov et al., 2001a, b) and further implicated in not only modification but also in initial assembly of the ECM (Collighan and Griffin, 2009).

During cellular stress, TG2 is exported to the ECM, binds FN, and in that heterocomplex is deposited into the wound area (Jonhson et al., 1999; Verderio et al., 2004). However, once deposited, it can also interact with heparan sulfate proteoglycans chains that are located at the cell surface (Verderio et al., 2003). This process has implications in the adhesiveness functions of the enzyme which will be detailed in chapter 5. The affinity between TG2 and HS (heparan sulfate), which are known ECM components, has already been suggested (Verderio et al., 2003; Telci et al., 2008). Predominantly one of the HS proteoglycans receptors localised at the cell surface of osteoblasts and fibroblasts (syndecan-4[S4]) has been indicated as a TG2 binding partner that together with the enzyme plays an important role in the cell adhesion process (Verderio et al., 2003, Telci et al., 2008; Scarpellini et al., 2007). Although S4 has been suggested to be the main HSPGs that TG2 binds to, the interactions between enzyme and other family members cannot be disqualified. Studies by Zehe et al. (2006) revealed a possible involvement of cell surface HSPGs in the trafficking and localisation of FGF-2 at the plasma membrane. FGF-2 is another molecule that similar to TG2 was suggested to be secreted via an unconventional pathway from cells (Shäffer et al., 2003; Beckhaus et al., 2003). Thus it is of importance to
establish if HSPGs can by some means influence the localisation of the TG2 at the cell surface as well as its deposition into the ECM.

The initial objective of this chapter is to gain a deeper insight into the influence of how specific mutations of TG2 can affect the association of the enzyme with the cell surface and to clarify the importance of the association of TG2 with its extracellular binding partners in its translocation into the ECM. A further objective is to establish the physiological importance of the two distinct peptide sequences within the N-terminal domain of the enzyme on its interaction with FN (Gaudry et al., 1999a; Hang et al., 2005).
4.2 METHODS

4.2.1 Biotinylation of cell surface TG2 and TG2 mutants

The association of the wild type enzyme and its constructs with the plasma membrane of HEK293/T17 cells and NIH3T3 cells was verified by the cell surface biotinylation of proteins using 0.8mM sulfo-NHS-LC-biotin (Sigma Aldrich, UK) dissolved in PBS, pH 8.0 (Materials and Methods section 2.2.10.1). Following three washes with 50mM Tris-Cl, pH 8.0 transfected cells were then lysed in 1% SDS (w/v) in PBS pH 8.0 and the amount of protein in each cell extract was estimated as detailed in the Materials and Methods (2.2.3.7). The amount of 200μg of proteins from each lysate was then pre-incubated overnight with 50μl of NeutrAvidin-Agarose resin. Next day to eliminate any possibility of non-specific intracellular protein binding, beads were washed three times with PBS pH 8.0 and further dissolved in 2xLaemmli buffer prior to denaturation. Equally prepared proteins were resolved by SDS-PAGE electrophoresis and the presence of TG2 antigen bound to the cell surface was verified by Western blotting as described in section (2.2.4 and 2.2.5).

4.2.2 Deposition of TG2 and TG2 constructs into the ECM

To verify whether the wild type enzyme and its constructs are distributed into the ECM, transiently transfected NIH3T3 cells (1 x 10^6 cells) were incubated for 72 hours at 37°C which included serum starvation using DMEM supplemented with 1% FBS (v/v) for 48 hours post-transfection as detailed in the general Materials and Methods (2.2.10.2). After discarding the medium, cells were detached with 2mM EDTA in PBS pH 7.4 and collected into the eppendorf tubes. Following two washes, with 2mMEDTA in PBS, pH 7.4 deposited matrix was further incubated with 0.1% (w/v) deoxycholate in PBS, pH 7.4 in order to eliminate all soluble ECM fractions and uncollected cells. Following one more
wash with PBS, pH 7.4, insoluble ECM fractions, were dissolved in 50μl of 2x Laemmli buffer and after denaturating were separated by SDS-PAGE electrophoresis using 8% resolving gels. The presence of TG2 antigen deposited into the ECM was then analysed by Western blotting as described under sections (2.2.4 and 2.2.5).

4.2.3 Detection of TG2 in the culture medium

After 72 hours incubation of NIH3T3 cells transfected with the wild type TG2 and TG2 constructs namely HS2 and the N-terminal mutant, as detailed under Materials and Methods (Section 2.2.3.5) the reaction was stopped. The medium was removed from every single well and checked for the presence of secreted TG2. Collected medium was centrifuged at 300xg to eliminate any dislodged cells. The proteins present in the medium were then precipitated in 300μl ice cold 10% (w/v) TCA by incubating the samples on ice for 30 minutes. Following centrifugation at 13000g for 10 minutes, protein pellets were washed once with ice cold 10% (w/v) TCA, ethanol-acetone (1:1) and acetone and left for 20 minutes at room temperature to dry out. Obtained pellets were then resuspended in 30 μl Laemmli loading buffer (2x concentrated), and then checked for the presence of TG2 antigen by SDS-PAGE electrophoresis and Western blot analysis using anti-TG2 antibody as described in section (2.2.5).

4.2.4 Detection of TG2 expression level in cell extracts

To verify the expression level of wild type TG2 and all TG2 mutants, transfected HEK293/T17 and NIH3T3 cells (See Materials and Methods 2.2.3.5) were lysed, denatured and then equal amounts of protein were resolved by SDS-PAGE electrophoresis and analysed for the presence of TG2 by Western blot as stated in Materials and Methods (2.2.4 and 2.2.5).
4.2.5 Measurement of TG2 activity on the cell surface, Biotin-X-cadaverine incorporation into fibronectin

This TG2-activity assay is based on measuring the incorporation of biotin-X-cadaverine into fibronectin as described by Jones and his collaborators (Jones et al., 1997). Briefly, wells of a 96-well plate were coated with 5 µg/ml of fibronectin in 50mM Tris-Cl, pH 7.4 and incubated overnight at 4°C. Trypsinized cells, transfected as described before in section (2.2.3.5) were resuspended in a serum free medium containing 0.132mM biotin X-cadaverine and 10mM DTT at a concentration of 1 x 10^5 cells/ml. The total volume of 100 µl was added into each well of 96-well plate and incubation was allowed to proceed for 2 hours at 37°C. In order to stop the reaction, the 96 well plate was three times washed with 2mM EDTA in PBS pH 7.4. Further to eliminate remaining cells, 0.1% (w/v) sodium deoxycholate in PBS pH 7.4 containing 2mM EDTA was added into the plate which was then gently agitated for 10 minutes at room temperature. After discarding the supernatant, the remaining fibronectin layer was washed three times with 50mM Tris-HCl, pH 7.4. To eliminate any non-specific binding, 100µl per well of a blocking solution composed of 3% BSA (w/v) in Tris-Cl, pH 7.4 was used. After three washes with 50mM Tris-Cl pH 7.4 buffer as described before, the incorporated biotin X-cadaverine was detected by incubation for 1 hour at 37°C with 100µl extravidin-peroxidase (Sigma-Aldrich, UK) diluted 1:5000 in 3% (w/v) BSA in TBS, pH 7.4. Following another wash, the TG2 activity was analysed using Sigma Fast OPD, tablets dissolved in 20ml of distilled H2O. The colour development was stopped by addition of 2.5M H2SO4 and the absorbance was read at 490nm using an Absorbance microplate reader ELx808™.

4.2.6 Measurement of TG2 activity in the total cell lysate, Biotin-X-cadaverine incorporation into fibronectin

This assay is based on a measuring the incorporation of biotin-X-cadaverine into fibronectin as described by Jones and his collaborators (Jones et al., 1997). Briefly, wells
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of 96-well plate were coated with 5 μg/ml of fibronectin in a 50mM Tris-Cl, pH 7.4 and incubated overnight at 4°C. After three washes with 50mM Tris-Cl, pH 7.4, any possibility of non-specific interactions was eliminated by blocking the 96 wells plate in a blocking solution composed of 3% (w/v) BSA in 50mM Tris-Cl pH 7.4 for 30 minutes at 37°C. Following three washes, 100 µl per well of cell extracts containing 50-60μg of proteins obtained from transfected cells as described before in section (2.2.3.5) were resuspended in 50mM Tris-Cl pH 7.4 buffer containing 5mM CaCl₂, 10mM DTT, 0.132mM biotin X-cadaverine and then added in a triplicate into 96-well plate. The activity of all mutants was compared to the wild type TG2, which under the test conditions was considered to be a positive control. The immobilisation of wild type TG2 and TGase mutant to fibronectin was allowed to proceed for 2 hour at 37°C. In order to stop the reaction, 96 wells plate was three times washed with 2mM EDTA in PBS pH 7.4. Further 0.1% (w/v) sodium deoxycholate in PBS pH 7.4 containing 2mM EDTA was added into the plate which was then gently agitated for 10 minutes at room temperature. After discarding the supernatant, the remaining fibronectin layer was washed three times with 50mM Tris-Cl, pH 7.4. Afterwards the incorporated biotin X-cadaverine was detected by incubation for 1 hour at 37°C with 100µl extravidin-peroxidase (Sigma-Aldrich, UK) diluted 1:5000 in 3% (w/v) BSA in TBS, pH 7.4. Following another washing, the TG2 cross-linking activity was analysed using Sigma Fast OPD, tablets dissolved in 20ml of distilled H₂O. The colour development was stopped by addition of 2.5M H₂SO₄ and the absorbance was read at 490nm using an Absorbance microplate reader ELx808™. Results were expressed as change in absorbance with time per mg of protein.

4.2.7 Measurement of recombinant human TG2 ability to bind to two FN amino terminal fragments as well as full length FN

To detect the saturation point of recombinant human TG2 (rhTG2) when bound to three different lengths of human plasma FN an ELISA was performed. The solid binding phase assay was conducted with a high binding affinity plate (Nunc, Thermo Fisher Scientific, Denmark) coated overnight with the three different fragments of fibronectin:
45kDa of fibronectin (54μg/ml), 70 kDa fibronectin (45μg/ml) and full length fibronectin (5μg/ml) (Sigma-Aldrich, UK) (Verderio et al., 2003) in a 50mM Tris-Cl, pH 7.4. To reduce the possibility of non specific reactions, the wells were blocked as detailed under Materials and Methods section (2.2.6.2). Following washing with PBS, pH 7.4, 100μl/well of the increasing concentration of rhTG2 (1μg/ml, 2.5μg/ml, 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml, 30μg/ml) was added into the wells of coated plates, and immobilisation of the enzyme on FN was allowed to proceed for 1 hour at room temperature. After three washes with PBS pH 7.4 plates were incubated for 2 hours with anti-TG2 monoclonal antibody (CUB 7402) diluted 1:1000 in the same blocking buffer followed by incubation with secondary antibody (See section 2.2.6.2). The colorimetric reaction between the peroxidase conjugated secondary antibody and Sigma Fast OPD substrate was stopped by addition of 2.5M H₂SO₄ and then amount of bound rhTG2 to FN was analysed spectrophotometrically at 490nm.

4.2.8 Competition between human recombinant TG2 and AEELVLERCDLELE (P4) and WTATVVDQQDCTLSLQLTT (P5) peptides for the binding to fibronectin

ELISA was performed in a high binding 96-well nunc microtiter plate. Microtiter plates were coated overnight with 50μl/well of the three different lengths of fibronectin: 45kDa of fibronectin (54μg/ml), 70 kDa fibronectin (45μg/ml) and full length fibronectin (5μg/ml) (Sigma-Aldrich, UK) (Verderio et al., 2003). The wells were then blocked for 30 min at RT with blocking buffer (1% BSA (w/v) in 1xTBS pH 7.6). After two washes with 0.05% Triton X-100 in TBS ph 7.6 and one wash with 1xTBS, 100μl per well of the peptides (P4, P4s and P5) diluted in 2mMEDTA in PBS pH 7.4 were added to the wells at 0.1μmM – 1mM concentrations and incubated for 1 hour at RT. Following three washes, 100μl per well of purified human recombinant TG2 was added to the same wells at a final concentration of 2μg/ml and incubated for 1 hour at room temperature. Bound TG2 was measured by a 2 hour incubation at room temperature with CuB7402 (diluted 1:1000 in blocking buffer) followed by 2 hour incubation at room temperature with peroxidase conjugated goat ant-mouse IgG antibody (diluted 1:1000 in blocking buffer). After three washes, Sigma Fast
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OPD substrate was added into the wells, and after few minutes the amount of bound TG2 was assayed spectrophotometrically at 490nm.
4.3 RESULTS

4.3.1 Detection of the wild type TG2 and TG2 mutants at the HEK293T cell surface

To explore the extracellular localisation of the wild type TG2 as well as TG2 constructs (HS1, HS2, GTP1, GTP2, FN1, N, and C) in transiently transfected HEK293T cells, cell surface proteins were biotinylated using sulfo-NHS-LC-biotin. This particular reagent is characterised by the presence of sulfonate groups and is unable to penetrate the cell membrane. Following labelling, biotinylated cells were cleared by centrifugation, lysed and 200μg of proteins were subjected to incubation with NeutrAvidin Agarose-resins. The proteins present in the whole cell lysate and the resin bound proteins were then analysed by SDS-PAGE and Western blotting for the presence of TG2 antigen.

The wild type enzyme and most of the TGase mutants but excluding the N-terminal deletion (Δ1-15TG2) could be found at the plasma membrane of HEK293T cells when analysed by Western blotting (Fig. 4.3.1). There is a detectable but a very small amount of the wild type enzyme found on the surface which could be due to its weaker expression by the cells when compared to the expression level of the other TG2 mutants (HS1, HS2, GTP2, C). As shown in Fig. 4.3.1 the HS1 mutant (K600A, R601A, K602A) which still bound to heparin (see chapter 3 section 3.3.13) appears to be present at the cell surface of HEK293T cells in a much greater amount in comparison to the HS2 mutant K205A, R209A (the novel heparan sulfate binding site on the TG2 molecule) even though both of these mutant proteins are similarly, expressed by cells. Additionally both of the GTP defective binding site mutants (GTP1 and GTP2) the FN1 mutant which is characterised by reduced affinity to FN and the C-terminal deletion mutant (Δ594-687TG2) are found in a greater amount on the plasma membrane. However when the visible differences in their expression level is taken into account the data suggests the lack of importance of each of these mutated or deleted TG2 regions in the localisation of TG2 at the cell surface.
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**Figure 4.3.1 Detection of cell surface TG2 antigen in HEK293/T17 cells transfected with wild type TG2 and TG2 mutants via biotinylation.** HEK293/T17 cells were transiently transfected with the wild type enzyme and TG2 mutant constructs then cell surface proteins labelled with sulfo-NHS-LC-biotin (Sigma-Aldrich, UK), further lysed with 1% SDS (w/v) in PBS pH 8.0 as described under 2.2.10.1 section in the Materials and Methods chapter. After denaturation in gel loading buffer and discarding of the membrane and cellular debris, by centrifugation, 200μg of proteins calculated per each sample were incubated overnight with NeutrAvidin-Agarose resins (Thermo-Fisher Scientific, UK). In addition to determining the cell surface presence also the expression level of the wt TG2 as well as each of the particular mutant TG proteins present in the whole cell lysate (WCL) was measured. A Proteins were separated by SDS-PAGE electrophoresis using equal protein loadings and the TG2 antigen bound to the resin was detected by Western blotting using anti-TG2 antibody (CUB 7402) followed by the incubation with secondary anti-mouse IgG conjugate as described under Materials and Methods (2.2.4 and 2.2.5). B Densitometry of the TG2 bands representing the expression level of TG2 as well as TG2 mutants in a whole cell lysate were normalised to the equal loading marker α-tubulin and measured using the ImageJ analysis software. Likewise cell surface levels of TG2 and TG2 constructs were normalised to the relative expression levels which was calculated from the densitometric analysis of Western blots.
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Figure 4.3.1

Densitometric analysis of Western blots

- Normalised expression level of wild type and mutant TG2 to tubulin
- Normalised cell surface level of wild type and mutant TG2 to the relative expression level

Figure 4.3.1
The fact that the N-terminal product is not detectable at the cell surface but is present in the whole cell lysate suggests that the first 15 residues of the N-terminus of the enzyme are very important for its distribution to the cell surface (Fig. 4.3.1). Due to negligible amounts of endogenous TG2 in the vector transfected HEK293T cells the enzyme could not be found either at the cell surface or in the cell lysate.

4.3.2 Cellular localisation of the wild type TG2 and TG2 mutants in NIH3T3 cells

To investigate the cell surface distribution of TG2 and TG2 mutants in NIH3T3 cells, transfected cells were labelled with sulfo-NHS-LC-Biotin and isolated by extraction and binding to NeutrAvidin-coated beads as described before in the Materials and Methods (2.2.10.1) and the amount of TG2 antigen was detected using SDS-PAGE followed by Western blotting. Performed analysis showed that the wild type enzyme can be found on the cell surface of NIH3T3 cells in a greater amount when compared to the HS2, GTP1 and N-terminal mutant and smaller amounts when compared to HS1 (K600A, R601A, K602A), GTP2 (K173L), FN1(D94A, D97A) and C-terminal mutant (Δ594-687TG2). Mutants that could be found in greater amounts on the cell membrane when normalised to that found in the cell lysate included the HS1, GTP2, FN1 and C-terminal deletion products (Fig.4.3.2). All these mutants were expressed at the similar level by cells when compared to the wild type as determined by densitometry analysis. Additionally the expression level of the HS2 (K205A, R209A) mutant was similar to the previously described mutations however its distribution level at the cell surface was remarkably smaller strongly suggesting that heparan sulfate proteoglycans are involved in the translocation of the enzyme to the cell surface as proposed by (Verderio et al., 2009; Scarpellini et al., 2009). On the other hand, the N-terminal mutant (Δ1-15TG2) could not be found at the cell surface, which stays in agreement with the previous result (Fig. 4.3.1) however the fact that the GTP1 (S171E) mutant is almost undetectable on the cell surface of NIH3T3 cells, contradicts the result obtained with the HEK293T cells.
Figure 4.3.2 Localisation of wt TG2 and TG2 mutants at the cell surface of NIH 3T3 cells.

The presence of the wild type enzyme and TG2 mutants at the cell surface of fibroblasts was determined via cell surface biotinylation. After 48 hours post-transfection, transfected NIH 3T3 cells were biotinylated with sulfo-NHS-LC-biotin (Sigma-Aldrich, UK) in order to label the cell surface proteins. NIH3T3 cells were then lysed with 1% SDS (w/v) and after centrifugation at 14 000g for 20 min 200μg of proteins were measured from the clarified sample and incubated with NeutrAvidin-Agarose resin (Thermo-Fisher Scientific, UK). A Affinity bound proteins were separated by SDS-PAGE electrophoresis and the amount of TGase in each sample was determined by Western blotting using monoclonal anti-TG2 antibody (CUB7402) followed by the incubation with secondary antibody conjugated with HRP and revealed by ECL detection system as stated in the Materials and Methods (2.2.4 and 2.2.5). B The expression of total TG2 (WCL) in cell lysates was evaluated by Western blotting and compared to the equal loading marker α-tubulin (Sigma-Aldrich, UK). The net area value for TG2 bands were normalised against tubulin after measurement by densitometry analysis using ImageJ analysis software. The cell surface level of wild type and mutant TG2 was then normalised to the relative expression levels of wild type and mutant TG2 in the cell lysates, respectively after densitometric analysis of Western blots.
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM

Densitometric analysis of Western blots

- Normalised expression level of wild type and mutant TG2 to tubulin
- Normalised cell surface level of wild type and mutant TG2 to the relative expression level

Figure 4.3.2
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM

Likewise HEK293T cells as are NIH3T3 cells are characterised by very low levels of endogenous TG2 thus no TG2 band could be found at the cell surface or in the cytosolic fraction of untransfected or transfected only with the empty vector pcDNA3.1/CT-GFP cells.

4.3.3 Effects of TG2 derived mutants on TG2 transamidating activity in cell lysates of transfected HEK293T cells and NIH3T3 cells

To investigate whether the different mutations of TG2 influences its cell surface transamidating activity, two cell types including HEK293T cells and NIH3T3 cells were chosen as cell models. TG2 intracellular activity was measured in a total cell lysate assaying 50-60μg of protein as described under Methods (4.2.6). The activity of all mutants was compared to the wild type TG2, which under the test conditions was used as a positive control. Under these conditions, four of the total number of seven TG2 mutants showed a decrease in the TG2 activity (change in absorbance over time per 50-60μg of cell lysate protein added) in respect to the wild type in both HEK293T and NIH3T3 cell lines. As shown in Figure 4.3.3 A, C both the GTP defective binding site forms (S171E and K173L) and the TG2 deletion product (Δ1-15TG2) demonstrated a significant reduction in the cell lysate activity in comparison to the wild type. This result together with another representing specific activity (TG2 activity per TG2 antigen obtained from Western blots) of the mutants (Fig. 4.3.3 B, D) indicates that GTP as well as the first 15 amino acids of the N-terminal domain of TG2 is important to the transamidating activity of the enzyme. Although the C-terminal deletion mutant (Δ594-687TG2) showed a significant decrease in the cell lysate activity in the HEK293T cells (specific activity low), it did not lead to a significant decrease in the TG2 activity in the cell lysate of NIH3T3 cells (specific activity comparable to wild type TG2) (Fig. 4.3.3 A, B, C, D, respectively). On the other hand, HS1 (K600A, R601A, K602A), HS2 (K205, R209A) and FN1 (D94A, D97A) mutants showed elevated or similar level of activity when compared to the wild type which again was observed to be a cell dependent process. These findings indicate that the HSPGs binding mutations and the FN binding site mutation site are presumably not implicated in the activity of the enzyme.
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Figure 4.3.3 A, B, C, D Activity of wild type TG2 and TG2 mutants in cell lysate of transfected HEK293/T17 cells and NIH3T3 cells. TG2 activity of the wild type enzyme and TG2 constructs expressed by A HEK293/T17 cells and C NIH3T3 fibroblasts was analysed by biotin-X-cadaverine incorporation into the FN. Transfected with TG2 and TGase mutants (HS1, HS2, GTP1, GTP2, FN1, N, and C) cells were lysed as described under Materials and Methods (2.2.3.6) and 50-60μg of proteins from cell extracts (WCL) were resuspended in a 50mM Tris-Cl, pH 7.4 buffer containing additionally 5mM CaCl₂, 10mM DTT, and 0.132mM biotin-X-cadaverine on wells of FN coated plate. Incorporated biotin-X-cadaverine into FN was analysed spectrophotometrically at 490nm using extravidin-peroxidase. Obtained values are shown as means +/-SD from the three independent experiments. The net values of wild type TG2 activity was used as a standard to determine the significant difference (* p < 0.05 and • p < 0.001) between the activity of wild type enzyme and all TG2 mutants. B, D The specific activity of the wt and TG2 mutants was assayed by normalisation of cell lysate activity to the normalised cell lysate antigen using ImageJ analysis software.
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Figure 4.3.3
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM

Figure 4.3.3
4.3.4 Measurement of TG2 derived mutants on TG2 cell surface activity in transfected HEK293T and NIH3T3 cells

Knowing that some of the TG2 mutants affect TG2 activity found in the cell lysate it was of further interest to examine their influence on the extracellular activity of the enzyme using the same epithelial and fibroblast cell models. The activity of cell surface TG2 was measured using FN coated plates as stated under Methods (4.2.5) according to the previously described method (Jones et al., 1997). The first part of the experiment (Fig 4.3.4 A) was performed on HEK293T cells that were transfected with the whole set of mutants together with the wild type TG2 as a reaction control. A significantly lower level of TG2 transamidating activity was noticed at the cell surface of HEK293T cells which expressed GTP defective binding site mutants (GTP1 and GTP2) as well as both N- and C-terminal deletion mutants (Δ1-15 and Δ594-687) (Fig. 4.3.4 A). In addition when the cell surface activity was normalised to that of cell lysate (result not shown), the activity of GTP mutants was found to correlate to the mutant activity in the cytosol (less surface activity because of smaller amount of activity of mutants in the cytosol). This however was not the case for the N-terminal mutant cell surface activity indicating that its reduced cell surface activity may be somehow associated with the smaller amount of protein on cell surface. These findings suggest the first 15 amino acids from the N-terminal domain of TG2 and the GTP binding mutants are affecting TG2 presentation on the cell surface with respect to activity.

The level of cell surface TG2 activity when compared to that found in the cell lysate was however found to be similar in wild type TG2 and HS1 (K600A, R601A, K602A), HS2 (K205A, R209A) as well as FN1 (D94A, D97A) mutants. These results indicate that these mutations are not able to influence extracellular TG2 activity. Likewise NIH3T3 cells transfected with all TG2 mutants (Fig. 4.3.4 B) showed a very similar pattern of TG2 cell surface transamidating activity. The only difference refers to the cell surface activity of the C-terminal mutant which in NIH3T3 cells showed comparable surface activity to wild type TG2, suggesting that the C-terminal domain does not affect the extracellular activity of the enzyme in this cell type.
Figure. 4.3.4 A, B Measurement of wt TG2 as well as TG2 mutant activity on the cell surface by biotin-X-cadaverine incorporation into the FN. A, B Briefly after 48 hours post-transfection transfected with wild type enzyme and TGase mutants (HS1, HS2, GTP1, GTP2, FN1, N, and C) cells (both HEK293/T17 cells and NIH3T3 cells) at a concentration of 1x10^5 cells/ml were resuspended in DMEM only containing 0.132mM biotin-X-cadaverine, 10mMDTT and seeded into the wells of 96-well plate coated with 5μg/ml FN for 2 hours at 37°C. The incorporated biotin-X-cadaverine into FN was determined spectrophotometrically at 490nm after removal of cells using extravidin-peroxidase as described under Methods (4.2.5). TG2 activity is presented as mean values +/- SD of three separate experiments. Net values obtained for wild type TG2 were used as control values in order to assess significantly different TG2 activity (* p < 0.05 and • p < 0.001) of the TG2 mutants.
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Figure 4.3.4
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM

4.3.5 Measurement of the TG2 derived mutants binding affinity to Fibronectin

As previously established (Lorand et al., 1988; LeMosey et al., 1992), TG2 when externalised to the ECM was shown to have a high affinity for FN which as further reported is independent on the transamidating activity of the enzyme. Therefore to estimate the recognition of FN by all TG2 mutants, the ELISA assay was applied. The level of binding affinity was compared between all generated mutants (HS1, HS2, GTP1, GTP2, FN1, N, and C) and the wild type TG2 using the same amount of protein (60μg of protein lysate) obtained from HEK293T cell homogenates as described under general Materials and Methods (2.2.6.2) and then normalised to the relative expression level of mutant TG2 in the cell lysates (result not shown). The ELISA assay shown in Figure 4.3.5 revealed remarkable differences in the TG2 antigen level bound to FN especially in relation to FN1 (D94A, D97A) and N-terminal (Δ1-15TG2) mutants. The statistically significant reduction in binding affinity to FN of both of these mutations was expected since they have already been characterised in Chapter 3 (Fig.3.14) and one of these mutations has been previously described to be important for FN-TG2 interaction (Hang et al., 2005). Likewise, FN1 mutant was shown to abolish around 50% binding while the N-terminal mutant around 70% of TG2 binding to FN indicating once more that both of these TG2 regions are important in its interaction with FN. The C-terminal mutant also showed a reduction in its binding strength to FN; however when compared to the mutant expression level, it was not significant enough to speculate any involvement of the C-terminal domain on the TG2-FN interaction. In contrast, both GTP binding defective forms GTP1 (S171E) and GTP2 (K173L) but not heparan sulfate binding site mutants HS1 (K600A, R6001A, K602A), and HS2 (K205A, R209A) demonstrated a significant increase in affinity for FN suggesting influence of any of these mutated TG2 regions on the complex formation between the enzyme and extracellularly present FN (Fig. 4.3.5B).
Figure 4.3.5 Measurement of wt TG2 and TG2 mutant binding affinity for FN. A Cell homogenates containing 50-60μg of proteins obtained from untransfected as well transfected with TG2 and TG2 mutants HEK 293/T17 cells were immobilised on a plate coated with 5μg/ml FN as described under Materials and Methods (2.2.6.2). After 1 hour incubation at 37°C, the amount of enzyme bound to FN was evaluated using anti-TG2 antibody (CUB7402) followed by HRP-conjugated anti-mouse IgG antibody and further determined by spectrophotometry analysis at 490nm as detailed under Materials and Methods (2.2.6.2). B The expression of total TG2 in cell lysates was evaluated by Western blotting and compared to the equal loading marker α-tubulin by densitometry analysis (result not shown). The binding strength to FN of wild type and mutant TG2 was then normalised to the relative expression levels of wild type and mutant TG2 in the cell lysates, respectively after densitometric analysis of blots. The results are presented as a mean value +/- SD of three separate experiments. The mean value for wild type TG2 was established as a standard for determining the significant difference (* p < 0.05 and • p < 0.001) of values represented by TG2 mutants.
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Figure 4.3.5
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM

4.3.6 Distribution of TG2 mutants in the extracellular matrix

The effect of TG2 mutants on the deposition of the enzyme into the ECM was measured by a Western blot analysis. Knowing that HEK293T cells do not lay down large amounts of ECM, NIH3T3 were chosen as an initial cell model for this particular experiment. Transfected with the wild type TG2 and all the TG2 mutants, cells were incubated for 24 hours in DMEM containing 10% (v/v) serum and then serum starved for another 48 hours in order to detect of ECM associated TG2. The presence of TG2 antigen in the ECM of NIH3T3 cells was compared to that found in the whole cell lysate (WCL). In Figure 4.3.6 it is shown that every mutation affected TG2 deposition in a different way. There is a detectable however not particularly large amount of deposited TG2 antigen in the ECM by the wild type enzyme. In contrast there is a noticeably much greater amount of the antigen deposition compared to the wild type TG2 in the HS1 (K600A, R6001, K602), GTP1 (S171E), GTP2 (K173L) and FN1 (D94A, D97A) mutants indicating that none of these TG2 regions play an important role in the distribution of the enzyme into the ECM. In addition the C-terminal mutant (Δ594-687TG2) which could be found in similar amounts in the ECM to the wild type is also considered to be insignificant in that process.

However as further shown in Figure 4.3.6 TGase deposition into the ECM was altered by the two mutants, the HS2 mutant (K205A, R209A) which happens to represent a novel HSPGs binding site on the TG2 molecule and the N-terminal deletion product (Δ1-15TG2) that lacks the first 15 amino acids from the first TG2 domain. Both of these mutations were shown to inhibit the deposition of TG2 antigen into the matrix indicating the importance of HSPGs as well as the presence of an intact N-terminal FN binding site in the distribution of the enzyme into the matrix.
Figure 4.3.6 Effect of TG2 mutations on the distribution of the enzyme into the extracellular matrix (ECM). NIH3T3 cells were transiently transfected with wild type TG2 and TG2 mutants using DMEM supplemented with 10% (v/v) serum which after 24 hours post-transfection was replaced with 1% (v/v) serum. The incubation was allowed to proceed for another 48 hours and afterwards transfected cells were detached leaving the deposited matrix which was further washed with 0.1% (w/v) deoxycholate in PBS, pH 7.4 to remove of all remaining soluble fractions (Materials and Methods 2.2.10.2). After denaturation, insoluble ECM fractions were separated by SDS-PAGE electrophoresis using 8% resolving gels and the presence of TG2 antigen in the ECM was detected by Western blot using anti-TG2 antibody CUB 7402 as described under Materials and Methods (2.2.4 and 2.2.5). In addition the expression level of the wild type TG2 and TG2 mutants present in cell lysates was determined using the same Western blot method as described above. The TG2 bands showed on the blot were further normalised to α-tubulin as a marker of equal protein loading using ImageJ analysis software. In addition the level of TG2 in the ECM was normalised to the relative expression levels of TG2 found in the cell lysates. The blots represent one standard experiment.
Chapter IV: Involvement of TG2 mutants in the localisation of enzyme to the cell surface and its deposition into the ECM

Figure 4.3.6

Densitometric analysis of Western blots

- Normalised expression level of wild type and mutant TG2 to tubulin
- Normalised ECM level of wild type and mutant TG2 to the relative expression level
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4.3.7 Detection of the TG2, HS2 mutant and N-terminal deletion product in the cell growth medium

In the previous experiment two different TG2 mutants (HS2 and N-) have been shown to impair the distribution of the enzyme into the ECM. Since the wild type TG2 secreted from NIH3T3 cells transfected with HS2 (K205, R209A) and N-terminal (Δ 1-15TG2) is not deposited into the ECM it may localise in the cell culture medium. Hence it was of most importance to investigate the presence of these two particular mutants in medium of NIH3T3 cells. So far this method has been shown difficult to apply (Gaudry et al., 1999a) however as described in the later publications (Balklava et al., 2002) not impossible. To minimize the possibility of TG2 interaction with FN which is known to be present in serum and to obviate the problems caused by the large amount of albumin in the SDS gels, after 24 hours post-transfection for which 10% (v/v) serum was required, cells were further incubated for another 48 hours in medium containing 1% (v/v) serum. After that the medium was collected, proteins were precipitated with TCA and the presence of TG2 antigen was detected by SDS-PAGE followed by Western blotting as described under Materials and Methods (2.2.4 and 2.2.5). Additionally to control the test conditions, 1ng of recombinant human TG2 (Zedira, Germany) was applied into to the medium collected from untransfected cells and after precipitation with TCA, the presence of purified TG2 was then analysed by Western blotting.

As shown in Figure 4.3.7, the positive control, represented by purified recombinant human TG2 showed a very strong band indicating that the method is efficient enough to detect TGase antigen present in the growth medium of NIH3T3 cells. Further analysis allowed detection of two weak bands indicating the presence of TG2 antigen in the medium of the NIH3T3 cells transfected with HS2 and N-terminal mutants. In contrast TG2 band could not be detected in the medium of cells transfected with the wild type enzyme as well as in the medium of untransfected cells which were used as a negative control. This could result from the fact that TG2 shows high affinity for ECM proteins, especially to associated with cell surface FN (Upchurch et al., 1991) which when bound to the enzyme could retain it at the cell surface and in the matrix further prevent its release into the cell growth medium.
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Figure 4.3.7 Detection of wild type TG2, HS2 (K205A, R209A) mutant and N-terminal deletion product (Δ1-15TG2) in the medium of cultured NIH3T3 cells. The Western blot result shows the presence of wt TG2 and TG2 mutants (HS2 and N) in a DMEM supplemented with 1% (v/v) serum following 72 hours incubation and compared to 1ng of human recombinant rhTG2 (Zedira, Germany) added to the wt cells a positive control as seen in (Methods section 4.2.3). The presence of protein was determined after precipitation with 10% (w/v) TCA and analysed by SDS-PAGE and western blotting using monoclonal antibody (CUB 7402) directed against TG2 antigen as stated in the Materials and Methods (2.2.4 and 2.2.5). Additionally the expression of total TG2 in cell lysates was estimated using Western blot analysis and then normalised against tubulin.
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Figure 4.3.7

Densitometric analysis of Western blots

% expression level

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- Normalised expression level of wild type and mutant TG2 to tubulin

Figure 4.3.7
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4.3.8 Detection of TG2 bound to the two amino terminal fragments of FN and full length of FN

In order to assess the optimum concentration of TG2 bound to FN, a solid binding assay was applied. The assay was based on using increasing concentration of recombinant human TG2 (rhTG2) (1μg/ml, 2.5μg/ml, 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml, 30μg/ml) on a high binding plate coated with 45kDa (54μg/ml), 70kDa (45μg/ml) and full length (5μg/ml) of FN as described under Methods section (4.2.7) (Verderio et al., 2003).

As shown in Fig 4.3.8, the full length from human plasma showed the highest affinity for rhTG2, with the net values not greatly higher when compared to the 45 kDa and 70 kDa amino terminal fragments of FN. The 70 kDa and 45 kDa FN fragments revealed no significant difference in binding strength when increasing concentrations of rhTG2 were applied even though the 70kDa fragment bound TG2 with a higher affinity in comparison to the 45 kDa FN fragment FN.

The ELISA revealed that the saturation level of TG2 bound FN was estimated to be 10μg/ml for full length FN, 15μg/ml for 45 kDa FN fragment and 20μg/ml for 70kDa FN fragment, respectively.

4.3.9 Localisation of P4 (²AEELVLERCDLELE¹⁵) and P5 (⁸⁸WTATVVDQQDCTLQLTT¹⁰⁶) peptides on the TG2 molecule

The NΔ1-15 and D94A, D97A mutants were assessed for their ability to bind to fibronectin in a solid phase binding assay utilising purified FN and transfected HEK293T cell lysates as detailed previously in Chapter 4 (Fig. 4.3.5). Both of the mutations were shown to reduce binding of the enzyme to FN compared to wild type TG2. Thus it was of importance to generate two peptides that will correspond to the deleted or mutated FN binding site on the TG2 molecule. As shown in Figure 4.3.9, the first peptide was generated at the N-terminus of TG2 (²AEELVLERCDLELE¹⁵) while the second peptide was found to be present in distinct part of the same domain (⁸⁸WTATVVDQQDCTLQLTT¹⁰⁶) as previously described by (Hang et. al., 2005).
Figure 4.3.8 ELISA detection of the relative amounts of recombinant human TG2 bound to the full length and two amino terminal fragments of FN (45kDa, 70kDa). The relative levels of human recombinant TG2 (rhTG2) (1μg/ml-30μg/ml) bound to the three different fragments of immobilised FN was determined using monoclonal anti-TG2 antibody CUB (7402) as stated in Methods (4.2.7). Data are expressed as mean +/-SDS absorbance at 490nm and was taken from three separate experiments, each performed in triplicate. The *, o, and ◊ represent the statistical difference ($p < 0.05$) for the increasing levels of TG2 when compared to the background (PBS + 2mMEDTA) level.
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Figure 4.3.9 Illustration of 3D fragment of TG2 structure pointing out possible FN binding sites with corresponding peptides localised on TG2 molecule kindly provided by Dr Russell Collighan from Aston University.
4.3.10 The involvement of the N-terminus of TG2 in its interaction with FN

Competitive peptides were synthesised corresponding to both mutations i.e. \( ^{88}\text{WTATVVDQDCTLSLQLTT}^{106} \) (P5) for D94A, D97A FN binding site according to Hang et al., (2005) and \( ^{2}\text{AEELVERCDLELE}^{15} \) (P4) for \( \Delta 1-15 \) TG2 which undergoes N terminal post-translational modification of the N-terminal methionine (Gaudry et. al., 1999) and further tested for their ability to inhibit wild type TG2 binding to FN in the same assay. The solid binding assay was based on using three different types of FN, including its two amino terminal fragments (45kDa and 70kDa) and full length of protein. Initially, P4 peptide, it’s negative control, scrambled peptide (P4s) and P5 peptide were added to the wells at increasing concentrations (10μM – 1mM) and pre-incubated on a FN coated plate, before immobilisation of TG2 which has been initiated as described under Methods (4.3.8). Interestingly, as shown in Figure 4.3.10 C peptide P5 when used in a test to check if it will compete for TG2 (assuming 100% binding of TG2 to the FN) did not cause any alteration of TG2 binding to FN, whereas peptide P4 resulted in a moderate inhibition of binding at the higher concentrations of the peptide (Figure 4.3.10A). This suggested that the N-terminal residues 1-15 of TG2 are important for FN binding, in addition to D94A, D97A. However, only the peptide directed against residues 1-15 was capable of inhibiting TG2 binding to FN, suggesting that a larger structural unit may be involved in FN binding. As expected no inhibition was noticed when P4s peptide the negative control for P4 peptide was used (Figure 4.3.10B).
Figure 4.3.10 Effect of soluble FN binding site peptides (P4 and P5) on TG2 interaction with FN. Interaction of A P4 peptide (\(^2\)AEELVERCDLELE\(^{15}\)), B P4s peptide (EECRLAEELLEDVL) C P5 peptide (\(^{88}\)WTATVVDQDDCTLSQLLT\(^{106}\)) with the two fragments (45kDa and 70kDa) and full length FN. The interaction between TG2 derived peptides and FN was tested by a solid phase binding assay using increasing concentrations of the 3 different peptides (10μM, 100μM, 250μM, 500μM, 750μM, 1mM) pre-incubated on a TCP plated coated with three different types of FN as described under Methods (4.2.8) The presence of TG2 bound antigen was further developed by incubation with anti-TG2 CUB 7402 monoclonal antibody, followed by secondary antibody as described under Methods (4.2.8) and colour development measured spectrophotometrically at 490nm. Each data represent the mean values +/- SD from three different experiments each performed in triplicate. The statistically significant differences of net values are shown as *, and • and calculated by means of student-t test when \( p<0.05 \) and \( p < 0.001 \), respectively and compared to that of the wild type TG2 as the control.
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Figure 4.3.10
Although tissue type transglutaminase (TG2) lacks the signal peptide, indirect evidence regarding its externalisation into the ECM and its association with the cell surface of different cells has been provided by many researchers (Martinez et al., 1994; Jones et al., 1997; Verderio et al., 1998; Gaudry et al., 1999a, b; Akimov and Belkin, 2001; Verderio et al., 2009; Scarpellini et al., 2009). Once released TG2 has been shown to further co-localise at the cells plasma membrane with fibronectin, however its functions has also been implicated in interaction with other transmembrane receptors (integrins or HSPGs) which like FN have been reported to be high affinity binding partners for the enzyme (Lorand et al., 1988; Gaudry et al., 1999a, b; Akimov and Belkin, 2001; Telci et al., 2008; Verderio et al., 2009; Scarpellini et al., 2009). Despite available data indicating its broad occurrence in the extracellular environment of tissues (Upchurch et al., 1991; Aeschlimann and Paulson, 1991; Schittny et al., 1997; Johnson et al., 1999) and its deposition by different cells (Martinez et al., 1994; Verderio et al., 1999; Lorand and Graham 2003) limited knowledge has been presented regarding the exact fate of TG2 externalisation as well as the involvement of its binding partners in that process with very little mention of the mechanism of its externalisation although the mechanism for its endocytosis has been suggested by Zemskov et al., (2007) to be likely involved in the regulation of the turnover of extracellular TG2. The present literature suggests that cells surface TG2 forms direct complexes with integrins and HS chains of HSPGs (Gaudry et al., 1999b; Akimov et al., 2000; Scarpellini et al., 2009) which may not be mediated by cell surface FN. However when localised in the extracellular environment TG2 non-covalently interacts with FN (Turner and Lorand, 1989) which targets the enzyme to the ECM compartments. Based on these findings investigations were undertaken to explore the influence of TG2 high affinity binding partners on the presence of the enzyme at the cell surface and its deposition into the ECM. The importance of the conformational state of TG2 for its translocation and further targeting into the ECM has also been investigated (Balklava et al., 2002). Since some proteins (FGF-2) that undergo unconventional secretion pathway have been suggested to be secreted in a folded state it was of
importance to assess how GTP- defective binding sites mutants GTP1 (S171E) and GTP2 (K173L) of which one (GTP1), is known to lock TG2 in an extended form, affects the extracellular distribution of the enzyme. Since previous discoveries indicated that the extracellular Ca2+ bound TG2 is in an open conformation and a recent publication showed that the closed GTP-bound form (Johnson and Terkeltaub, 2005) may also be found in the ECM it was of interest to find out what conformational state the enzyme adopts when present outside the cell which is known from its low-GTP and high calcium concentration.

To be able to gain a more in depth view on the subject a human cell model (HEK293/T17 cells) and mouse cell model (NIH3T3 cells) were applied in the experimental system as previously described. The initial experiment undertaken was to determine the cell surface localisation of the wild type TG2 and TG2 constructs (HS1, HS2, GTP1, GTP2, FN1, N-, C-) by cell surface biotinylation using sulfo-NHS-LC-biotin which in principle allows it to bind and biotinylate only proteins present at the plasma membrane without affecting the intracellular ones. Cell surface biotinylation of transiently transfected HEK293/T17 and NIH3T3 cells showed some discrepancy in the pattern of TG2 cell surface expression (Fig. 4.3.1 and Fig. 4.3.2). Of the two TG2 mutants generated at distinct possible heparan sulfate binding sites the one found to be the novel HSPGs binding site HS2 (K205A, R209A) mutant, did not appear to greatly impair the association of the enzyme with the surface of HEK293 cells. However because of its noticeably smaller amount on the cell surface of NIH3T3 it strongly suggested the involvement of HSPGs in trafficking of the enzyme to the cell surface in a cell dependent mechanism. This might be the case since the only available studies regarding TG2 – HSPGs interaction, identified syndecan-4 as one of the HSPGs transmembrane receptors of HSPGs as a fundamental binding partner for cell surface binding and translocation at the cell surface (Scarpellini et al., 2009; Telci et al., 2008). The cell-surface association of the enzyme has also been shown to be unaffected by one of the FN binding site mutants of TG2 -FN1 (D94A, D97A) which suggests that FN is not involved in this process. However as shown by previous studies, FN may interact with TG2 possibly at two distinct sites located within the N-terminal domain of the enzyme (Jeong et al., 1995; Gaudry et al., 1999a; Hang et al., 2005) thus its involvement on TG2 localisation to the cell membrane cannot be ruled out.
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categorically. In fact it was confirmed by the N-terminal deletion product (Δ1-15TG2) which completely abolished cell surface TG2 indicating that the intact N-terminus of TG2 is required for the cell surface TG2-FN co-localisation (Fig 4.3.5) (Gaudry et al., 1999a). No effect on the localisation of the enzyme at the cell surface was shown by C-terminal mutation indicating that the C-terminal domain is not important in this process. However, a recent publication suggesting that integrin binding sites are located in the N-terminal domain rather than C-terminal domain (Hang et. al., 2005) does not rule out their involvement in TG2 secretion and matrix localisation. Furthermore the fact that GTP1 mutant (S171E) (open conformation) was found on the cell surface of NIH3T3 cells in much smaller amounts whereas the GTP2 mutant (K173L) (uncharacterised conformation) was secreted in much larger amounts in both cell types suggests that externalisation of the enzyme is also dependent on its conformation rather than GTP-binding.

A series of studies have revealed that TG2 possesses transamidation activity which is important for the cross-linking of many ECM proteins. This enzymatic, calcium dependent activity of the enzyme however was shown not to be required for its interaction with FN, integrins, and syndecans when mediating the cell-matrix interactions (Lorand et al., 1988; LeMosey et al., 1992; Akimov et al., 2000; Telci et al., 2008; Collighan and Griffin, 2009). To investigate the role of HSPGs, FN, integrins as well as GTP on the cell surface cross-linking activity of TG2 in living cells and further in the cell lysates a well known assay based on biotin-X-cadaverine incorporation into FN was applied (Jones et al., 1997; Balklava et al., 2002). In this study we showed that the effect of some mutations e.g. HS1 (K600A, R601A, K602A), HS2 (K205A, R209A), FN1 (D94A, D97A) did not reduce TG2 activity within the cellular compartment or at the cell surface of both cell types. In contrast significantly lower activity was obtained in both GTP-defective binding mutants and the N-terminal mutant both at the cell surface and in the cell lysate suggesting the importance of these GTP binding sites and TG2 intact N-terminal tail for the extra- as well as intracellular activity of the enzyme. However the findings regarding the activity of the GTP mutants that lose their GTP binding function is inconsistent with previous results (Ismaa et al., 2000) which indicated that impairment in GTP binding caused by substitution of glutamate in S^{171} and leucine with K^{173} did not affect TG2 activity. However these disagreements may be due to the fact that the authors were using purified proteins
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whereas in this approach cell lysates were used from transiently transfected living cells. Moreover, visualisation of in situ activity of the enzyme in the absence of the novel heparan sulfate binding site (K205, R209A) and in one of the TG2 FN binding site (D94A, D97A) mutants indicated that the HSPGs and FN binding domains are presumably not implicated in determining TG2 cross-linking when associated with the cellular or membrane compartments. However the engagement of HSPGs cannot be left out completely since no experiments have been done to estimate the interaction strength between the HS2 mutant and the syndecan-4 molecule. In addition the discovered HSPGs binding site within the TG2 structure might be one of a few (as with FN) present on the TG2 molecule as previously suggested by Verderio et al., (2009) therefore may not be directly involved in TG2 syndecan-4 interactions and therefore able to influence TG2 activity. Although a visible discrepancy between the TG2 cell-surface and cell lysate activity in both cultured cells was observed when the activity of the C-terminal mutant (Δ594-687TG2) was assayed, the influence of the C-terminal domain on the activity of the enzyme cannot be completely disqualified.

Given that the different TG2 mutations can affect TG2 distribution in different ways at the cell surface as well as influencing its activity and being consistent with previous findings indicating that impairment in the TG2 active site and also the need for special conformation requirements can affect its localisation into the ECM, it was of interest to stimulate further investigation as to how every single mutation influences deposition of the enzyme into the ECM. Since TG2 has been suggested to localise into the ECM in complex with FN (Upchurch et al., 1991; Griffin et al., 2002) which was shown to be important in this process, all of the mutants were first analysed for their ability to bind FN (Fig 4.3.5). Only two of them FN1 (D94A, D97A) and the N-terminal (Δ1-15TG2) that has already been characterised as FN binding regions significantly reduce binding to FN. When verified for its interaction with FN, mutants were tested for their ability to localise into the ECM. In this particular experiment NIH3T3 fibroblasts were used since only these cells from both cell models were able to lay down ECM. No impairment in TG2 deposition was observed by HS1 (K600A, R601A, K602A), both the GTP-defective binding site mutants and the FN1 mutant indicated that neither GTP nor FN binding are required for that process. No alteration in the distribution of the C-terminal mutant could also be found
suggesting no involvement of the last TG2 domain in ECM deposition. The most significant change resulting in negligible levels of TG2 deposition were by the HS2 (K205A, R209A) and the N-terminal (Δ1-15TG2) mutants suggesting that HSPGs as well as the intact N-terminal TG2 domain contributes to the distribution of the enzyme into the ECM. This novel discovery was further investigated by analysing the presence of these two particular mutants in the culture medium where they were both present in increased amounts when compared to the wild type enzyme. This finding stays in agreement with the hypothesis that HSPGs can act across all cells and binds associated cell surface ligands (as shown here for TG2) which further could contribute to the binding of these ligands by distinct ECM components thus for example being involved in increasing the local concentration of TG2 in the ECM (Bishop et al., 2007; Verderio et al., 2009). Additionally it throws light on the importance of the intact N-terminal FN binding site of TG2 that was previously shown by Gaudry et al., (1999a) to be necessary for the TG2-FN interaction.

To further explain the disagreement between the importance of the so far two described TG2 motifs responsible for its interaction with FN, two peptides were applied. In vitro binding studies have already located the putative high affinity binding sites for human TG2 at the N-terminus, gelatin binding site of FN (LeMosey et al., 1992; Radek et al., 1993). As shown by Verderio et al., (2003) the putative high affinity binding sites on FN including the 70kDa (heparin and gelatine binding site) and 45 kDa (gelatin binding site) can substitute for the full length FN. Hence it was decided to use these FN fragments in a FN binding ELISA system. The concentration of FN fragments was chosen according to Verderio et al., (2003). The immobilisation of the relative levels of rhTG2 showed a saturation point around 10-20 μg/ml of the enzyme dependently on length of the FN fragments. However because of the conditions of the experiment the saturation of the enzyme was not the most important issue. Sufficient interaction between TG2 and the FN fragments was seen with a concentration of rhTG2 of 2.5μg/ml which was selected for further investigation.

The purpose was to determine whether any of synthetic peptides generated from the N-terminal tail of TG2 (P4, P4s) or its β5/β6 hairpin (P5) affect the interaction between the enzyme and FN. The peptide P4, representing intact N-terminal site ^2AEELVLERCDLELE^15, but excluding its scrambled analogue was able to moderately inhibit the interaction of
TG2 with the different FN fragments indicating once more the importance of that N-terminal fragment for their complexation. However no inhibition was noticed when P5 peptide, $^{88}$WTATVVDQDCTL$LQ$LT$^{106}$ was used in the same experimental system. In general this result could suggest the unimportance of that particular TG2 binding site for FN but this would be in disagreement with a previous finding (Hang et al., 2005). Despite that result, the final conclusion cannot be formed since the authors encountered unexpected resistant of synthesised P5 peptide for its dissolution in DMSO and further suggested hydrophilic solutions.
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5.1 INTRODUCTION

Cell phenotype is characterised by a multitude of events which may include communication with and composition of the extracellular matrix (ECM). So far much has been revealed about the organisation and assembly of this complex network through studying the interactions of matrix macromolecules with themselves and with distinct molecules that are present at the cell surface. Assembly is a difficult process that was shown through analysis of one of the most abundant ECM proteins fibronectin (FN) which requires the cooperation of extracellular and intracellular mediated signalling pathways. Once initiated the soluble dimers of FN are deposited into a dense fibrillar FN network which provides a dynamic environment for cell adhesion, migration, growth, survival and differentiation (Wierzbicka-Patynowski and Schwarzbauer, 2003). As previously indicated, under physiological conditions, FN assembly into the ECM takes place on the surface of particular cells in vitro (Mosher, 1993) and expands during pathological circumstances like wounding or commonly during tumorigenesis and fibrosis (Gailit and Clark, 1994). FN matrix assembly and the interaction of cells with pericellular FN do however require the presence of additional components. The main cell surface receptors that fibronectin binds to are integrins, mainly the α5β1 integrins. This interaction takes place through the III8-III10 modules present on FN, although the most important integrin binding site on FN has been mapped to the Arg-Gly-Asp (RGD) sequence that is localised within module III10 (Mosher, 1984). FN however is not an exception, other extracellular proteins including fibrinogen, vitronectin, laminin, osteopontin, collagens also possess RGD recognition motifs (Rouslahti and Pierschbacher, 1987). Hence this particular sequence has been speculated to play an important role in regulating cell-matrix interactions which was further confirmed by many investigators.

However integrin presence is not the only factor that is required for FN matrix assembly and the subsequent formation of focal cell adhesions. The role of heparan sulfate proteoglycans in cell adhesion on FN has been brought to attention by initial studies on two HSPGs mutant cell lines characterised by lack or impairment in HSPGs expression (Couchman et al., 1988; LeBaron et al., 1988). Also earlier data indicated the
importance of heparan sulfate activity in the formation of focal contacts by fibroblasts, which suggests that in addition to their role in cell adhesion HSPGs are also involved in cell spreading (Woods et al., 1986). This was further confirmed by studies on fibroblasts indicating that these cells were able to spread in the presence of fibronectin containing RGD motifs; however could not form well defined focal contacts in the absence of glycosaminoglycan (GAG) binding domains (Burridge et al., 1992). There are two main heparin-binding sites localised within the FN domain structure, the Hep I site situated at the N-terminal tail and the Hep II binding site situated near the C-terminal tail of FN (Barkalow and Schwarzbauer, 1991; Lin et al., 2000). Although Hep II was initially suggested to be the main heparin binding site, Lin and collaborators (2000) revealed the Hep I played a crucial role in this interaction as well. However as later demonstrated, soluble heparin-binding peptides derived from the Hep II (WQPPRARII) were shown to significantly reduce the formation of focal adhesions on FN indicating the importance of this particular binding site in mediating cell-matrix adhesions (Chon et al., 2001).

So far syndecans have been characterised as the major cell surface transmembrane proteoglycan possessing affinity for FN. Thus it is not surprising, that some of the family members have been implicated in the regulation of FN-matrix assembly and cell-matrix interactions (Woods et al., 1988). There are four different receptors among the syndecan subfamily of HSPGs, from which two have been shown to affect matrix assembly. Syndecan-2 a major proteoglycan receptor of fibroblasts when expressed by stably transfected Chinese Hamster ovary (CHO) cells in a full length had no affect on matrix formation. However when expressed in a truncated state, lacking residues from the cytoplasmic tail, it was shown to negatively regulate assembly of FN and laminin matrices indicating its indirect role in this process (Klass et al., 2000). The other major proteoglycan receptor, Syndecan-4, widely distributed within different cell types that forms cell adhesions, has been shown to influence fibrillogenesis of FN in a more direct way. Studies on fibroblasts seeded on the cell-binding domain of FN (CBD), the one that comprises only RGD and the PHSRN binding motifs demonstrated that cells were able to attach however failed to form focal adhesions or actin stress fibres (Woods et al., 1986; Saoncella et al., 1999). Only when treated with antibodies against the extracellular domain of syndecan-4 did cells form mature focal adhesions and stress fibres thus indicating syndecan-4 as the
main HSPGs involved in the assembly process (Saoncella et al., 1999). Further analysis showed formation of focal adhesions and stress fibres is promoted by the cooperation of syndecan-4 with integrin α5β1 which has been shown to act via a Rho-dependent mechanism (Saoncella et al., 1999). In addition studies on syndecan- null fibroblasts when compared to wild type cells suggested syndecan-4 was able to increase phosphorylation of focal adhesion kinase FAK Tyr(397), a condition that has been shown to favour FN matrix assembly (Wilcox-Adelman et al., 2002; Telci et al., 2008). Further indication of Syndecan-4 in this process has also been associated with activation of protein kinase Calpha (PKCalpha ) which was shown to localise with focal adhesions in fibroblasts (Barry and Crichtley, 1994). The activity of this kinase is persistently regulated (Keum et al., 2004) in the downstream pathway directly influencing Rho kinases (RhoA) (Dovas et al., 2006), which can mediate stress fibre formation and focal adhesion assembly through phosphorylation of myosin II and myosin phosphatase (Pellegrin and Mellor, 2007).

Studies assaying the interaction of human osteoblasts with fibronectin revealed that both the RGD binding sequence and the heparin-binding domain of FN plays an important role in osteoblast attachment (Verderio et al., 1998; Verderio et al., 2000; Sim et al., 2004). Additionally during cell binding, fibronectin has been shown to be a substrate and binding protein for different enzymes externalised from cells into the extracellular matrix. The ability of TG2 to co-localise with FN at the cell surface or pericellular matrix has been widely described (LeMosey et al., 1992; Gaudry et al., 1999a; Hang et al., 2005). The cross-linking activity of the enzyme has been believed to play a role in enhancing the attachment and spreading of various cell types (Chau et al., 2005). However TG2 has also been presented as a novel cell adhesion protein which does not require its transamidating activity to promote cell adhesion (Akimov et al., 2000). Association of the enzyme with fibronectin and its deposition in FN-complex into the ECM was found to be greater during wounding (Telci et al., 2006). Therefore it is not surprising that many investigators decided to explore the effect of FN-bound TG2 matrix on mediating cell adhesion. Early data on HOB cells and Swiss 3T3 fibroblasts indicated that TG2 binds to FN with high affinity and when bound promotes RGD-independent cell adhesion (Verderio et al., 2000; Verderio et al., 2003). However further suggestions of the possible influence of heparan sulfate proteoglycans on FN-TG2 mediated cell adhesion resulted from applying
glycosaminoglycan-degrading enzymes into the experimental system (heparinase and its control chondroitinase) which demonstrated that only heparinase that digest cell surface heparan sulfate was able to significantly reduce RGD-independent cell adhesion on a FN-TG2 matrix indicating the importance of HSPGs (Verderio et al., 2003). Among members of the HSPGs family, syndecan-4 has been suggested to be involved in TG2 mediated cell adhesion. Thus it was not surprising when Telci et al., 2008 revealed that the binding of the TG2-FN complex to the chains of syndecan-4 promotes RGD-independent cell adhesion.

However TG2 is not an exception with respect to its high affinity for HSPGs. As stated before, fibronectin possesses two heparin binding sites which could compete with TG2 for binding to HSPGs. As reviewed by Verderio et al., (2009) even treatment with a peptide that impairs immobilisation of TG2 on FN (Hang et al., 2005) did not reduce the amount of TG2 in syndecan-4 immunoprecipitates thus indicating a direct interaction between TG2 and S4. Knowing that TG2 interacts directly with syndecan-4, and this interaction need not require the presence of FN it was of most importance to investigate how TG2 binds to HSPGs and how this affects the influence of the enzyme on cell adhesion. Therefore the work contained in this particular chapter was focused on looking at the effects on cell adhesion of different peptides where the amino acid residues were mapped to the newly found potential heparin binding site of TG2. This included a long peptide P1 (NPKFLKNAGRDCSRRSS) and two short peptides: P2 (NPKFLKNA) and P3 (GRDCSRRSS) which were used in the experimental cell model using HOB cells (Verderio et al., 2000; Heath et al., 2001; Verderio et al., 2003).
5.2 METHODS

5.2.1 Docking studies

The crystal structures 1KV3 and 2Q3Z were downloaded as pdb files from the Protein Data Bank (http://www.rcsb.org/) and opened in the software CAChe WorkSystem Pro version 7.5.0.85 (Fujitsu Ltd). Hydrogens were added and water and ions were deleted. Docking sites were defined by selecting all the amino acid residues within either 5 Å or 8 Å of residues K202, K205, R209, R213 and R222. Both structures contained missing residues but these were far enough away from the defined docking sites such that their absence would not interfere with the docking studies. Using the same software, three ligand structures (a dimer, a pentamer and a hexamer) were defined by taking residues 2-3, 2-6 and 2-7 respectively from the glycosaminoglycan structure 1HPN.pdb.

Using the ProjectLeader module and the ActiveSite docking component of the same software, the three ligands were each docked three times into the defined docking sites of both proteins using the flexible ligand and flexible active site side chain options. Other parameters and options included: Use Amber van der Waals; population size 50; maximum generations 3000; crossover Rate 0.8; mutation Rate 0.2; elitism number 5; local search rate 0.06; maximum iterations local search 300.

5.2.2 Cell adhesion and spreading assay

Wells of TCP were coated with 5μg/ml of human plasma fibronectin in Tris-Cl, pH 7.4 overnight at 4°C and then blocked for 30 min with 3% (w/v) marvel in PBS. Exponentially growing HOB cells were trypsinised and then washed in serum free medium in which they were further incubated for 20 min at 37°C with increasing concentrations (100-500μg/ml) of long (P1) and two short (P2, P3) peptides corresponding to the newly discovered heparan sulfate TG2 binding site and their analogue P1s peptide. Additionally to find out the effect of the RGD containing peptide on HOB cell adhesion, 150μg/ml of RGD peptide...
was also added into the cell suspension that contained the different concentrations of the previously described peptides. Pre-treated cells were then seeded into the FN coated wells with and without immobilised guinea pig TG2 (used at a concentration of 20µg/ml) and allowed to adhere for 30 minutes at 37°C to minimize the externalisation of any endogenous proteins (Verderio et al., 2003). Attached cells were washed once with PBS, pH 7.4 and then fixed with 3.7% paraformaldehyde, further permeabilised with 0.1% (v/v) Triton-X in PBS, and co-stained with May-Grunwald and Giemsa stain as described under Materials and Methods (Section 2.2.9). Images of stained cells from non-overlapping fields were visualized by digital camera at 20 x magnification and analyzed using the Image analysis program written at the National Institutes of Health.

5.2.3 Competition between long (P1), and short peptide (P3) mapped to HS2 mutant binding site and the wild type enzyme for binding to heparin

The resulting supernatants (total cell lysates) (~150µl containing 4mg/ml of TG2) obtained from HEK293/T17 cells transfected with wtTG2 as described before (Section 2.2.3.5) were dissolved in around 450 µl of 50mM Tris-Cl, 1mM EDTA, 1mM DTT, pH 7.5 buffer and further pre-incubated on ice with P1, P1s, and P3 peptides to a final concentration of 1mg/ml. After 15 minutes pre-incubation of the heparin sepharose column with the desired peptide, such prepared supernatants were applied to the 1ml bed volume Heparin sepharose column (Amersham Biosciences, Sweden) equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer only. The column was eluted at 2ml/min with the same buffer using a peristaltic pump at room temperature. The column was then washed with 15ml of the same buffer before linear gradient elution with salt (1M NaCl in 50mM Tris-Cl, 1mM EDTA, and 1mM DTT, pH 7.5) was initiated. Both flow-through and resulting fractions were collected into 96 well microtiter plates. Content of the resulting fractions were verified by SDS-PAGE electrophoresis and Western blotting using anti-TG2 monoclonal antibody for the presence of TG2 bands (Materials and Methods, Section 2.2.4 and 2.2.5).
5.3 RESULTS

5.3.1 A heparin derived oligosaccharide docks into a characteristic heparin binding pocket in TG2

The HS1 and HS2 regions were docked with heparin derived oligosaccharides. An iduronic acid-2-sulphate-glucosamine-2, 6-disulphate disaccharide which is the most common repeating unit of heparin docked well with the HS2 region of TG2. In addition a pentasaccharide (2-6) and hexasaccharide (2-7) consisting of the same repeating units were checked for binding affinity to HS2 derived binding pocket localised on TG2 molecule (Table 5.3.1). However undertaken studies revealed differences in the docking scores (kcal/mol) dependently on conformation of the crystal structure of TG2. The more negative the score, the better the enthalpy change on binding thus the pentasaccharide docked in TG2 which was locked in a close conformation (1KV3) was shown to be most effectively bound within the area of the desired binding pocket which was further illustrated on a three-dimensional image (Figure 5.3.1).

<table>
<thead>
<tr>
<th></th>
<th>1KV3 5A site</th>
<th>1KV3 8A site</th>
<th>2Q3Z 5A site</th>
<th>2Q3Z 8A site</th>
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<tbody>
<tr>
<td>IDS – SGN</td>
<td>-548</td>
<td>-602</td>
<td>-574</td>
<td>-627</td>
</tr>
<tr>
<td>2-6</td>
<td>-836</td>
<td>-710</td>
<td>+1407</td>
<td>-349</td>
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<tr>
<td>2-7</td>
<td>-790</td>
<td>-679</td>
<td>+3706</td>
<td>+9460</td>
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</tbody>
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Table 5.3.1 Table represents the scores (kcal/mol) of di-, penta-, and hexasaccharide docked into, or within the nearest localisation of the novel heparin binding site pocket localised on TG2 molecule. Using Cache WorkSystem Proversion 7.5.0.85 (Fujitsu Ltd) software, downloaded crystal structures adopted to a close (1KV3) and an open (2Q3Z) conformations were treated with IDS-SGN (disaccharide), 2-6 (pentasaccharide) and 2-7 (hexasaccharide) and further verified for the binding efficiency into the HS2 binding pocket by Dr Dan Rathbone from Aston University. The more negative the score, the higher binding efficiency.
Figure 5.3.1 Three dimension analysis of the novel heparan sulfate binding site pocket interaction with pentasaccharide designed and kindly provided by Dr Russell Collighan and Dr Dan Rathbone from Aston University. Illustration represents the electrostatic surface potential of TG2 with red indicating negative charges and blue indicating positive charges. In addition it points out positively charged amino acid residues (K202, K205, R209, R213, K387) surrounding the novel heparin binding site pocket where pentasaccharide (carbon is indicated by green; hydrogen by white; oxygen by red; sulphur by yellow) was docked.
5.3.2 Tissue transglutaminase supports RGD-independent HOB cell adhesion to a FN-TG2 matrix

The TG2-FN interaction and its involvement in promoting cell surface adhesion has already been widely described in the literature. As shown by immunogold electron microscopy TG2 co-localisation at cell surface of Swiss 3t3 cells could be found in association with FN (Gaudry et al., 1999a). Moreover initial investigations in this field suggested that binding between the enzyme and human plasma FN can take place through the N-terminal tail of TG2 (Radek et al., 1993; Gaudry et al., 1999a) which was further confirmed by our studies with N-terminal deletion mutant (Δ1-15TG2) as shown in chapter 4. Thus to verify previous findings of Verderio et al., (2003) regarding FN-TG2 interactions, guinea pig TG2, human plasma FN and soluble RGD peptide were used in an experimental system. The saturation point of TG2 when immobilised on FN has already been measured by Verderio et al., (2003) and established at 20μg/ml. As an initial cell model HOB cells were used because of being rich in the appropriate cell surface integrin receptors, which mainly possess RGD-binding β1 subunit linked with different α subunits (α1, α2, α3, α5, and αV) (Gronthos et al., 1997). Moreover this particular cell line shows increased cell spreading on biomaterials coated with FN-TG2 in comparison to FN alone (Heath et al., 2002).

HOB cells were grown to 80-90% confluence and when ready were treated with increasing concentrations of RGD (GRGDTP) synthetic peptide and its control RAD (GRADSP) peptide and further seeded onto FN and FN-TG2 matrices. HOB cell attachment and spreading was then visualised by camera (Fig. 5.3.2a). To indicate the differences between attached and spread cells colourful arrows were applied on each of the images. Cell attachment was shown as the total amount of cells attached to particular matrix, while cell spreading was described as the number of cells characterised by an extended flattened shape. The results showed visual differences in the amount of cells attached to both matrices with no significant difference between cells treated with increasing concentrations (up to 150μg/ml) of RAD peptide and only a small but significant decrease in either cell attachment or cell spreading at concentrations of 200μg/ml and
Figure 5.3.2 Adhesion of HOB cells on FN and FN-TG2 matrices influenced by the presence of RAD and RGD peptides. A Visualisation of four different images representing attachment and spreading of HOB cells treated either with RAD or RGD peptides. Examples of differences between attached and spread cells are signified by arrows, black points on attached (spread) cells whereas red points on non-spread cells; B effect of RAD peptide; C effect of RGD peptide. Analysis of cell attachment and cell spreading was performed according to the previously described method (Materials and Methods 2.2.9). Each data point representing attached or spread cells on FN or FN-TG2 matrices is shown as a mean percentage +/- SD of three separate experiments performed in triplicate. Mean number of cells on FN, was used as percentage of control values and established at 100%. The mean value of attached cells on FN cells was calculated as 108.3 +/- 1.5 and represented 100%. The same rule applied to spread cells. To show any statistical differences between obtained data points when compared to the control value they were calculated by Mann-Whitney test and shown as * (p < 0.05) including both RAD and RGD treatment.
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Figure 5.3.2a
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Figure 5.3.2b
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Figure 5.3.2c
250μg/ml RAD peptide on FN-TG2 and FN matrices when compared to the value of control cell adhesion on FN. This result is consistent within both data panels (upper and lower panels in Fig. 5.3.2b). However, differences in cell adhesion were noticeable when HOB cells were treated with increasing concentrations of RGD peptide. A significant reduction of cell attachment and cell spreading was seen when cells were seeded on both matrices when compared to untreated cells. Although, HOB cell treatment with RGD peptide caused approximately 20-25% (at 50μg/ml - 250μg/ml of RGD) reduction in attachment and spreading of cells on FN-TG2 matrix, it was fairly consistent when compared to control values on FN matrix. The decrease observed on FN matrix was much greater and varied between 26% - 59% (at 50μg/ml - 250μg/ml of RGD) in cell attachment and 35% - 78% (at 50μg/ml - 250μg/ml of RGD) in cell spreading. However, there was also a significant decrease in attachment and spreading observed between cells when treated with the same peptide when seeded on FN in comparison to the FN-TG2 matrix (Fig. 5.3.2c). Cell attachment to FN-TG2 in the presence of 150μg/ml of RGD was approximately 28% higher when compared to cell attachment to FN. Similarly, cell spreading on FN-TG2 at the same concentration of RGD was 30% higher when compared to cell spreading on FN. At higher concentrations RGD peptide there is likely to nonspecific affects since RAD peptide was shown to cause a small reduction in cell adhesion starting at concentration of 200μg/ml. Thus 150μg/ml of RGD and its control peptide were chosen for further experiments. All together these results indicate the involvement of TG2 in promoting RGD-independent cell adhesion when immobilised on FN matrix which stays in agreement with previous finding of Verderio et al., (2003) and Telci et al., (2008).
5.3.3 Effect of P1 (NPKFLKNAGRDCSRRSS) peptide on cell binding to FN and FN-TG2 in the presence and absence of RGD peptide

To verify whether cell surface proteoglycans can be involved in mediating cell adhesion to FN-TG2, HOB cells were subjected to treatment with P1 peptide (NPKFLKNAGRDCSRRSS) which corresponds to a binding pocket in the core domain of TG2 where the novel heparan sulfate binding site has been found. Previous studies have shown that TG2 when bound to FN can compensate for the loss of integrin-mediated cell adhesion in the presence of RGD peptides in a process requiring cell surface heparan sulfate chains, but not requiring the transglutaminase enzymatic activity (Verderio et al., 2003). It has also been shown that the cell surface proteoglycan syndecan 4 is critical in this process (Telci et al., 2008). Since TG2 can interact with both FN and HSPGs, the HSPG binding properties of TG2 are most likely involved in this process Telci et al., (2008).

Therefore the P1 peptide was tested for its ability to interfere with TG2-mediated RGD-independent binding of HOBs to FN in a simple adhesion and spreading assay. To initiate the experimental procedure HOB cells when ready were treated separately with competitive concentrations of the P1 peptide or in parallel with the same P1 peptide supplemented with 150μg/ml of RGD peptide and then plated on FN and FN-TG2 matrices in the presence of the peptides. For clarity only images representing two distinct concentrations of P1 peptide (100μg/ml and 500μg/ml) has been chosen for visualisation. As seen in Figure 5.3.3a P1 peptide did not influence the morphology of the cells, which when attached showed a round form whereas when spread displayed a flat extended shape. This appearance was however changed by additional treatment with RGD peptide, which caused impairment in cell spreading in particular on the TG2-FN matrix. Once incubated with P1 peptide at a concentration of 100μg/ml, cells showed no significant differences in adhesion (both attachment and spreading) whether seeded on FN or TG2-FN matrices when compared to the control value on FN (Figure 5.3.3b upper and lower panel). In the presence of 200μg/ml of P1 peptide cell attachment and spreading was slightly reduced to around 11% when compared to that of FN-TG2, whereas a significant reduction of 17% was seen on the FN matrix. Additionally at the concentration of 300μg/ml of P1 peptide a significant reduction of 24% to 20% of cell attachment and 25%
to 21% of cell spreading was observed on FN-TG2 and FN alone, respectively. However a great impairment in cell attachment and spreading was noticed with 400μg/ml and 500μg/ml concentration of P1 peptide. At concentration of 400μg/ml, P1 peptide led to reduction of 48% to 30% of cell attachment and 50% to 32% of cell spreading on TG2-FN and FN alone, respectively. At concentration of 500μg/ml, P1 peptide caused even greater reduction of 60% to 44% of cell attachment and 65% to 45% of cell spreading on TG2-FN and FN alone, respectively (Fig. 5.3.3b). Hence at these higher concentrations P1 peptide may work non-specifically. The reduction of cell adhesion and spreading could also be seen in the additional presence of RGD peptide. On the FN-TG2 matrix, at 100μg/ml concentration of P1 and 150μg/ml of RGD HOBs showed an approximately 20% decrease in cell attachment and spreading whereas no significant difference was noted in cell adhesion on FN when compared to the untreated control on FN. At concentration of 200μg/ml of P1 peptide and in the presence of RGD peptide, the reduction of around 25% of cell attachment and 29% of cell spreading on FN-TG2 was noticed. The significant decrease to approximately 20% in cell attachment and spreading was also observed on FN alone when compared to the FN control. Greater reduction in cell adhesion has been noticed with higher concentrations of P1 peptide supplemented with RGD peptide both on FN-TG2 and FN alone. At concentrations of 300μg/ml and 400μg/ml of P1 peptide HOBs cells showed around 35% to 40% and 50% to 55% decrease in cell attachment and spreading on FN-TG2, respectively. In addition at these concentrations of P1 peptide and in the presence of RGD peptide also 22% to 25% reduction in cell attachment and spreading on FN alone was observed. However at 500μg/ml concentration of P1 in the presence of 150μg/ml of RGD, cells showed a 60% to 32% decrease in cell attachment and 64% to 34% in cell spreading on TG2-bound FN and FN matrices, respectively. The data therefore indicates that peptide NPKFLKNAGRDCSRSS reduces cell adhesion and spreading on FN-TG2 independently of the presence or absence of RGD peptide in a dose dependent manner. In addition it suggests that P1 peptide is able to compensate for the RGD induced loss of adhesion on FN alone (even at the lowest concentrations) and seems to be substituting for the heparin binding site and compensation itself.
Figure 5.3.3 Effect of P1 peptide on attachment and spreading of HOB cells on FN and FN-TG2 matrices. **A** Illustration of attachment and spreading of HOB cells treated with two distinct (100μg/ml and 500μg/ml) concentrations of P1 peptide without and with the presence of RGD peptide; **B** effect of P1 peptide alone and in the presence of RGD. Adhesion and spreading of HOB cells on FN and FN-TG2 matrices was analysed in the presence of 100μg/ml to 500μg/ml P1 peptide in the presence or absence of 150μg/ml RGD peptide. Cells were preincubated with the peptides and incubated in the presence of the peptides on the different matrices as previously described under (Methods 5.2.2). Cell adhesion (attachment and spreading) is expressed as a mean percentage of attached or spread cells +/- SD, respectively and shown as a mean of triplicate measurements. Total amount of cells from a control sample (cells on a FN matrix) established as 100% was assessed as 129.47 +/- 2.95 for attached cells and 126.86 +/- 4.2 for spread cells. The * symbol represents statistical significance between the control sample and each data point corresponding to attached and spread cells in the presence of P1 synthetic peptide alone or with RGD peptide. The significant different cell adhesion values were evaluated by Mann-Whitney test with (p<0.05). In addition to indicate significant differences between the single data points symbol ◊ (p< 0.05) was used.
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Figure 5.3.3a
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Figure 5.3.3b
5.3.4 Effect of P2 (NPKFLKNA) peptide on cell binding to FN and FN-TG2 in the presence and absence of RGD peptide

Since P1 (NPKFLKNAGRDCSRRSS) peptide was shown to inhibit the cell adhesion pathway mediated by TG2 bound to fibronectin it was of further interest to investigate whether this pathway could be differently influenced by two small peptides randomly generated from the P1 peptide. The first short peptide named P2 (NPKFLKNA) was synthesised from the first eight amino acids within P1 peptide thus containing the first residue (Lys205) that was substituted with Ala within the TG2 HS2 mutant. The second short peptide P3 (GRDCSRRSS) was synthesised containing the ninth to the seventeenth residue of the P1 peptide and in contrary to the P2 peptide consisted of the second amino acid (Arg209) residue that was substituted with Ala within the same TG2 HS2 mutant.

To explore the possibility that cell adhesion to FN-TG2 may be mediated by one of the two short peptides, first P2 (NPKFLKN) peptide was applied into the experimental system using a similar approach to the one that was previously described with the longer P1 peptide. Exponentially growing HOB cells when confluent were trypsinised, washed with serum free medium as described under Materials and Methods (Section 2.2.9.3) and further 2.5 x 10^5 cells/ml cell suspension was incubated with increasing concentrations (100μg/ml - 500μg/ml) of P2 peptide alone or complemented with 150μg/ml RGD peptide. Cell adhesion of the HOB cells was then compared on FN-TG2 and FN matrices. Due to clarity only images representing two most distinct concentrations (100μg/ml and 500μg/ml) of P2 peptide in the absence or presence of RGD peptide are shown in this section. When seeded, HOB cells were analysed for attachment and changes in their morphology. As seen in Figure 5.3.4a the P2 peptide did not influence the appearance of the cells which when spread on either FN-TG2 or FN matrices presented extended shapes with a dark nucleus in the middle and bright cytoplasm surrounding this. Following treatment with RGD, cells adopted more round shape that was much more evident on FN. When visualised, cells were counted and the influence on cell adhesion of the P2 peptide with or without RGD on FN-TG2 and FN matrices was compared to the control value of untreated cells on FN. On the FN-TG2 matrix, HOB cells treated with 150μg/ml of RAD
(control peptide) showed increase of around 14% to 8% in cell attachment and spreading when compared to the control value on FN alone. In addition small significant difference in the amount of adhered cells was noticed in the presence of 150μg/ml RGD peptide when compared to that of FN alone supporting once more the hypothesis of the importance of FN bound TG2 in RGD-independent cell adhesion. In the presence of 100μg/ml and 200μg/ml of P2 peptide attachment and cell spreading appeared to be slightly (8% to 10%) greater than that of TG2-FN control (Figure 5.3.4.b) whereas no effect was seen on the FN matrix. When treated with 300μg/ml and 400μg/ml of P2 peptide, cell adhesion (attachment and spreading) of HOBS remained almost unchanged on FN-TG2, however was significantly reduced to around 25% and 33% of cell attachment and 28% to 36% of cell spreading on FN alone, respectively. Additionally at the concentration of 500μg/ml of P2 no difference was observed in cell adhesion on FN-TG2, while on FN, cell adhesion (attachment and spreading) was significantly reduced to around 55% of that of control cell adhesion to FN. This result could suggest that like P1, P2 peptide when used at high concentration may work none specifically. Further treatment of HOB cells with 100μg/ml and 200μg/ml of P2 peptide and 150μg/ml of RGD peptide resulted in no significant difference on cell attachment and spreading on FN-TG2, but significant decrease of around 42% to 38% of cell attachment and 47% to 42% of cell spreading when cells were plated on FN, respectively. At concentration of 300μg/ml of P2 peptide and in the presence of RGD peptide approximately 17% decrease in cell attachment and 23% decrease in cell spreading on TG2-FN and around 40% decrease in cell attachment and spreading on FN alone was noted. In addition in the presence of 400μg/ml of P2 peptide supplemented with RGD approximately 22% decrease in cell attachment and 25% in cell spreading was noticed on TG2-FN, whereas 37% reduction in cell attachment and around 40% in cell spreading was observed on FN alone when compared to the control FN. When P2 was used at concentration of 500μg/ml in the presence of RGD peptide this caused a significant decrease in cell attachment and spreading estimated at 20 - 22% on TG2-FN and 46% - 59% on FN matrix, respectively. This result could suggest the involvement of P2 peptide in promoting TG2 mediated RGD-independent cell adhesion.
Figure 5.3.4 Adhesion of HOB cells on FN and FN-TG2 matrices in the presence of P2 synthetic peptide. A Visualization of 8 different images representing HOB cell adhesion in the presence of two different concentrations of P2 peptide alone or/and in the presence of RGD peptide respectively. Images of non overlapping fields were captured by digital camera at 20x magnification and further analysed by a Scion Image program as outlined in the experimental procedures (Materials and Methods 2.2.9); B effect of P2 peptide in the absence or presence of RGD peptide on the attachment and spreading of HOB cells. Prior to the cell adhesion, cells were pre-incubated for 20 min with 150µg/ml RGD peptide and/or 100µg/ml to 500µg/ml P2 peptide respectively and the assay was performed in the absence of serum using FN and FN-TG2 matrices as stated in the Materials and Methods (5.2.2). Each data points corresponds to the mean percentage of attached cells (cell attachment) or the mean percentage of spread cells (cell spreading). Mean values presented as the percentage of control (attached or spread cells on FN) +/- SD of triplicate measurements were taken as 100%. The mean percentage attachment value +/- SD normalised to 100% was 129.47 +/- 2.95 while the mean percentage spreading value +/- SD was 126.86 +/- 4.2. The statistically significant difference was shown as * (p<0.05) in the presence or absence of the RGD peptide in the test conditions. The ◊ symbol indicates the statistically different cell adhesion values (p<0.05) of points representing P2 treated cells with or without RGD peptide on FN-TG2 and FN matrices.
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Figure 5.3.4a
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Figure 5.3.4b
5.3.5 Effect of P3 peptide on cell binding to FN and FN-TG2 in the presence and absence of RGD peptide

So far the effect of both P1 (NPKFLKNAGRDCSRRSS) and P2 (NPKFLKNA) on RGD-independent pathway mediated by TG2-FN has been evaluated. Neither of these peptides affected cell adhesion on the TG2-FN matrix when RGD was present in the expected manner. Therefore it was of importance to test the influence of the second short P3 (GRDCSRRSS) synthetic peptide on the adhesion of HOB cells using the same experimental approach. As previously described, confluent HOB cells were trypsinised and after washing were preincubated with increasing concentrations (100 - 500μg/ml) of P3 peptide alone or P3 peptide substituted with 150μg/ml of RGD peptide.

The reason for using P3 peptide complemented with RGD peptide was to assess whether the P3 peptide can block the adhesion of HOB cells on FN-TG2. The first part of the investigation was to explore the effect of P3 peptide on the morphology of HOB cells. As seen in images (Figure 5.3.5a), like P1, and P2 peptides, P3 peptide used in a lower concentration did not change the appearance of attached cells which was only affected by additional presence of RGD peptide. However at the higher concentration of P3 peptide, cells showed a slightly more rounded appearance. In the absence of P3 and RGD peptides, but in the presence of 150μg/ml RAD peptide attachment and spreading increased to around 13% to 8% on FN-TG2 when compared to the FN control. Also treatment with 150μg/ml RGD peptide resulted in a significant decrease in cell adhesion on FN-TG2 but a much greater reduction to around 50% of cell attachment to 60% of cell spreading on FN in comparison to the control values. When HOB cells were plated on the TG2-FN matrix in the presence of 100μg/ml of P3 peptide they exhibited a 10% increase in cell adhesion (cell attachment and spreading) in comparison to the untreated FN control. Increase in the P3 concentration to 200μg/ml, and 300μg/ml caused no significant effect on cell attachment and spreading on FN-TG2 and FN alone when compared to the FN control. Additionally at the concentration of 400μg/ml only moderate however no significant decrease in cell adhesion (attachment and spreading) was observed on both matrices. When used at the concentration of 500μg/ml P3 peptide
caused significant decrease of attachment and spreading to around 36% to 35 on FN-TG2 and around 20% to 23% on FN alone, respectively. These results suggest that at the highest concentration P3 peptide may be non-specific. Additional treatment of HOB cells with RGD peptide at the lower concentration of P3 peptide (100μg/ml) did not affect attachment and cell spreading on FN-TG2 but led to a significant decrease, estimated as 26% of cell attachment and 28% of cell spreading on FN alone. In the presence of 200μg/ml of P3 peptide supplemented with RGD peptide, 12% to 25% decrease in cell attachment and 21% to 32% decrease in cell spreading was observed on FN-TG2 and FN alone, respectively. Under the same conditions but at the concentration of 300μg/ml of P3 peptide approximately 17% decrease in cell attachment and 26% decrease in cell spreading on FN-TG2 and around 26% decrease in cell attachment and 36% in cell spreading on FN alone was noted. Notably, RGD peptide did affect cell attachment and spreading on FN-TG2 in the presence of the higher concentrations of P3 peptide which was observed to decrease to around 38% to 40% (400μg/ml) and 52% to 59% (500μg/ml), respectively when compared to that of a control FN. In addition cell adhesion (attachment and spreading), in the presence of these higher concentrations of P3 peptide with RGD peptide, was also significantly reduced by around 26% (400μg/ml) and 20% (500μg/ml) when compared to cell adhesion when cells were incubated with the P3 peptide alone but in the absence of RGD on FN-TG2 matrix. The data therefore suggests that HOB cells treated with higher concentrations of P3 peptide did not restore the compensatory effect of FN-TG2 when cell adhesion was disrupted in the presence of RGD peptide. However P3 peptide like P1 peptide seemed to restore the loss of cell adhesion induced by the RGD peptide on FN alone.
Figure 5.3.5 Effect of soluble P3 peptide on the adhesion of HOB cells to FN and FN-TG2 matrices in the presence of RGD peptide. A Illustration of eight independent images of HOB cells on FN and FN-TG2 matrices treated with P3 alone and with the same peptide but additionally supplemented with RGD peptide; B effect of P3 peptide on TG2 mediated RGD-independent HOB cell adhesion on FN and FN-TG2 matrices. HOB Cells were analyzed for attachment and spreading to FN and TG2 immobilised FN matrices as indicated under Materials and Methods (Section 2.2.9). Where indicated, cells were pre-treated with 100µg/ml to 500µg/ml P3 peptide alone or in addition with 150µg/ml RGD peptide for 20-25 min and then plated in the presence of these peptides. Each data point representing attached or spread cells on FN or FN-TG2 matrices is shown as a mean percentage +/- SD of two separate experiments performed in triplicate. The points in the upper and lower graphs representing different variables were expressed as percentage of control attachment or control spreading to FN +/-SD, respectively which stands for 100%. The mean value for cell attachment on FN was calculated as 129.47 +/- 2.95 while the mean number of cells spreading on FN +/- SD was 126.86 +/- 4.2 and considered as 100%. To show any statistical differences between obtained data points when compared to the control point they were calculated by Mann-Whitney test and shown as * (p < 0.05) including both the presence and absence of RGD peptide on cell adhesion mediated by P3 peptide. In addition to indicate significant differences between the single data points symbol ◊ (p< 0.05) was applied.
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Figure 5.3.5a

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Figure 5.3.5b
5.3.6 **P1s (FNRADLKPRCGSSNKSR) peptide does not affect TG2 mediated RGD-independent cell adhesion on fibronectin**

To further confirm any influence of P1, P2, or P3 peptides on FN-TG2 mediated cell adhesion, scrambled peptide encompassing the novel heparan sulfate binding site was designed. Scrambled peptide P1s (FNRADLKPRCGSSNKSR) was generated from the P1 peptide, by random combination of amino acids residues within the P1 sequence. This peptide was then tested in cell adhesion experiments, as previously described (see Section 5.2.2). After reaching confluence, HOB cells were trypsinised and when ready treated with increasing concentrations (100 - 500μg/ml) of P1s alone and/or in the presence of 150μg/ml of RGD peptide. However images representing only results characterised by the lowest and highest concentrations of P1s peptide were presented in this chapter. As for previous peptides (P1, and P2) P1 scrambled did not affect the appearance of the cells when attached to either FN-TG2 or FN matrices (Figure 5.3.6a). Cell adhesion on both matrices in the presence of RGD peptide and its RAD control peptide showed a similar pattern when compared to the untreated control of FN. At all concentrations the P1s peptide demonstrated no effect on cell adhesion (attachment and spreading) on FN-TG2 and only a moderate decrease in cell attachment (estimated at around 10%) when used at the lower concentrations and significant decrease to approximately 20% of cell attachment when used at the highest concentration (500μg/ml) on FN when compared to the control of FN. In addition at concentrations of 100μm, 200μm and 300μg/ml the P1s peptide caused no significant difference in cell spreading on FN alone; however at higher concentrations of peptide (400μg/ml to 500μg/ml) cell spreading was significantly reduced to around 20%. When treated with the whole range of concentrations (100μg/ml to 500μg/ml) of P1s peptide with the addition of 150μg/ml RGD peptide, HOB cells showed no differences in attachment and spreading on FN-TG2 when compared to the FN control but demonstrated a significant decrease of 40% in cell attachment and 46% in cell spreading on FN at the same P1s and RGD peptide concentrations.
Figure 5.3.6 Effect of P1 scrambled analogue (P1s) on the attachment and spreading of HOB cells on FN and FN-TG2 matrices. A Visualisation of different images of HOB cells on FN and FN-TG2 in the presence of scrambled peptide alone and with addition of RGD peptide; B effect of P1s on TG2 mediated RGD-independent HOB cells adhesion on both matrices. The adhesion experiment and further analysis of obtained images was performed according to procedures described under the Materials and Methods (Section 2.2.9) and Methods (5.2.2). Each data point represents the mean percentage of attached cells (cell attachment) or the mean percentage of spread cells (cell spreading) and is expressed as the mean percentage of the control number of cell attachment on FN +/- SD, or cell spreading on FN +/- normalised to 100%, respectively. The mean percentage for attachment of the two separate experiments was 135.2 +/- 3.475, which was considered as 100%. The mean percentage of spreading for the two separate experiments was 132 +/- 4.2, which was established at 100%. The * symbol represents statistical significance (p < 0.05) between the control sample and each data point corresponding to attached and spread cells in the presence of P1s synthetic peptide alone or supplemented with RGD peptide. In addition to indicate significant differences between the single data points symbol ◊ (p< 0.05) was used.
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Figure 5.3.6a
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Cells

RAD

RGD

P1s

Figure 5.3.6.b
The noticeable reduction in cell adhesion when cells were treated with different concentrations of scrambled peptide (P1s) in the presence of RGD peptide on FN suggest that this peptide does not compensate for the loss of cell adhesion mediated by RGD on FN.

5.3.7 P1 peptide does not compete off TG2 from the Heparin binding column

It has already been shown by many investigators (Signorini et al., 1988; Gambetti et al., 2005; reviewed by Verderio et al., 2009) and further confirmed by our studies that TG2 has a high affinity for heparin which is an analogue of heparan sulfate. Moreover cell binding ECM proteins including fibronectin and vitronectin have been demonstrated as heparin binding partners. Since some of the TG2-mediated protein cross-linking is known to be enhanced by glycosaminoglycans (GAGs) such as heparin, it may suggest its supportive role in this process (Sane et al., 1990; Verderio et al., 2009). Therefore it was of importance to test whether peptides generated within the novel heparan sulfate binding site loop were able to interact with heparin and what is even more important compete off the wild type TG2 from the heparin column. So far the physiological importance of each of the three peptides (P1, P2, and P3) has been tested in cell adhesion and spreading assays using HOB cells as the initial cell model. Given that the P2 peptide showed to be insignificant for interfering with TG2-mediated RGD-independent cell adhesion on fibronectin it was of no importance to verify its affinity for heparin. Therefore only the long peptide P1 and short peptide P3 were used on the Heparin column. Cell extracts rich in TG2 protein (Materials and Methods 2.2.6.1 A) were applied to the 5ml bed volume column prior peptide treatment. For this, P1 peptide (10mg/ml) was first diluted into the cell extract to a final concentration of 200μg/ml and incubated for 15 minutes. The column was also pre-incubation with the same peptide for 15 min at the same concentration, prior to initiation of the experiment by addition of the preincubated cell extract to the column. Bound proteins were eluted with an increasing salt gradient (1MNaCl in equilibrating buffer).
Figure 5.3.7 Analysis for competition between the P1 peptide and the wild type TG2 for binding to Heparin. All preparations for the experiment were performed as stated under Materials and Methods (2.2.6.1 A, B). The only difference was that cell lysate containing wt TG2 was pre-incubated for 15 min on ice with 200μg/ml of P1 peptide before loading on the 5ml bed volume Heparin column that was also preincubated with peptide before being equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer. Following gradient elution with 1MNaCl in equilibrating buffer, obtained fractions (1-25) were assayed for the presence of TG2 antigen by SDS-Page and Western blotting (Materials and Methods, Section 2.2.4 and 2.2.5).
Once eluted, obtained fractions were resolved by SDS-PAGE electrophoresis followed by Western blot analysis as detailed in Materials and Method (2.2.4 and 2.2.5). As shown in Figure 5.3.7, TG2 bound to the column with a low and high affinity indicating as previously described in chapter 3 the existence of two distinct populations of the enzyme. However no big difference in the protein elution pattern was noticed when peptide was used. P1 peptide did not compete off the enzyme from the column which bound to it with low and high affinity. The only noticeable difference concerned the bands in the flow-through indicating that some of the enzyme remained unbound to the column. However the result was not credible enough to speculate any ability of the P1 peptide to compete off the TG2.

5.3.8 Ability of the P3 peptide to compete off TG2 from the Heparin binding column

To explore if the heparan sulfate binding site peptide might be more efficient when used in a smaller bed volume of affinity column the 5ml bed volume column was replaced with 1ml bed volume column using the same supplier (Amersham Biosciences, Sweden). This time, not only P1 peptide but also its scrambled analogue P1s peptide and the P3 peptide were applied into the experimental system. Peptide solutions were prepared by dissolving each of the peptide in the cell extract containing around 4mg/ml of TG2 protein mixed with equilibration buffer to a final concentration of peptide 1mg/ml. Such prepared peptide solutions were preincubated for 15 min before being applied on the Heparin column which was also pre-treated with 1mg/ml of each of the peptide for the same period of time. Proteins were eluted using a linear gradient of high salt buffer (100mM-1.0M NaCl in equilibrating buffer) at a flow rate of 2ml/min with a 45min gradient reaching 35% high salt buffer. All collected fractions (flow-through, wash and elution) were assayed for the presence of TG2 antigen using SDS-PAGE followed by Western blotting (Materials and Methods, section 2.2.4 and 2.2.5). Initially this linear salt gradient elution revealed a big proportion of control sample containing only TG2 with low and high binding affinity of enzyme for the column (Figure 5.3.8 A). In contrary to the control sample, it showed some remaining unbound proteins in the column flow-through.
as well as in the wash of the sample containing the P3 peptide (see red arrow on Fig. 5.3.8 A). The presence of unbound proteins in the wash could suggest P3 is interfering with TG2 for binding to Heparin. However due to its very small amount, fractions containing these proteins were concentrated by freeze-drying and then once more verified for the presence of TG2 by immunoblotting. The same approach was used for the P1 peptide and for the control samples containing scrambled peptide and the other sample containing only TG2. As seen on every image in Fig. 5.3.8 B none of the peptides caused any differences in the TG2 elution pattern when compared to the sample containing only the wild type TG2. Each single blot showed bands representing unbound proteins in the flow-through fractions, with additional proteins present in the wash and proteins which bound to the column with low and high affinity. These results indicate that neither the P1 nor P3 peptide has enough binding strength to bind to Heparin and compete off the wild type enzyme. However their binding affinity to Heparin cannot be ruled out completely since there might be some special conformational requirements that are necessary for such a binding. Furthermore heparin-like material still present in the cell extract that was initially pre-treated with each of the peptides may possibly interfere with binding to Heparin.
Figure 5.3.8 A Western blot analysis of TG2 eluted fractions from Heparin sepharose column demonstrating negligible competition between the wild type TG2 and the short peptide P3 (GRDCSRRSS). Cell extracts (~150µl) containing only TG2 and additionally P3 peptide at a final concentration of 1mg/ml were applied to a 1ml bed volume Heparin sepharose column equilibrated in 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer and run through the column as previously described under Methods (Section 5.2.3). The column was however pre-incubated with 1mg/ml of P3 peptide before loading with peptide solution was initiated. Proteins were eluted with linear gradient of salt dissolved in equilibrating buffer. Both flow-through and resulting fractions (7-16) were assayed by SDS-PAGE electrophoresis and Western blotting using anti-TG2 monoclonal antibody (CUB 7402) for the presence of TG2 antigen as stated under Materials and Methods (2.2.4 and 2.2.5).
Figure 5.3.8 B Comparison showing TG2 binding to the Heparin Sepharose column in the presence of P1 and P3 peptides and the scrambled P1 counterpart. Cell lysates from transfected with wild type TG2 HEK293/T17 cells mixed with 50mM Tris-Cl, 1mM EDTA, 1mMDTT pH 7.5 buffer were pre-incubated with P1, P3, and P1s peptide respectively, to a final concentration of peptide 1mg/ml and then loaded onto a 1ml Heparin sepharose column prepared as described previously (Methods 5.2.3). Proteins were eluted with increasing gradient of 1MNaCl in 50mM Tris-Cl, 1mM EDTA, and 1mM DTT, pH 7.5. All fractions including flow-through and eluted proteins (7-16) were analysed for the presence of TG2 using SDS-PAGE electrophoresis followed by Western blotting as stated in Materials and Methods (2.2.4 and 2.2.5).
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![Diagram showing protein bands and fraction numbers](image)

Figure 5.3.8 B
5.4 DISCUSSION

It is known that many features of cell behaviour are regulated by the interaction of the cell with the extracellular matrix. To provide the integrity and structure of the cell, it is essential for it to adhere to the matrix. Since FN is one of the major ECM proteins that binds to cells and TG2 has a high affinity for FN it was not surprising that in vitro cell adhesion studies have shown that cell adhesion to FN in some cells is enhanced when in complex with TG2 (Akimo et al., 2000; Verderio et al., 2000; Verderio et al., 2003). The initial findings on HOB and Swiss 3t3 cells described that TG2 promotes cell adhesion to fibronectin through an RGD-independent cell adhesion mechanism (Heath et al., 2002; Verderio et al., 2003).

To verify these findings, an initial experiment with TG2 immobilised on human plasma FN, using competitive concentrations of RGD peptide was performed. Peptides when assayed for their inhibitory functions, can however cause non specific effects, thus it was essential to use in addition to GRGDTP inhibitory heparaxapeptide, an inactive GRADSP control hexapeptide (Verderio et al., 2003). In the cell adhesion assay the effect of RGD peptide on HOB cells seeded on FN and FN-bound TG2 matrices was studied. As suggested by the same investigators Verderio et al., (2003) the incubation of cells with RGD peptide should not exceed a certain period of time due to the risk of contamination by endogenously secreted ECM proteins, therefore peptide treatment was restricted to around 25 minutes. Under these conditions, the RGD peptide caused inhibition of cell attachment and spreading on FN, which was however rescued in the presence of TG2. These results were in agreement with previous findings, confirming the importance of FN bound TG2 as an enhancer of cell adhesion and its involvement in an RGD-independent adhesion pathway (Heath et al., 2002; Verderio et al., 2003).

Although the participation of the integrin binding domain (RGD) has been shown to be crucial for cell adhesion to FN, the involvement of the heparin binding domain HepII, has also been suggested (Woods et al., 1986). The results from experiments on immobilised FN or different FN fragments revealed that the binding of cell-surface proteoglycans to FN
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is necessary for cell-adhesion (Woods et al., 1986, Saoncella et al., 1999; Park et al., 2000). Other data presented by Sim et al., (2004) indicated the direct role of cell-surface HS in the binding of FN to primary human osteoblasts (HOBS). Recently TG2 was also shown to have a high affinity for heparin/HS (Gambetti et al., 2005; Verderio et al., 2009) and this TG2-HS interaction was further demonstrated to affect cell-matrix adhesion (Verderio et al., 2003; Telci et al., 2008). Till now no detailed information has been presented regarding the heparin/HS binding site on the TG2 molecule. However our finding with the HS2 mutant allowed us to speculate where such a binding place could be localised. As previously described (Verderio et al., 2009; Telci et al., 2008) it is known that TG2 can directly interact with the HS chains of syndecan-4. Although HS chains are characterised by the high degree of structural diversity, they are classified as dissacharide chains (Kirkpatrick and Selleck, 2007). Therefore the interaction between the different length saccharides and the proposed heparan sulfate binding loop of TG2 which additionally was locked in two distinct conformations was investigated. The docking studies reported the pentasaccharide to fit perfectly into the loop surrounded by the different positively charged amino acid residues including residues suggested to be important for the TG2-HS interaction when the enzyme was adopted in a close conformation. Obtained results indicated that this part of TG2 is involved in the binding of HS chains and in addition revealed the conformational requirements for efficient binding.

Since the docking studies supported the discovered novel heparin/HS binding site on TG2 it was of importance to generate a P1 peptide containing the possible heparin binding consensus sequence (NPKFLKNAGRDCRSSS) and further evaluate its physiological significance in cell adhesion assays. In an attempt to examine whether P1 peptide will contribute to inhibition of HOB cell attachment and/or spreading on a FN bound TG2 matrix, competitive concentrations of this peptide were first tested in the experimental system. Additionally to emphasise the inhibitory effect on RGD independent cell attachment and spreading the P1 peptide was supplemented with the RGD peptide. The data reported here shows a significant and dose-dependent reduction of cell attachment and spreading on both FN and FN-bound TG2 matrix in the presence of P1 peptide. The same results for cell attachment and spreading were also noted on FN-TG2 when RGD peptide was used in the system. Therefore these data indicates that P1
peptide inhibits the TG2-HS interaction thus abolishing the TG2 mediated RGD-independent cell adhesion; however because of its strong inhibitory effect on cell adhesion on FN alone it is possible that this peptide is also affecting other elements important to cell adhesion.

To verify whether the length of the peptide could somehow cause it to be non specific, two short peptides derived from the P1 peptide were generated and applied in the cell adhesion assay. Similar to the previously described approach, increasing concentrations of P2 peptide (NPKFLKNA) and P3 peptide (GRDCSRRSS) were applied to FN alone and FN matrix with immobilised TG2. The influence of the peptide on HOB cell adhesion either alone or in the presence of RGD peptide was examined. The levels of cell attachment and spreading when treated with P2 peptide only appeared to be unchanged on FN bound TG2 which still retained the same TG2 supportive role in the presence of RGD peptide. This finding indicates that this particular part of the peptide does not block the heparin binding site on TG2 hence still allowing TG2 to promote cell adhesion on FN independently in the presence or absence of RGD. Surprisingly cell adhesion on FN alone with higher peptide concentrations showed a significant reduction suggesting the P2 peptide may be acting by blocking the cell surface heparan sulfate proteoglycan receptors thus impairing osteoblasts adhesion to FN when present at high concentrations. However the opposite result was noticed when the P3 peptide was used. This peptide when incubated with cells seeded on a FN-TG2 matrix supplemented with RGD peptide suppressed TG2 mediated cell adhesion on FN in a dose-dependent manner indicating its disrupting influence on the interaction between TG2 and cell surface HS. In addition on FN alone it brought the loss of cell adhesion induced by RGD peptide. Firstly these results suggest P3 peptide like P1 peptide may be able to mimic the heparin binding site on TG2 thus representing a novel HS binding motif within the TG2 structure. Secondly it is in agreement with previous findings of Verderio et al., (2003) suggesting that TG2 promotes RGD-independent cell adhesion on FN, which takes place via interaction of the FN bound TG2 with cell surface heparan sulfate proteoglycans.

As stated earlier to avoid the potential non-specific reduction of cell attachment and spreading, control peptide should be applied in the cell adhesion assay. In this chapter, scrambled peptide P1s containing a random combination of amino acid residues within
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the P1 peptide was chosen as control peptide to rule out non-specific effects. P1s did not interfere with cell adhesion on a FN bound TG2 matrix. Although the RGD peptide caused inhibition of cell adhesion on FN, it was restored when cells were seeded on FN-TG2. These results suggest that a relatively small non-specific reaction may take place especially when used with cells binding to FN alone, however it is not great enough to speculate any major influence of the control peptide on cell adhesion.

In vitro binding studies have identified heparin (heparan sulfate analogue) as a high affinity binding partner for tissue transglutaminase (Signorini et al., 1988; Gambetti et al., 2005). Hence in the initial experiment, the ability of P1 long peptide to bind and further compete off TG2 from the Heparin sepharose column was investigated. In the presence of P1 peptide, TG2 could still bind to the heparin with low and high affinity indicating P1 peptide not to have strong enough affinity to displace TG2. Since P3 peptide like P1 was shown in a physiological assay to inhibit the heparan sulfate binding site on TG2 it was further chosen for its interaction with heparin. In addition as a control for the reaction, P1s peptide was applied. It was very surprising when the same or a very similar binding pattern was obtained for TG2 when all the peptides used to compete off TG2 were loaded onto the column. TG2 was not displaced from the heparin column and TG2 still bound with the same affinity. Although these results suggest that P3 peptide does not compete for the heparin binding site on TG2, its affinity for heparin cannot be ruled out completely. It is possible that it did not displace TG2 because it was bound out by the heparin binding -like impurities in a cell extracts containing TG2. The other possibility is due to the conformational requirements for binding to the column. As shown in a chapter 3, TG2 binds tightly to the column and needs to be in a closed conformation; however even in an open form it still has affinity for the column. Because of the nature of the peptide we cannot confirm what conformation it adopts when it interacts with heparin. In addition the P3 peptide is a short peptide thus it could be very easily washed away from the column resulting in no effect on TG2 binding to it.

To sum up a single linear peptide may not compete with a 3 D structure of TG2 which may be necessary for binding to Syndecans but not to heparin.
Chapter VI: Final discussion
Chapter VI: Discussion

6.1 DISCUSSION

Available evidence suggests that tissue transglutaminase is mainly a cytosolic protein with around 70-80% of the enzyme localised in the cytoplasm (Griffin et al., 2002; Lorand and Graham, 2003). Meanwhile a smaller but significant fraction of membrane associated and extracellularly present TG2 has also been reported (Gaudry et al., 1999a, b; Griffin et al., 2002; Fesus and Piacentini et. al. 2002; Zemskov et al., 2006). The physiological reason for such a location of TG2 is still poorly understood. A variety of different investigations have been undertaken in order to explain how TG2 is targeted to the cell surface and further into the ECM since it has no leader sequence and is not glycosylated. Some suggest that the presence of the enzyme outside the cell is due to the cell damage (Upchurch et al., 1991; Kawai et al., 2008) while the others showed that TG2 can be externalised from the healthy cells (Vederio et al., 1998; Gaudry et al., 1999). Although, limited information is available in this field, some findings indicate the potential involvement of TG2 binding partners in the targeting of the enzyme to the cell membrane. Depending on its subcellular localisation, TG2 has been shown to interact with different molecules that are considered to be enzyme substrates. They may include GTP in the intracellular environment and in the extracellular environment cell surface receptors such as integrins and HSPGs (heparan sulfate proteoglycans), and the most ubiquitous ECM molecule, fibronectin (Achyutan and Greenenberg, 1987; Akimov et al., 2000; Verderio et al., 2009; Lorand et al., 1988; LeMosey et al., 1992). It is therefore possible that the alterations in TG2 binding sites corresponding to some of these proteins could have implications in targeting and further subsequent fate of its externalisation. In order to define a role of known TG2 binding partners, one of the aims of this thesis was to generate specific TG2 mutants and further evaluate their role in the cell secretion of the enzyme to the cell surface and its subsequent deposition into the ECM.

Defining the binding sites for all TG2 binding partners was not straight forward. So far only GTP binding sites as well as possible FN binding sites have been mapped within the structure of the enzyme (Ismaa et al., 1997, 2000; Liu et al., 2002; Begg et al., 2006a; Hang et al., 2005). Thus it was not surprising, that two main GTP binding site residues
localised within the core domain and two main FN binding site amino acids within the N-terminal site including the first 15 amino acids of the same TG2 N-terminal tail were chosen for mutagenesis (Ismaa et al., 2000; Gaudry et al., 1999a; Hang et al., 2005). The binding sites for both integrins and HSPGs within the structure of TG2 are still not mapped. With the major focus on discovering a novel heparan sulfate binding site for TG2, examination of the crystal structure of TG2 was undertaken using the consensus sequence XBBXBX. As a result two likely candidates such as $^{590}KIRILGEPKQRK^{602}$ (HS1) localised at the C-terminal tail of TG2 and $^{202}KFLKNAGRDCSSPVYVGR^{222}$ (HS2) restricted to the core domain of enzyme were chosen. The findings presented in this thesis reveal for the first time a potential novel heparan sulfate binding site which is localised within the catalytic core domain of TG2 where the HS2 mutant (K205A, R209A) was generated.

Inside the cell where the calcium concentration is low, TG2 binds GTP (Achyutan and Greenberg, 1987). This interaction is independent of the enzyme active site and can link it to a signalling pathway via the binding of alpha 1-adrenergic receptors (Nakaoka et al., 1994). The binding of GTP forces conformational changes of TG2 which when in its GTP-bound form adopts a closed conformation (Smethurst and Griffin, 1996; di Venere et al., 2000). In contrast calcium binding is essential for TG2 to allow access to the catalytic triad at the active site thus facilitating the transamidating activity of the enzyme to take place. It is therefore possible that modulation of conformational changes, provoked by TG2-GTP interactions, may be important in the regulation of TG2 presence either at the cell surface or outside the cell. So far GTP binding sites have been mapped within the structure of the enzyme. A number of reports identified different residues to be important for GTP-binding and hydrolysis in rat and human TG2 (Ismaa et al., 1997, Ismaa et al., 2000; Begg et al., 2006). Residues Ser171 and Lys173 localised within the catalytic core domain and Arg580 present within the barrel 1 have been indicated to be important for GTP binding (Ismaa et al., 2000; Begg et al., 2006). Unlike other investigations, this study was based on using two TG2–GTP defective binding forms GTP1 (S171E) and GTP2 (K173L) to provide insights into the translocation pattern of the enzyme released at the cell surface and into the ECM. Both GTP mutants reduced the activity of the enzyme either on the cell surface or in cell lysates suggesting that TG2 activity is destabilised by these GTP mutations. This
finding is not necessarily consistent with early reports (Ismaa et al., 2000) which indicated that impairment in GTP binding caused by S171E and K173L did not affect TG2 activity. Further investigations of Antonyak et al., (2001) on transiently tranfected NIH3T3 cells revealed however that the human S171E mutant is poorly expressed and significantly reduces TG2 activity suggesting that TG2 activity is critically dependent on the presence of this GTP binding site. Ser171 does not seem to interact directly with GTP (Begg et al., 2006a); therefore the lack of activity may be peculiar to that mutation rather than its ability to bind GTP. The poor expression of GTP1 and the almost undetectable level of the mutant at the NIH3T3 cell surface was shown to be opposite to the result obtained with HEK293 cells. Nevertheless the GTP1 mutant (open conformation) was found in smaller amounts on the cell surface of both cell types and was deposited less into the ECM, when compared to GTP2 mutant (uncharacterised conformation). However the GTP2 seemed to be secreted in much larger amounts. These findings suggest that externalisation of the enzyme is affected by the conformational changes caused by substitution of Ser with Glutamic acid rather than a lack in GTP binding.

Despite lacking a typical ER targeting signal peptide and the characteristics of a cytoplasmic molecule, TG2 presence was detected in the membrane fractions (Griffin et al., 1978; Barnes et al., 1985; Tyrrell et al., 1985) and in the ECM (Aeschlimann and Paulsson 1994, Jones et al., 1997; Johnson et al., 1997 and 1999; Verderio et al., 1998). It is known that different cell types express a variety of transmembrane proteins and receptors such as integrins, selectins and CAMs (cell adhesion molecules) as well as HSPGs (heparan sulfate proteoglycans) (Akimov et al., 2000; Zemskov et al., 2006). Some of them are recognized from their ability to directly or indirectly associate with TG2 and are thus believed to be involved in targeting of the enzyme to the cell membrane. So far both integrins and HSPGs were suggested to be high affinity binding partners of TG2 (Gaudry et al., 1999b; Akimov et al., 2000; Johnson et al., 2008; Verderio et al., 2009). Therefore applying the suggestions of previous investigators (Akimov et al., 2000) regarding a possible integrin binding site within the β-barrel 2 domain of TG2, the C-terminal deletion mutant (C) was generated. Analysis of the lysates of HEK293 and NIH3T3 cells transfected with the C-terminal deletion mutant revealed the expression level of this mutant to be greater than that of the wild type TG2 or comparable to it.
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Although the C-terminal deletion mutant showed a significantly reduced activity than that of the wild type TG2 on the cell surface of HEK293 cells, and a comparable activity to the wild type enzyme on the cell surface of NIH3T3 cells it was still secreted to the cell surface of both cell types and further distributed into the ECM. This result could suggest that the C-terminal domain of TG2 is not implicated in the enzyme externalisation process. However, a recent publication suggesting that the integrin binding sites are likely to be located within the N-terminal domain of TG2 (Hang et al., 2005) does not rule out their involvement in TG2 secretion and its localisation within the ECM.

Moreover this study has also provided insight into the expression of the two novel mutants HS1 (K600A, R601A, K602A) and HS2 (K205A, R209A) identified as possible TG2 heparan sulfate binding mutants. The Ala substitution in the amino acids consensus of HS1 and HS2 mutants was considered likely to disturb the protein folding according to the protein structure viewed by a modeling program. Indeed, analysis of lysates expressing both of the mutants revealed their expression level to be comparable or greater than that of wild type. However to verify whether any of these mutants were folded correctly, their interaction with heparin was examined. There is evidence indicating high affinity between heparin which is considered to be an analogue of heparan sulfate and TG2 (Signorini et al., 1988; Gambetti et al., 2005). Experiments with Heparin Sepharose columns have shown that the binding of the enzyme to heparin does not interfere with the enzymatic activity of TG2 but protects it from thermal unfolding and proteolytic degradation (Gambetti et al., 2005). In keeping with these findings, this study demonstrated a high and low affinity of the wild type enzyme to heparin. Further examination revealed that this affinity to heparin is conformation dependent and is much greater when TG2 is in a GTP-bound form than in the open conformation when bound to the irreversible inhibitor of TG2 activity. It is also greater than that of the inactive Cys277 mutant form which stabilises the enzyme in an open conformation. Moreover no difference in the binding pattern to heparin between the HS1 and wild type enzyme suggested this particular mutation is not involved in TG2-HSPGs interactions. However, great discrepancies in the binding pattern of the HS2 mutant to that of wild type indicated the HS2 mutation site to represent a novel heparan sulfate binding site on TG2. Additional verification of the mutants’ activity confirmed that the low affinity of HS2 for the column resulted from the
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substitution of two distinct amino acids with Ala and not from protein degradation. Further analysis of the triple and double mutants revealed that both of them had no greater influence on TG2 activity either at the cell surface or in the cell lysates. In addition the HS2 mutant was found in much smaller amounts than that of wild type enzyme when released to the cell surface or distributed into the ECM suggesting once more that this particular binding site is involved in the enzyme’s association with HS given earlier data (Verderio et al., 2009).

Since the enzyme is known to be deposited into the ECM under normal physiological conditions (Verderio et al., 1998) as well as during the stress or inflammation caused by damage to the cell (Johnson et al., 1999; Upchurch et al., 1991) it’s affinity for the matrix proteins especially for FN, which is abundant at wound sites and the most ubiquitous component of the ECM is not surprising. Thus a number of reports have analysed the possible FN binding sites on TG2 and revealed that the likely intact N-terminal tail of TG2 (Gaudry et al., 1999a) and the two residues encompassing Asp94 and Asp97 (Hang et al., 2005) are involved in TG2-FN interactions. The deletion of the first 15 amino acids (Δ1-15) from the TG2 N-terminus and substitution of these two amino acids with Ala (FN1 D94A and D97A) resulted in either poor or good expression of the mutated TG2 in the HEK293 and NIH3T3 cells, respectively. The double point mutant FN1 and the Δ1-15 N-terminal deletion mutant (N) both showed reduced binding to FN. However a greater reduction of binding to FN was caused by the Δ1-15 N-terminal deletion (N) when compared to the double point mutant (FN1). The FN1 mutation did not affect enzyme activity either intracellularly or at the cell surface. It is evident that although FN1 was suggested to be the main FN binding site on TG2 (Hang et al., 2005), the mutation has retained a similar distribution pattern to the wild type whether it was released at the cell surface or distributed into the ECM. Conversely, the N-terminal deletion mutant (N) had decreased TG2 activity on the cell surface and in cell lysates and was detected in greatly reduced amounts at the cell surface and in the ECM (Figure 6.1). In addition when compared to the FN1 mutant it was characterised by a low capacity to interact with Heparin an analogue of HSPGs which are present at the cell surface and in the ECM. All together these results suggest that the first 15 amino acids of the N-terminal tail of TG2 are important for both enzyme activity and its distribution on the cell surface and into the
matrix. In support of this hypothesis, previous data has demonstrated that TG2 when released from cells co-localises at the cell surface in association with FN, and that the intact N-terminus of TG2 mediates this interaction. Early experiments with SDS-denatured TG2 fragments identified the first seven amino acids to be important in this process (Jeong et al., 1995) which was further confirmed using expression vectors encoding truncated TG2 lacking these amino acids from the N-terminal tail of TG2 (Gaudry et al., 1999a). Furthermore Hang et al., (2005) designed a synthetic peptide mapped to the first 12 amino acids from the N-terminal tail and additionally produced a TG2 deletion mutant with the first 15 amino acids deleted. Given that no direct binding of the synthetic peptide to fibronectin took place and the removal of the first 15 amino acids of TG2 did not appear to significantly reduce the interaction between truncated TG2 and the 42kDa FN fragment, the new fibronectin binding site on TG2 encompassing 88WTATVVDQQDCTL506 was proposed. However according to results presented in this thesis, the truncated TG2 lacking the first 15 amino acids greatly inhibited its binding to human plasma fibronectin. Therefore in an attempt to explain these discrepancies the synthetic peptide 2AEELVLERCDLEEL5 based on the proposed truncation was generated and examined for its ability to reduce and/or further compete with the enzyme for binding to fibronectin. Additionally for the scrambled peptide 2EECRLAEELLEDVL15 it was necessary to use the synthetic peptide 88WTATVVDQQDCTL506 as a control, so any changes in TG2-FN interaction could be attributed to the 2AEELVLERCDLEEL5 peptide. The peptide P4, 2AEELVLERCDLEEL5, but not its scrambled analogue and other control peptide was able to moderately inhibit interaction between the enzyme and fibronectin. This moderate inhibition could result from the fact that only the small fragment of this peptide is extended beyond the N-terminal domain and its second part is localised within the β-sheet and further partially covered α-helix, thus it makes it hard for it to be fully accessible by the fibronectin molecule. All together these results suggest that the first 15 amino acids of the N-terminal tail of TG2 represents a recognition motif on the TG2 molecule involved in the interaction of the enzyme with fibronectin.

Understanding how TG2 can have so many pleiotropic functions in cells remains a challenging assignment. TG2 is believed to associate with many molecules present in the different cellular compartments which in turn influence cellular behaviour and its
physiology. Once externalised from cells, TG2 is removed from the cell surface and localised within the ECM, where it was shown to play a role in the cross-linking and stabilisation of matrix proteins (Aeschlimann and Paulson 1991; Raghunath et al., 1996; Zemskov et al., 2006). The extracellular matrix is an insoluble, adhesive complex supported by various structural and functional proteins released by cells themselves. These adhesive molecules associated with the solid matrix include, laminin, vitronectin, fibronectin and collagens, from which fibronectin have been particularly shown to be a good substrate for TG2 (Lorand and Turner, 1988; LeMosey et al., 1992; Gaudry et al., 1999a; Hang et al., 2005).

Figure 6.1 Cellular distribution of wild type TG2 and TG2 constructs including HS1 (K600A, R601A, K602A), HS2 (K205A, R209A), GTP1 (S171E), GTP2 (K173L), FN1 (D94A, D97A), N (Δ1-15), and C (Δ594-687).
When outside the cell, TG2 interaction with FN may be mediated via the cross-linking activity of the enzyme (Jones et al., 1997; Verderio et al., 1998) or via TG2 binding to FN (Baliklava et al., 2002; Verderio et al., 2003) in a manner independent of its transamidating activity leading to increased cell attachment to the ECM. Thus later studies revealed that TG2 may act as a novel adhesion receptor for cell surface FN (Akimov et al., 2000; Belkin et al., 2001; Kabir-Salmani et al., 2005; Zemskov et al., 2006) and this association was shown to be further involved not only in adhesion of cells to the matrix (Akimov et al., 2000; Akimov and Belkin, 2001) but also in cell migration, signalling pathways and matrix assembly (Akimov et al., 2000; Belkin et al., 2001; Akimov and Belkin 2001; Akimov and Belkin, 2001; Verderio et al., 2003).

Consistent with findings that TG2 is not only released from healthy cells but also in response to cell damage or injury (Upchurch et al., 1991; Haroon et al., 1999; Johnson et al., 1999) which causes up-regulation and enhanced deposition into the ECM where it associates with FN fibrils or plasma FN (LeMosey et al., 1992; Gaudry et al., 1999a; Verderio et al., 1998), a cell model based on imitating normal and pathological conditions in vivo was generated. This model was based on the hypothesis that the TG2-FN association with cell surface heparan sulfate proteoglycans could mediate cell survival during matrix turnover when RGD containing peptides are released via proteolysis (Verderio et al., 2003). Initial investigations examined the function of TG2-bound FN matrix in cell-matrix interactions when inhibition of the classical RGD-dependent adhesion mechanisms was initiated. To achieve this the synthetic peptide GRGDTP containing an RGD consensus as well as anti-integrin function blocking antibodies directed against α5 and β1 subunits on osteoblasts were applied. The results were straightforward, obstructed RGD cell binding motifs on a FNIII10 domain, prevented its interaction with cell surface α5β1 integrins and further cell-matrix interaction, which however was restored upon the seeding of cells on a TG2-FN matrix (Verderio et al., 2003). In support of previous findings, the data presented in this thesis confirm that TG2 can support RGD-independent osteoblast adhesion when bound to FN. In addition the examination of other cell types including mouse 3T3 fibroblasts and the epithelial-like cells ECV-304 revealed the same restoration effect verifying once more the adhesive function of TG2 (Verderio et al., 2003).
Available data also suggested that the FN-bound TG2 RGD-independent cell adhesion, could not take place through other integrin receptors localised at the cell surface (Verderio et al., 2003; Telci et al., 2008) since the involvement of α4β1 integrins, that are found in negligible amounts in osteoblasts (Gronthos et al., 1997) and which are not involved in the formation of stress fibres (Sechler et al., 2000) seemed unlikely. Initial studies on the distribution of transglutaminase, demonstrated that TG2 in rat lung is strongly connected with its insoluble matrix which was found to contain high levels of heparan sulfate (Cocuzzi and Chung, 1986). Further investigations based on using the competitive substrate (mono-dansylcadaverine), demonstrated the formation of high molecular weight dermatan sulfate proteoglycans in bovine aortic endothelial cell cultures (Kinsella and Wight, 1990). Additionally by using in vitro purification techniques for TG2 involving Heparin sepharose columns (Gambetti et al., 2005) and more physiological assay such as surface plasmon resonance (SPR) (Scarpellini et al., 2007) the affinity of TG2 to heparin, an analogue of heparan sulfate (Signorini et al., 1988) was confirmed. Moreover the treatment of HOB cells with heparinase and chondroitinase ABC revealed that only digestion of HSPGs with heparinase but not digestion of chondroitin sulphates with chondroitinase ABC, reduced FN-bound TG2 mediated RGD-independent cell adhesion. This indicated that HS chains are particularly important in supporting cell attachment to the ECM by TG2 (Verderio et al., 2003). Even though a vast amount of information has been revealed regarding TG2-HSPGs interactions, so far the binding site for HSPGs on the TG2 structure has not been found. Such a binding site has however been mapped within fibronectin, indicating that the HepII/IIICS region was mainly involved in its interaction with HSPGs and integrins and implicated in the cell adhesion process (Woods et al., 2001; Chon et al., 2001). In addition the effect of GAGs and FN fragments on cell attachment to FN coated surfaces showed that the binding to cell surface proteoglycan receptors was necessary for successful cell adhesion (Woods et al., 1986; Saoncella et al., 1999; Woods et al., 2000; Park et al., 2001; Sim et al., 2004). Although HSPGs have been shown to be necessary, they are not fully engaged in the cells binding to FN alone. Thus it was not surprising when Verderio et al., 2003 observed in HOB and Swiss 3T3 cells that the additional presence of TG2 and its possible interaction with HSPGs caused increased cell binding to the FN matrix (Verderio et al., 2003).
Given that the supported mechanism of adhesion was shown to be TG2, FN and HSPGs dependent, it was hypothesised that the heparin-binding peptide derived from TG2 could significantly impair interaction between TG2 and the common high-affinity binding sites on the heparan sulfate chain thus reducing FN-bound TG2 mediated RGD-independent adhesion mechanism. Since in this thesis a clear indication of such a binding site within the TG2 molecule has been proposed, it was of interest to generate a synthetic peptide encompassing the novel heparin binding site. However to generate such a peptide, protein modeling studies using three dimensional TG2 structures were undertaken. The data presented previously, showed osteoblast cell lines to be commonly used in adhesion experiments (Gronthos et al., 1997; Heath et al., 2002; Verderio et al., 2003) because of possessing a well defined pattern of integrin receptors, mainly β1 integrins and HS receptors (Verderio et al., 2003; Sim et al., 2004). It was therefore sensible to use this cell line as a cell model. Our results suggest that the possible heparin-binding peptide 200 NPKFLKNAGRDCRSS216 derived from the indicated sequence within the catalytic core domain of TG2 may act non specifically thus decreasing cell attachment not only to the FN bound TG2 matrix but also to the FN matrix alone as assessed by the physiological assay. These findings were consistent with an observed dose-dependent decrease in cell-matrix adhesive strength (See chapter 5). In addition it was noticed that the capacity of the heparin-binding peptide to reduce cell adhesion on FN-TG2 was not changed in the presence of RGD synthetic peptide. Such a strong inhibition by the generated peptide can only suggest its indirect effect on cell surface heparan sulfate proteoglycan receptors thus altering osteoblasts adhesion to FN which also takes place when integrin receptors are blocked by the presence of RGD peptide. Recent observations have identified one of the HS proteoglycans, syndecan-4 as the only member of the syndecan family that is able to enhance focal adhesion assembly (Woods and Couchman, 1994; Saoncella et al., 1999; Woods et al., 2000) and to interact through its heparan sulfate chains with integrins during focal adhesion formation (Woods and Couchman 1994; Couchman 2003). The influence of syndecan-4 and integrins on cell adhesion to fibronectin domains involves a series of downstream signalling pathways including the activation of tyrosine kinases by the integrins. In addition, the syndecan-4 cytoplasmic domain was shown to bind phosphatidylinositol 4, 5, biphosphate thus initiating conformational changes that could lead to activation of the protein kinase PKCα (Shin et al., 2001; reviewed by Xian et al., 2004).
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2010) further promoting cell spreading. Cell attachment and spreading studies suggested syndecan-4 as the possible co-receptor for the TG2 adhesive functions (Verderio et al., 2003). As shown by Telci et al., (2008) fibronectin associated TG2 can interact with syndecan-4 heparan sulfate chains thus promoting RGD-independent cell adhesion and further activating PKCα leading to its interaction with β1 integrins and downstream signalling pathways. Therefore it is plausible that in addition to the targeted inhibition of TG2 heparan sulfate interactions by soluble P1 peptide, it’s possible interaction with the cell surface syndecan-4 heparan sulfate chains could lead to strong (> 50%) impairment of osteoblast attachment to FN possibly via the HEP II binding site. Such a non specific binding of the peptide with syndecan-4 chains could further affect β1 integrin-mediated cell adhesion to the RGD cell binding motif on FN thus leaving it unaffected by the additional presence of RGD peptide as shown in the experimental system.

Hence to avoid this unwanted interaction, the long peptide was divided into two fragments. Further work presented in this thesis revealed that one of the short peptides derived from the 200NPKFLKNAGRDCRSS216 effectively reduces cell attachment and spreading to the FN bound TG2 matrix without having additional large non specific effects on cell adhesion to FN alone. The P3 peptide 208GRDCRSS216 but not its scrambled longer analogue showed a dose-dependent decrease, in the cell adhesion to FN-TG2 in the presence of the RGD peptide. Interestingly the P3 peptide also showed a compensation of the RGD induced loss of cell adhesion on FN alone possibly by mimicking the heparan sulfate binding site on TG2. The results presented here for the first time strongly suggest that this specific peptide is able to mimic the heparin binding site on TG2 hence representing the novel heparan sulfate binding motif on enzyme. This discovery is in agreement with the previous presumptions of Verderio et al., (2009) indicating clusters rich in Lys and Arg residues as possible TG2 heparan sulfate binding motifs. In addition the fact that two positively charged residues within the P3 peptide (Arg 209 and Arg 213 from which one was selected to generate the HS2 mutant), are conserved within different species (human, monkey, bovine, mouse, rat) and are not just specific for human TG2 (Figure 6.2) also supports this finding.
Figure 6.2 TG2 sequence alignment. Alignment is showing conserved (green) positively charged amino acids in position 202, 205, 209, 213, 222, and 387 among different species expressing TG2 as well members of transglutaminase family. Non-conserved residues are indicated by red.

An unresolved aspect of these findings is the lack of the ability of the long peptide 200NPKFLKNAGRDCSRRSS216 and the short 208GRDCSRRSS216 peptide capacity to bind and further compete off the wild type TG2 from the Heparin Sepharose column. Since both peptides consisted of clusters of positively charged amino acids, namely Arg and Lys, they were considered to be able to easily interact with the negatively charged GAG chains of heparin. However their limited association with the column could result from the fact that both sequences of the long and short peptides did not contain the putative heparin – binding motif (XBBXB consensus) characterised previously by Cardin and Weintraub, (1989) as important for association of heparan sulfate derived peptides with heparin. In addition previous studies indicated that synthetic peptides may represent a small part of a bigger contiguous binding motif therefore be frequently less active than the intact protein they were derived from (Rouslahti and Pierschbacher, 1987; Mooradian et al., 1992). This might be the case since results with the Cys277 mutant indicated the active site conformation of TG2 to play an important role for its interaction with Heparin. Moreover during synthesis changes of secondary structure can appear (Rouslahti and
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Pierschbacher, 1987) thus causing significant alterations to the stabilisation of the protein folding and reduced association with the column which enables it to compete off the parental protein. Additionally the limited interaction with the column may be due to the competition between the peptide and HSPGs that could be still present in HEK 293T cell extract (Schoefield et al., 2003).

Consistent with the data presented in previous reports, our findings suggest that TG2 through its interaction with FN and HSPGs contributes to maintaining cell-matrix interactions. Moreover identification of an intact N-terminal tail as a key element involved in TG2 association with cell surface FN and a novel HS binding site within the TG2 structure further indicated the conformational requirements necessary for this enzyme interaction with Heparan sulfates and with heparin.
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