RGD-independent Cell Adhesion via a Tissue Transglutaminase-Fibronectin Matrix Promotes Fibronectin Fibril Deposition and Requires Syndecan-4/2 and α5β1 Integrin Co-signaling*[S]

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Fibronectin (FN) deposition mediated by fibroblasts is an important process in matrix remodeling and wound healing. By monitoring the deposition of soluble biotinylated FN, we show that the stress-induced TG-FN matrix, a matrix complex of tissue transglutaminase (TG2) with its high affinity binding partner FN, can increase both exogenous and cellular FN deposition and also restore it when cell adhesion is interrupted via the presence of RGD-containing peptides. This mechanism does not require the transamidase activity of TG2 but is activated through an RGD-independent adhesion process requiring a heterocomplex of TG2 and FN and is mediated by a syndecan-4 and β1 integrin co-signaling pathway. By using α5 null cells, β1 integrin functional blocking antibody, and a α5β1 integrin targeting peptide A5-1, we demonstrate that the α5 and β1 integrins are essential for TG-FN to compensate RGD-induced loss of cell adhesion and FN deposition. The importance of syndecan-2 in this process was shown using targeting siRNAs, which abolished the compensation effect of TG-FN on the RGD-induced loss of cell adhesion, resulting in disruption of actin skeleton formation and FN deposition. Unlike syndecan-4, syndecan-2 does not interact directly with TG2 but acts as a downstream effector in regulating actin cytoskeleton organization through the ROCK pathway. We demonstrate that PKCα is likely to be the important link between syndecan-4 and syndecan-2 signaling and that TG2 is the functional component of the TG-FN heterocomplex in mediating cell adhesion via its direct interaction with heparan sulfate chains.

Fibronectin (FN)[3] mediates the cell adhesion process by interacting with cell surface receptors through its different cell binding sites. It is essential to embryogenesis and found associated with the extracellular matrix in large quantities during wound healing and angiogenesis. The amino acid sequence Arg-Gly-Asp (RGD) found within FN and many other matrix proteins is the most widely occurring cell-adhesive motif (1). It is the most important recognition site for about half of all known integrins, such as α5β1, αVβ1, αVβ3, and αVβ5 (2). However, this cell adhesion process can easily be inhibited by the presence of competitive peptides containing the RGD sequence, often leading to apoptosis (anoikis) (3, 4). Such peptides are commonly released during matrix remodeling, a process that is essential to both wound healing and angiogenesis (5–7). Of the integrins, α5β1 integrin is probably the major cell surface integrin that interacts with the RGD-cell binding site on FN, initiating the cell adhesion process (8), whereas the binding of αvβ3 integrin to the RGD domain can also support cell adhesion on FN (9). In addition to the RGD-cell binding domains, the heparin binding sites within FN have also been reported to be involved in cell adhesion because, although cell binding to the RGD-cell binding domains of FN can initiate the cell adhesion process, it is not sufficient to fully support actin cytoskeleton formation, an observation that led to the discovery of the cell surface heparan sulfate proteoglycan syndecan-4 (10, 11). Syndecan-4 is the most ubiquitous member of a larger syndecan family that interacts with the heparin binding domains of FN and supports the formation of a cytoskeletal network (12, 13). A further role of another syndecan family member, syndecan-2, was demonstrated as a downstream molecule in the syndecan-4 signaling pathway (14), although the intermediate protein(s) mediating this signaling have not been characterized. Apart from supporting the cell adhesion process, cell surface receptors are also involved in the assembly of inactive soluble fibronectin dimers into insoluble fibronectin fibrils to further support the cell adhesion process, among which α5β1 integrins are proba-

[3] The abbreviations used are: FN, fibronectin; TG, transglutaminase; TG-FN, TG2-fibronectin complex; FAK, focal adhesion kinase; MEF, mouse embryonic fibroblast(s); CHO, Chinese hamster ovary; gplTG, guinea pig liver TG; ROCK, Rho kinase.

bly the most efficient regulators and the most widely studied (15). Cell contractility mediated via the actin-myosin cytoskeleton and stimulated via activation of GTPase Rho (16) is needed to stretch the soluble fibronectin dimers, making the cell surface receptor binding sites available.

A further cell surface protein also implicated in cell adhesion and matrix deposition is the enzyme tissue transglutaminase (TG2). This Ca^{2+}-dependent enzyme catalyzes the post-translational modification of proteins either via their cross-linking through ϵ (γ-glutamyl) lysine bridges or by the incorporation of primary amines (17). Although mainly found intracellularly, during stress/injury situations, TG2 is up-regulated and secreted into the extracellular space by an uncharacterized mechanism, which requires an active site conformation (18) and may involve binding to cell surface FN and heparan sulfate proteoglycans (19, 20). When present at the cell surface and in the extracellular matrix, TG2 is tightly associated with fibronectin, for which it has a high affinity (19). In our previous work, we demonstrated that formation of the TG-FN heterocomplex can modulate cell behavior, including cell adhesion and spreading and their relevant signal transduction pathways (7, 18, 21). We demonstrated that the TG-FN heterocomplex can compensate for the RGD peptide-induced loss of cell adhesion through a syndecan-4 and β1 integrin co-signaling pathway supporting both cell adhesion and cell survival (22). We illustrated how this mechanism could be physiologically important by showing that a TG2-rich conditioned matrix produced by TG2-transfected Swiss 3T3 cells could also compensate for RGD peptide-induced loss of cell adhesion (22). We also illustrated how this compensation mechanism is particularly important for cell survival during situations involving matrix remodeling (*e.g.* wounding and scarring, angiogenesis, and tumor metastasis), where high levels of TG2 and RGD peptides are released during the matrix remodeling process (23–25).

In this paper, we have extended this work to explore the involvement of the RGD-independent adhesion mediated by TG-FN matrix in fibronectin matrix assembly, an event central to matrix remodeling and key to the process of many physiological and pathological situations where TG2 is found (26). We also explore the involvement of other cell surface receptors in addition to β1 integrin and syndecan-4, including syndecan-2 and α5, α4, and β3 integrins, in this process. Our findings suggest that cell spreading mediated by the TG-FN heterocomplex can lead to fibronectin matrix assembly even in the presence of RGD-containing peptides by a process involving cross-talk between the cell surface receptors syndecan-4, syndecan-2, and α5β1 integrin linked by the intracellular signaling molecule PKCα.

EMPERIMENTAL PROCEDURES

Reagents and Antibodies—Human plasma fibronectin was purchased from Sigma-Aldrich or Calbiochem. The FN synthetic peptides GRGDTP, GRADSP, and Rho kinase (ROCK) inhibitor Y27632 were from Calbiochem. Sulfo-NHS-LC-Biotin was obtained from Pierce. The GK21 peptide (GENPIYK-SAVTTVVNPIYEGK) and the scrambled control peptide (GTAKINEPYSVTVPYGEKNKV) in tandem with the anten-

napedia third helix sequence (PQIKIWFQNRRMKWKK) and the A5-1 peptide (VILVLF) were chemically synthesized by Peptide Protein Research. Anti-TG2 antibody CUB7402 was from Neomarkers. The rabbit anti-α5 integrin, rabbit anti-β1 integrin, mouse anti-human FAK, and mouse anti-PKCα were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); the anti-mouse β3 integrin antibody was purchased from Pharmingen; the mouse anti-α-tubulin antibody, mouse anti-cellular FN antibody, and mouse anti-vinculin antibody were from Sigma-Aldrich; and anti-human Tyr(P)[397] and Tyr(P)[861] were from Upstate Cell Signaling Solutions and BIOSOURCE, respectively. The Armenian hamster anti-β1 integrin antibody (HMβ1-1) and its IgG isotype control antibody and rat anti-mouse integrin α4 antibody and its rat IgG isotype control antibody were obtained from Biolegend. The rabbit polyclonal anti-syndecan-4 and syndecan-2 antibodies, which recognize the intracellular domains in the core proteins of these receptors, were from Zymed Laboratories Inc. Invitrogen. Cy[TM]5-conjugated streptavidin was from Jackson ImmunoResearch. The rabbit polyclonal anti-phosphotyrosine antibody was purchased from BD Biosciences. Vectashield mounting medium was purchased from Vector Laboratories. Purified guinea pig liver TG2 (gplTG) was purified according to Leblanc *et al.* (27). The site-directed irreversible transglutaminase inhibitor 1,3-dimethyl-2-imidazolium derivative R283 (28) was synthesized at Aston University. Specific siRNAs targeting mouse syndecan-2 and the universal negative control siRNA were purchased from Qiagen, whereas the scrambled siRNAs were synthesized by Sigma-Aldrich.

Cell Lines—EA5, EA5/α5, β3 wild type, and β3 null MEF cells were cultured according to Huveneers *et al.* (15). Wild type T98G glioblastoma cells and transfected T98G cells with syndecan-2 or syndecan-4 vectors were grown as reported previously (29). Wild type, syndecan-4 null, and β1 integrin null mouse embryo fibroblast (MEF) cells were grown as described previously (22). Parental Chinese hamster ovary (CHO) and CHO-K1 cells were purchased from ATCC and grown in Ham's F-12 medium according to the supplier's instructions.

Establishment of the TG2-transfected MEF (tg2-MEF) Cell Line—Transfection Of wild type MEF cells with the pSV40/Zeo2 expression vector containing wild TG2 cDNA was achieved by transfecting cells with the 5 μg of vector using a Nucleofector system (AMAXA Biosystems) according to the manufacturer's protocol. Clones resistant to 800 μg/ml Zeocin (Geneticin, Calbiochem) were screened for overexpression of TG2 by Western blotting as described below.

Cell Adhesion Assay—Cell adhesion assays were performed as described previously (7, 22). Unless otherwise stated, cells were detached with 0.25% (w/v) trypsin, and the trypsin was neutralized either with trypsin inhibitor or by the addition of serum-containing medium. Following serum starvation for 16 h, cells were detached as described above and then washed three times with serum-free medium to remove any traces of serum or trypsin inhibitor and pretreated with GRGDTP peptide or its control peptide GRADSP in serum-free medium for 20 min. Based on previous toxicity findings (7, 22), adhesion experiments were performed using 100 μg/ml RGD synthetic

peptide, leading to partial inhibition in cell adhesion. Cells were then seeded onto 96-well plates (2.5×10^4/well) coated with human plasma FN (FN matrix) with or without immobilized guinea pig liver TG (gplTG) (TG-FN matrix) as described previously (7, 22). Briefly, the TG-FN matrix was prepared by incubating 20 μg/ml gplTG in a 96-well plate precoated with 5 μg/ml FN as optimized previously (7, 22). In order to block the heparin binding sites within FN (30) or TG2 molecules (31), 300 μg/ml heparin in 50 mM Tris-HCl, pH 7.4, was used before and after the immobilization of gplTG as described previously (22). In certain experiments, the cells were pretreated with specific antibodies, inhibitors, or peptides for 30 min to 1 h. For inhibition of α4 integrin-mediated cell adhesion, cells were pretreated with anti-α4 integrin antibody 9C10 for 1 h in the presence of a 100 μg/ml concentration of either GRADSP or GRGDTP peptide. In order to block the cell adhesion process mediated by β1 integrins, cells were pretreated with 25 μg/ml HMβ1-1 for 1 h. For the ROCK inhibition experiment, cells were pretreated with the specific ROCK inhibitor Y27632 (10 μM) for 30 min (32). GK21 peptide (which blocks the interaction between PKCα and β1 integrin at the cytoplasmic domain) (22) or A5-1 peptide (which binds to and inactivates α5β1 integrin) (33) was used for incubations with MEF cells for 30 min, and the treated cells were used in cell adhesion assays.

Cells were allowed to attach for 20–40 min to minimize the secretion of any endogenous proteins. Cells were fixed, permeabilized, and stained, and digital images per each sample were acquired as stated before (7, 22). Cell attachment and spreading were quantified, and the number of cells per image was assessed as described previously (22).

siRNA Transfection—The siRNA solutions were prepared according to the manufacturer's instructions and stored at -20 °C. Before transfection, 3×10^5 cells were seeded into each well of a 6-well plate for 24 h to reach 50–80% confluence. 150 ng of siRNAs were used for each transfection as directed by the manufacturer. Following an approximately 30-h siRNA transfection, cells were used in Western blotting or cell adhesion assay. To visualize the presence of syndecan-2 in the syndecan-2 siRNA-treated MEF cells, the treated cells were seeded into 8-well chambers and incubated with rabbit anti-syndecan-2 antibody (recognizing the intracellular domain of the syndecan-2 core protein) and FITC-conjugated anti-rabbit secondary antibody after fixation and permeabilization. The nuclei were detected with propidium iodide, and the signals were detected via confocal microscopy. The sequences of the siRNAs were as follows: SDC-2 siRNA 1, TGGAATTTAATTTGTA-GAATA; SDC-2 siRNA 2, CAAAGGCAAATTAATGTGTAA; scrambled siRNA1, GTAAATTATGTAAGTTAGTAT; scrambled siRNA2, GATAATCAAAGCGAAATGTAT.

Analysis of FN Matrix Assembly via Fluorescence Staining—Fibronectin fibril formation was measured by the assembly of exogenously added biotin-labeled FN by a modification of Huveneers *et al.* (15). 6×10^4 cells/well of wild type, syndecan-4 null, β1 integrin null, EA5, and EA5/α5 cells were seeded onto FN or TG-FN in 8-well glass chamber slides. In some experiments, the cells were pretreated with RAD or RGD peptide as described above in the presence or absence of

1 mg/ml heparin. The cells were incubated for 1 h in serum-free medium, and following washing of the adherent cells with serum-free medium, the cells were incubated with 50 nM exogenous biotinylated FN (prepared according to the manufacturer's protocol) in serum-free medium. After a specified incubation period(s) (1, 3, 6, or 16 h), fixed cells were blocked with 3% bovine serum albumin (BSA) in PBS, pH 7.4 (BSA blocking buffer), and the cell matrices were stained with 1 μg/ml Cy5-streptavidin. Slides were mounted with Vectashield mountant (Vector Laboratories) and examined by confocal fluorescent microscopy (Leica Lasertechnik). To detect the deposition of the biotin-labeled FN via Western blotting, 6×10^5 cells (with or without the syndecan-2 siRNA treatment) were seeded onto FN- or TG-FN-precoated 6-well plates. After a certain time course, cell lysates containing both cells and the deposited matrices were scraped into cell lysis buffer (22) Equal amounts of protein from each sample (20 μg) were separated by SDS-PAGE, and after Western blotting, the biotin-labeled FN was detected by extravidin-peroxidase. The membranes were reprobed for α-tubulin. The amount of deposited fibronectin was quantified by densitometry and normalized to α-tubulin and used as a measure of fibronectin deposited/cell.

Detection of the Presence of Fibrillar Cellular FN or FN-bound TG2—Wild type MEF cells were detached and washed twice with serum-free medium as described above. Cell suspension with different cell densities (5,000, 1×10^4, or 2×10^4 cells/well) were prepared in serum-free medium in the presence of the RGD or RAD peptides and allowed to adhere on FN or TG-FN matrices for 3 h in 96-well plates. After incubation, the cells were detached with 5 mM EDTA in PBS, pH 7.4, and the remaining matrices were washed three times with PBS, pH 7.4, and blocked with BSA blocking buffer for 30 min at room temperature. Anti-mouse cellular FN monoclonal antibody and FITC-conjugated anti-mouse secondary antibody were used to detect the presence of deposited cellular FN. To visualize the cellular FN fibril formation, immunofluorescence staining was performed by using anti-mouse cellular FN antibody after seeding the cells on FN or TG-FN matrix in the presence of the RGD or RAD peptides for 3 h. Confocal microscopy was performed to detect the signals. Matrix-bound TG2 was detected after incubating the cells with FN or TG-FN matrices for 16 h after detaching the cells with 5 mM EDTA in PBS, pH 7.4. A modified ELISA (7) was used to measure the TG2 signal by using anti-TG2 antibody Cub7402 and anti-mouse secondary antibody. Development of the reaction was performed by the addition of OPD solution (Sigma-Aldrich). The color development was terminated by the addition of 50 μl of 2.5 M H_2SO_4, and the absorbance was read at 450 nm using a Spectrafluor plate reader. Results were expressed as absorbance at 450 nm.

Fluorescence Staining—Subconfluent MEF cells were serum-starved for 16 h, harvested, and pretreated with 100 μg/ml GRADSP or GRGDTP peptides as described above. Cells were seeded in 8-well glass chamber slides (8×10^4 cells/well) previously coated with FN or TG-FN and allowed to attach and spread for 20–40 min. Cells were fixed and permeabilized as described previously (7) and then blocked with BSA blocking buffer for 30 min. For the actin stress fiber

staining, the cells were incubated with FITC-labeled phalloidin (20 μg/ml) in blocking buffer. For the focal contact staining in MEF cells, after treatment with blocking buffer, the cells were incubated with anti-vinculin antibody (1:100 dilution) followed by anti-mouse IgG-FITC. For the syndecan-2 staining, the cells were seeded into 8-well chambers, and after fixation and permeabilization, the wells were blocked with BSA blocking buffer for 30 min at room temperature and then incubated with anti-syndecan-2 antibody at 37 °C for 2 h and with FITC-conjugated anti-rabbit secondary antibody at 37 °C for 2 h. Slides were mounted with Vectashield mountant (Vector Laboratories) and examined as mentioned above.

Western Blotting and Co-immunoprecipitation—Biotinylation of cell surface protein with EZ-link Sulfo-NHS-Biotin (Sigma) was performed according to the manufacturer's protocol. As described above, after a certain treatment, cells were collected and lysed by the addition of 70 μl of solubilization buffer (Santa Cruz Biotechnology, Inc.). Following a preclearing procedure by centrifuging at $300 \times g$ for 10 min, the samples were stored at -70 °C until use. Western blotting was performed as described previously (7). For the co-immunoprecipitation, cells seeded onto heparin-treated TG-FN or non-treated FN matrices were collected in lysis buffer as described previously (22). According to the target protein and antibody selected, either protein A- or G-Sepharose beads and negative control antibodies were used to preclear the cell lysates. The immunocomplexes were obtained via incubating the precleared samples with protein A or G beads and relevant specific antibody against the target protein and collected in 30 μl of Laemmli buffer. The immunoprecipitated proteins were detected via Western blotting by using their specific antibodies.

Statistics—Data are expressed as mean $\pm$ S.D. The data shown were derived from one representative experiment ($n >$ 3) undertaken in triplicate. The comparisons between the data sets were performed using Student's t test (two-tailed distribution with equal variance). Statistically significant difference between data sets was defined as by $p < 0.05$ (two-sided).

RESULTS

RGD-independent TG-FN-mediated Cell Adhesion Can Influence FN Deposition, but This Role Is Independent of the Role of Cell Surface TG2 as an Integrin Co-receptor for Fibronectin—We first investigated whether TG-FN-mediated cell adhesion can influence FN fibril formation in both the presence and absence of RGD peptides, given the potential importance of this adhesion mechanism in matrix remodeling. A prerequisite for fibronectin fibril formation is the formation of focal adhesions (15). We therefore investigated focal adhesion formation in MEF cells treated with RAD or RGD peptides when plated on FN or TG-FN matrices. Immunofluorescence staining of vinculin shown in Fig. 1A demonstrates that cells could not form focal adhesions when plated on FN alone in the presence of the RGD peptides when compared with the non-treated cells on FN and the cells seeded on TG-FN. Once seeded on TG-FN, this matrix restores the

formation of focal adhesions even in the RGD peptide-treated MEF cells, which is in keeping with our earlier findings for the activation of GTPase RhoA in this event (7). Moreover, the focal adhesions present in cells plated on the FN-TG seemed much sharper compared with those plated on FN alone (Fig. 1A) when measured 40 min after plating.

We next investigated the ability of TG-FN to facilitate cell FN fibril formation and deposition in the presence and absence of RGD peptides. First, in the presence of the RGD and RAD control peptides, wild type MEF cells were seeded on FN or TG-FN matrices with the addition of soluble exogenous biotin-labeled FN and incubated for 1, 3, and 6 h. In the RAD-treated cells seeded on TG-FN, after 1 h of incubation, FN fibril formation could be observed, and within 6 h, extended fibrils were easily detectable (Fig. 1B). In contrast, the same process appeared much slower in the cells plated on FN alone although equal numbers of cells were adhered (see Fig. 3A). Importantly, when biotinylated fibronectin was added to plates without cells, no fibril formation occurred (data not shown), confirming the need for cells in this process (34). Western blotting of the assembled biotinylated fibronectin confirmed this increase in fibril formation in the cells plated on FN-TG during a 1-, 3-, and 6-h time course (Fig. 1C). This increase in fibril formation became less apparent at 6 h, suggesting that TG-FN enhances the FN deposition at the early stage of FN fibril formation. After the longer incubation time of 16 h, the fluorescent images showed little difference (see Fig. 3A), suggesting that matrix deposition was reaching completion. Importantly, the localization of little biotin-labeled FN in the RGD-treated cells on FN, where both cell adhesion and focal adhesion formation was reduced (Figs. 1A and 3A), indicated a reduction in fibronectin fibril formation, an observation consistent with previous reports (35). This delayed deposition of fibronectin by the RGD peptide treatment was, however, compensated for by the TG-FN complex (Fig. 1B), which is in keeping with the maintenance of cell adhesion, the formation of sharp focal adhesions (Fig. 1A), and the activation of RhoA (16).

We next investigated whether FN fibril formation mediated by TG-FN required transamidating activity by using the site-directed irreversible TG inhibitor R283 of TG2, which selectively acetonylates the active site cysteine (28), to block the transamidating activity. As shown in Fig. 1D, no difference was observed in the R283-treated and control cells, suggesting that when present within the TG-FN complex, TG2 functions as a matrix protein instead of a transamidating enzyme (7).

Considering that there are two major sources of globular FN present in any physiological system, the extracellular FN from serum, which is both in solution and surface-bound, and the cellular secreted FN from fibroblasts, we undertook to investigate the effect of TG-FN matrix on cellular FN deposition. The deposition of cellular FN was detected by using an anti-mouse cellular FN antibody, which only recognizes the deposited FN of cellular origin. The quantitative assay was based on detecting the deposited FN by ELISA after removal of cells by 5 mM EDTA in PBS, which can detach the cells without disrupting the matrix (Fig. 2A). Detection of the deposited FN was also detected by immunofluorescence (Fig.
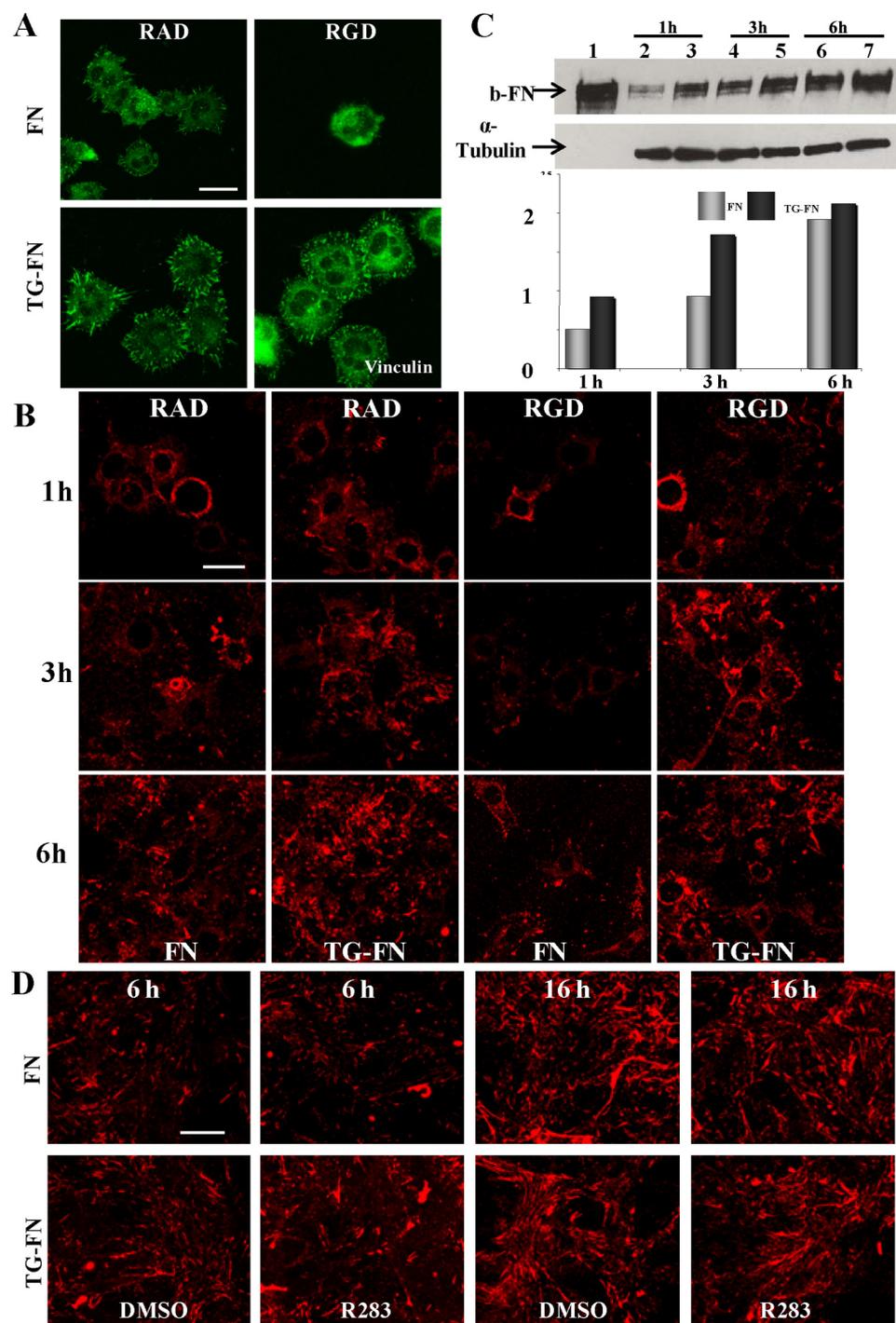
FIGURE 1. **TG-FN promotes RGD-independent deposition of exogenous fibronectin.** *A*, WT MEF cells treated with RAD or RGD peptide was seeded on FN or TG-FN matrix. After a 1-h incubation, focal contacts were visualized via fluorescence staining of vinculin. *B*, biotin-labeled FN was applied to the monolayer of WT MEF cells on FN or TG-FN matrix in the presence of RAD or RGD peptide. Following 1-, 3-, and 6-h incubation, biotinylated FN was detected via fluorescence staining, as described under "Experimental Procedures." *C*, biotin-labeled FN was detected via Western blotting as described under "Experimental Procedures." *Lane 1*, biotin-labeled FN marker; *lanes 2, 4*, and *6*, samples from the cells seeded on FN matrices; *lanes 3, 5*, and *7*, samples collected from cells plated on TG-FN matrices. The histogram shows representative data (*n* = 2) of the densitometric values obtained from the bands when normalized using the densitometric values of the α-tubulin bands. *D*, deposition of fibronectin is independent of TG2 cross-linking. A monolayer of WT MEF cells was incubated with 500 μM TG2 inhibitor R283 or DMSO (0.1%) in the presence of biotin-labeled FN for 6 or 16 h, respectively. Biotinylated FN fibril staining was as described above.

2*B*). The assay was undertaken on cells seeded on FN or TG-FN in the presence of RGD or RAD peptides after 3 h of incubation. As shown in Fig. 2, *A* and *B*, the RGD peptides not only blocked the exogenous FN deposition (see Fig. 1, *B* and *C*) but also inhibited the deposition of the cellular FN, which

could be compensated for by the seeding of the cells on the TG-FN matrix.

One question that aroused our interest is whether the TG2 in the TG-FN matrix complex can still be available to the cells after the formation of the newly deposited FN matrix. Hence,
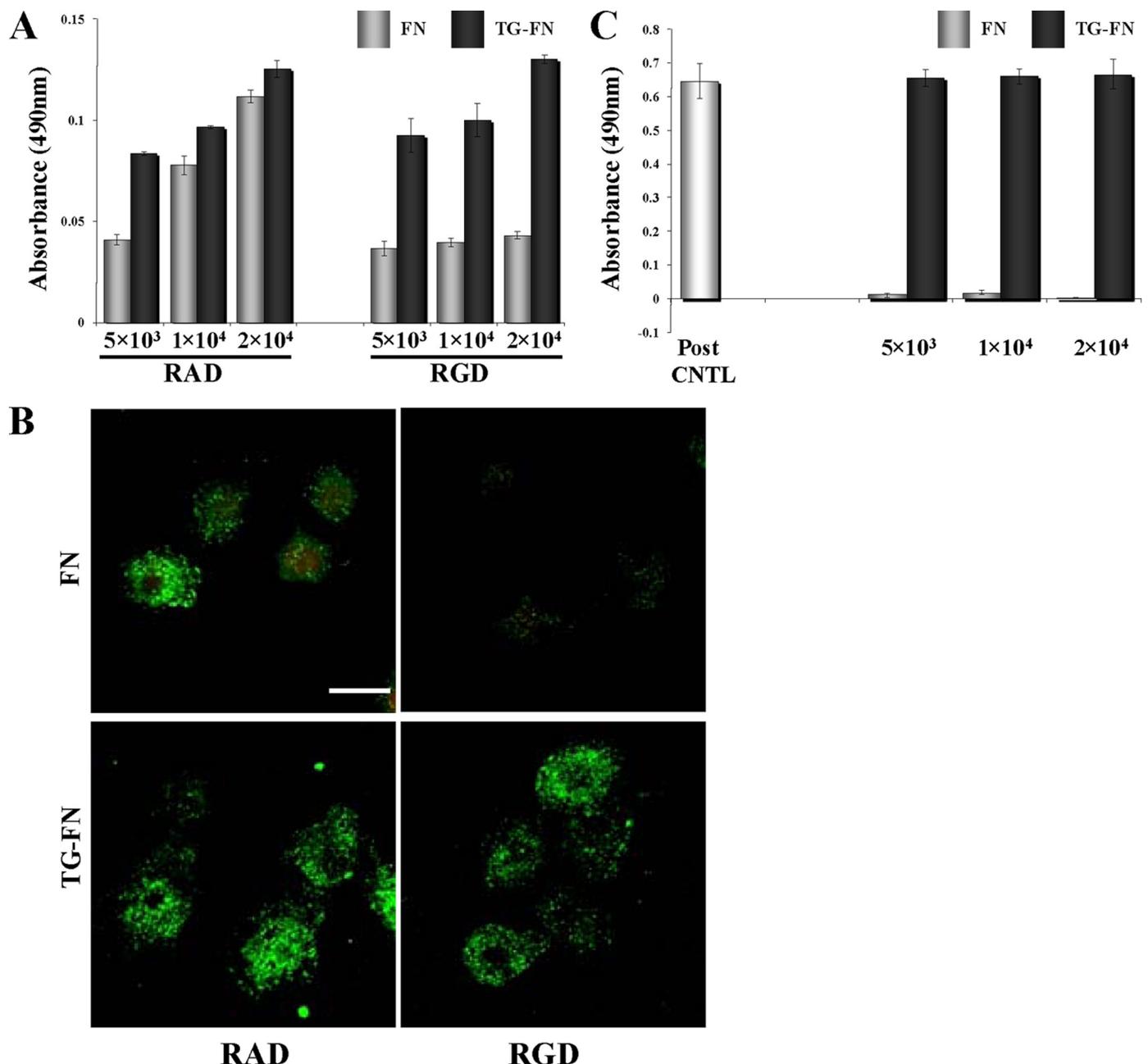
FIGURE 2. **Deposition of cellular fibronectin by the TG-FN complex in an RGD-independent manner and detection of matrix-bound TG2.** *A*, deposition of cellular FN was detected by an ELISA assay. The cells (at a density of 5×10^3, 1×10^4, or 2×10^4 cells/well) were allowed to adhere on FN or TG-FN matrix in the presence of RGD or RAD peptides for 3 h and then detached with 5 mM EDTA in PBS, pH 7.4. The deposited cellular FN in the remaining matrices was detected by ELISA using anti-mouse cellular FN antibody as described under "Experimental Procedures." *B*, to visualize the cellular FN fibril formation, immunofluorescence staining was performed by using anti-mouse cellular FN and FITC-conjugated secondary antibodies after seeding the cells on FN or TG-FN matrix in the presence of the RGD or RAD peptides for 3 h. The nuclei were detected with propidium iodide, and confocal microscopy was performed to detect the signals as described under "Experimental Procedures." *Bar*, 20 μm. *C*, the presence of matrix TG2 after incubating the cells (at a density of 5×10^3, 1×10^4, or 2×10^4 cells/well) with the FN or TG-FN matrix complex was detected via a modified ELISA by using anti-TG2 antibody Cub7402 after seeding the cells for 16 h. *CNTL*, control.

a modified ELISA (7) was performed to detect the TG2 matrix after seeding the cells onto the TG-FN matrix for 16 h. Fig. 2*C* demonstrates that the TG2 remained in the matrix and was available to antibody binding after incubation with the cells for 16 h, suggesting that the matrix-bound TG2 is able to sustain a long term effect of the TG-FN complex on both cell adhesion and FN deposition.

To test our hypothesis for the involvement of syndecan-4 in FN fibril formation, we investigated FN fibril formation in fibroblasts seeded on FN or TG-FN matrices using syndecan-4 knock-out (KO) MEF, Y188L mutant MEF (syndecan-4 KO cells transfected with syndecan-4 cDNA mutated at the PKCα binding site), and GK21 peptide-treated MEF cells (previously shown to block the interaction between PKCα and β1 integrin at the cytoplasmic domain) (21). As shown in Fig. 3*A*, in these different cells where syndecan-4 function has either been knocked out or altered, leading to reduced cell attachment and spreading (22), no well organized FN fibrils
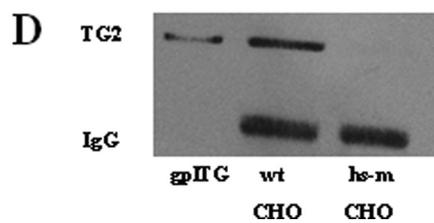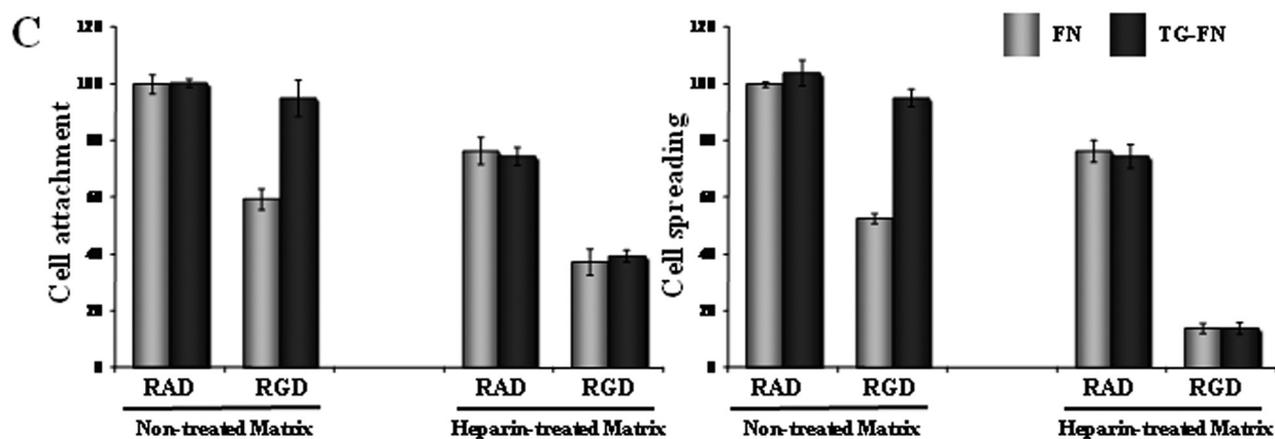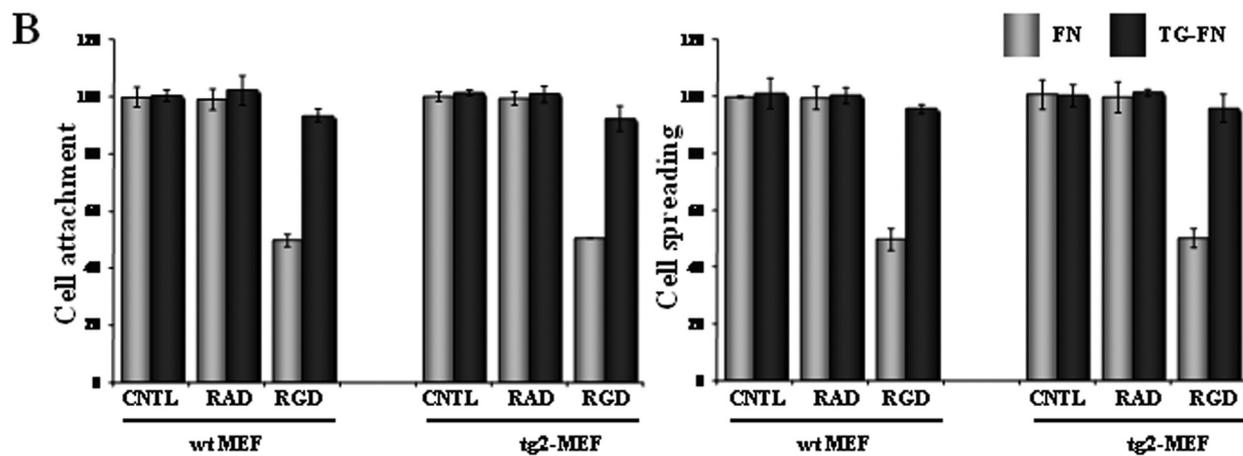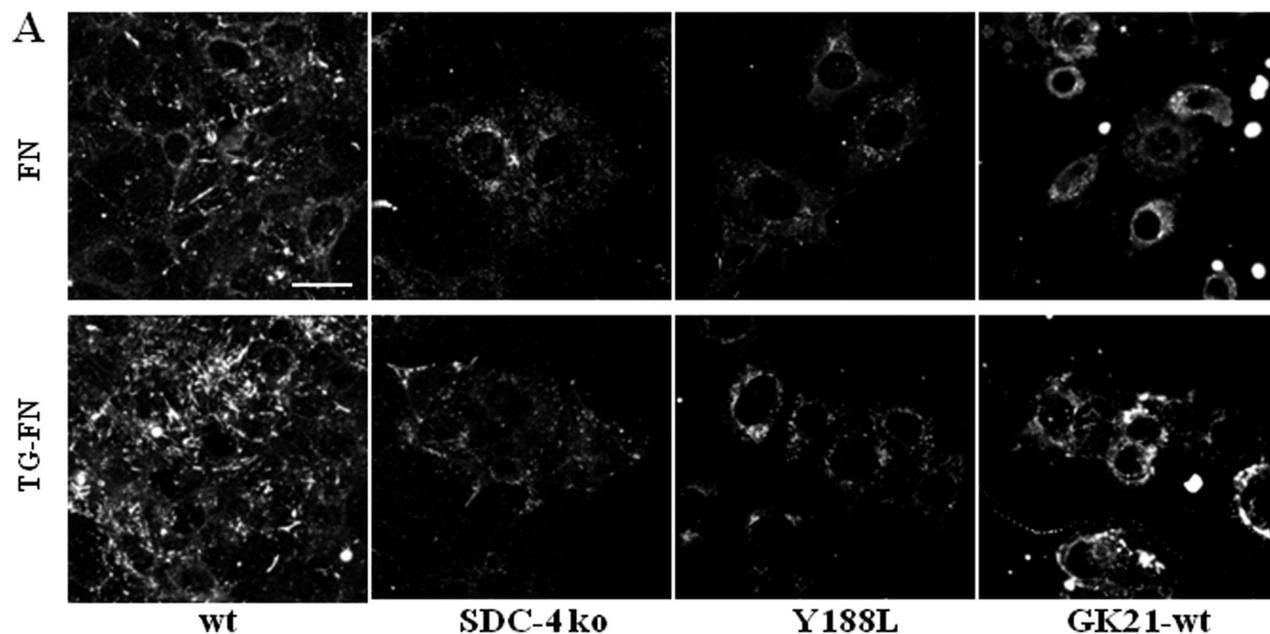
were observed. Moreover, this loss of FN fibril formation could not be restored when cells were seeded on the TG-FN complex and compared with wild type cells. This indicates for the first time that syndecan-4 is involved in FN fibril assembly particularly in the early stages following cell spreading, and this function requires its downstream signaling effector molecule PKCα to mediate this process when cells are adhered to TG-FN.

Our next objective was to distinguish the role of matrix-bound TG2 from its proposed role as a cell surface integrin co-receptor for fibronectin, which has also been shown to enhance fibronectin fibril formation but in an integrin-dependent manner. To do this, we stably transfected TG2 cDNA into wild type MEF cells to establish the tg2-MEF cell line, which expresses high levels of TG2 at the cell surface. As shown in supplemental Fig. 1A, no detectable cell surface or total TG2 antigen was found in wild type MEF cells, whereas high levels were detectable in tg2-MEF cells. Despite the increased cell surface enzyme expression of TG2 in these cells, they did not support cell adhesion and spreading in the presence of RGD peptides on a FN matrix and showed no advantages in this respect over wild type MEF cells (Fig. 3B) during a 20–40-min incubation time. Only in the presence of a pre-deposited TG-FN complex could the TG2-overexpressing cells rescue the effect of the RGD peptides, with no significant differences ($p > 0.05$) observed between wild type and tg2-MEF cells. This clearly suggests that cell surface TG2 is incapable of mediating the RGD-independent cell adhesion and confirms that its proposed role as a cell surface integrin-dependent co-receptor for fibronectin is different from that when it is bound to matrix fibronectin in mediating both cell adhesion and fibronectin assembly (22).

The Direct Interaction between the Heparan Sulfates of Syndecan-4 and TG2 Is Important for TG-FN to Regulate Cell Adhesion in the Presence of RGD Peptides—We previously showed that even when the heparin binding site within FN is blocked, TG-FN can still compensate for the loss of RGD-induced cell adhesion, suggesting that TG2 is the functional component in the TG-FN heterocomplex (22). As a continuation of this study, we next used heparin, a high affinity binding partner for TG2 (31), to treat the already assembled TG-FN complex to mask the potential heparin binding site(s) within TG2 itself. As shown in Fig. 3C, heparin treatment completely blocked the compensation of TG-FN in RGD-induced loss of cell adhesion, further proving that the heparin-binding site(s) within TG2 is the functional component for this TG-FN heterocomplex to exert its effect.

It has been reported that the core protein of syndecan-4 can also mediate cell spreading (36). Although our previous data using heparinase treatment to digest cell surface heparan

sulfate chains and the use of heparan sulfate-deficient CHO cells in cell adhesion studies (22) demonstrated the importance of cell surface heparan sulfates, the potential involvement of the core protein of syndecan-4 in this process was not ruled out. We therefore performed co-immunoprecipitation assays using the CHO-K1 cells and the heparan sulfate mutant CHO cells (devoid of cell surface heparan sulfates but containing the syndecan-4 core protein) (36). Anti-syndecan-4 antibody was used to pull down the syndecan-4-associated complex, and anti-TG2 antibody was used to detect the presence of TG2 antigen within this complex using Western blotting. As shown in Fig. 3D, unlike in the wild type cells, no TG2 was detected in the heparan sulfate mutant cells, indicating that no direct interaction between TG2 and the core protein of syndecan-4 takes place during the RGD-independent cell adhesion process mediated by TG2 when bound to FN.

α5β1 Integrin Is Required for TG-FN to Exert Its Function in Regulating RGD-independent Cell Adhesion and FN Fibril Formation—Our previous work demonstrated that β1 integrins are required for TG-FN to mediate its intracellular signaling pathway that facilitates RGD-independent cell adhesion. Further experiments were therefore undertaken in MEF cells to investigate the role of the β1 integrin signaling pathway and also the importance of the major FN binding companion of β1, α5, in the RGD-independent cell adhesion process on TG-FN.

Treatment of MEF cells with the β1 integrin functional blocking antibody, HMβ1-1 (37), led to a 54% decrease in cell attachment and blocked 57% of cell spreading on FN when compared with non-treated cells seeded on FN. The isotype control antibody did not show any effect on either cell adhesion or spreading. In contrast, when cells seeded on TG-FN matrix were treated with the function-blocking antibody, very little loss (~10%) of cell attachment and spreading was observed (Fig. 4A). HMβ1-1 is thought to exert its blocking effect by inhibiting the signaling transduction of the β1 integrin outside-in pathway (37), which can be confirmed via detecting the phosphorylation of FAK, a downstream signaling molecule of β1 integrin. As shown in Fig. 4B, in HMβ1-1-treated MEF cells, the antibody inhibited the phosphorylation of FAK at Tyr^{397} in the cells seeded on FN matrix but not in the TG-FN group, whereas the phosphorylation of Tyr^{861} (suggestive of inside-out signaling) was not significantly affected in both FN and TG-FN groups. Previously, we demonstrated that TG-FN can compensate the phosphorylation of both Tyr^{397} and Tyr^{861} in the RGD peptide-treated cells, suggesting the requirement of FAK by TG-FN to exert its function (21). Merging the discoveries together, we now show that the signaling pathway mediated by β1 integrin in TG-FN-mediated cell adhesion is that of inside-out signal transduction

FIGURE 3. **TG2 is the functional component within the TG-FN complex and directly interacts with heparan sulfates of syndecan-4.** *A*, after incubation with biotin-labeled FN for 6 h, biotinylated FN fibril formation produced by WT, syndecan-4 KO, Y188L syndecan-4 mutant (Y188L), and GK21 peptide-treated wild type MEF cells (*GK21-wt*) was detected as described under "Experimental Procedures." Bar, 20 μm. *B*, cell adhesion of WT MEF (*wt MEF*) and tg2-MEF to FN and TG-FN matrices in the presence of 100 μg/ml RAD or RGD peptide was undertaken as described under "Experimental Procedures." The non-treated cells were used as the control group (*CNTL*). *C*, heparin (300 μg/ml) was applied after the formation of the TG-FN complex, and cell adhesion was observed on non-treated and heparin-treated-TG-FN matrices. The percentages for cell attachment and spreading on non-treated FN matrix in the presence of RAD peptide were set as 100%. *D*, co-immunoprecipitation of syndecan-4 and TG2 was performed by using wild type (*wt*) and heparan sulfate mutant (*hs-m*) CHO cells, as described under "Experimental Procedures." gplTG was used as the internal standard.
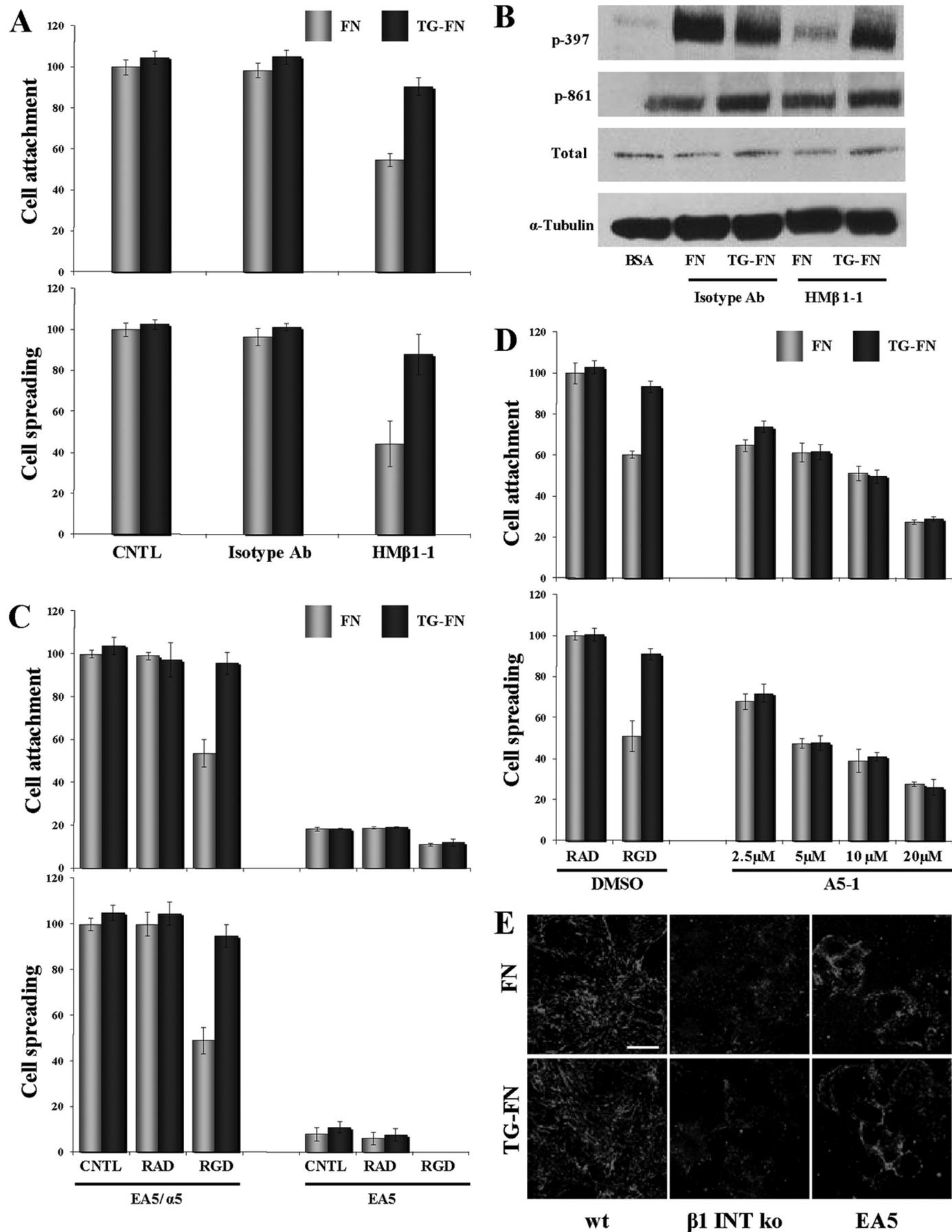
because the phosphorylation at Tyr[861] was not influenced by the HMβ1-1 antibody (38).

The role of α5 integrin in association with β1 in FN-dependent cell adhesion is well documented. Our hypothesis that α5 integrin is also the major companion of β1 integrin in mediating RGD-independent cell adhesion on TG-FN was first tested by using α5 integrin knock-out EA5 embryo cells and control EA5/α5 cells (EA5 transfected back with α5 integrin) (supplemental Fig. 1*B*) (15). Without α5 integrin, the EA5 cells adhered poorly compared with the control cells, and the adhesive response of the knock-out cells was much more susceptible to RGD peptide than that of its control cells (Fig. 4*C*). As expected, TG-FN failed to restore the loss of cell attachment and spreading caused by the presence of RGD peptides. To further confirm our observations for α5β1 involvement, an inhibiting peptide, A1-5, was used in MEF cell adhesion experiments. The peptide A5-1, which inhibits cell adhesion and proliferation via its specific blocking and interaction with α5β1 integrin (33), inhibited cell attachment and spreading in a dose-dependent manner, and at a concentration of 10 μM, it blocked cell attachment and spreading by ∼50%. Importantly, use of the peptide A5-1 led to the failure of the TG-FN heterocomplex to restore RGD-independent cell adhesion, confirming the importance of the α5β1 integrin in this process (Fig. 4*D*).

To confirm the involvement of these integrin members in FN fibril formation on TG-FN in the presence of RGD peptide, we used α5 and β1 integrin null cells, respectively. As expected, given the poor cell adhesion properties of these cells, even after a 16-h incubation, the ability of these cells to form fibronectin fibrils without either of these two cell surface receptors was negligible (Fig. 4*E*). Importantly, even those cells that did adhere did not form well organized fibrils that could not be compensated for by plating the cells on the TG-FN complex.

Syndecan-2 Is the Novel Cell Mediator in FN Fibril Formation When Cells Are Plated on TG-FN in the Presence of RGD Peptides—As a further member of the syndecan family, syndecan-2 is expressed in fibroblasts and has been shown to be involved in actin cytoskeleton formation when cells are seeded on fibronectin. We therefore hypothesized that syndecan-2 might also be involved in the syndecan-4-mediated RGD-independent cell adhesion on TG-FN and, given its importance in actin cytoskeletal formation, may also be key to the ability of TG-FN to mediate RGD-independent fibronectin fibril formation.

Syndecan-2 involvement was first investigated by using syndecan-2 siRNA-treated MEF cells in cell adhesion assays.

As shown in Fig. 5*A*, the syndecan-2 siRNA treatment significantly reduced the expression of syndecan-2 to around 50% compared with non-treated and the different control siRNA groups. Importantly, the expression of both syndecan-4 and β1 integrin was not significantly affected. Fig. 5*B* shows the inhibition of the siRNA in syndecan-2 expression in MEF cells by immunofluorescence staining of syndecan-2. This indicated that there was a fairly even knocking down effect on the expression of syndecan-2 in the different MEF cells by the specific targeting siRNAs. The syndecan-2 siRNA treatment led to an ∼25% loss of cell attachment and ∼60% loss of cell spreading on FN and a further 30% reduction of cell attachment and 20% reduction in cell spreading in the presence of RGD peptide. Importantly, TG-FN failed to compensate for the effect of the RGD peptide on cell attachment and spreading, indicating the requirement of syndecan-2 in the TG-FN adhesion mechanism (Fig. 5*C*).

Fluorescent visualization of the actin stress fibers by confocal microscopy indicated that syndecan-2 siRNA-treated MEF cells could not form well organized actin cytoskeleton structures compared with the cells treated with the negative non-silencing siRNA when plated on FN, confirming the importance of syndecan-2 in the formation of the actin cytoskeleton. Moreover, the TG-FN complex can only rescue the effect of the RGD peptide inhibition on actin stress fiber formation in the MEF cells treated with the non-silencing siRNA and not in the syndecan-2 siRNA-treated MEF cells (Fig. 5*D*).

Next we confirmed the importance of syndecan-2 in mediating fibronectin fibril formation in cells plated on TG-FN in the presence of the RGD peptides. In keeping with the ability of the syndecan-2-targeted siRNA to inhibit both cell spreading and actin cytoskeleton formation (Fig. 5, *C* and *D*), FN fibril formation was also found to be significantly reduced when both visualized (Fig. 6*A*) and measured using Western blotting (Fig. 6*B*). As shown in Fig. 6, *A* and *B*, the siRNA2 treatment resulted in around 50% reduction of FN deposition in the cells seeded on both FN matrix and TG-FN groups using Western blotting and normalized per cell using α-tubulin, a figure that equates well with the siRNA knockdown of syndecan-2 in these cells (Fig. 5*A*). Hence, not only are there fewer cells adhered after siRNA treatment (Fig. 5*C*), but those cells remaining are also less efficient in fibril formation, which probably accounts for the increased reduction in fibril formation visualized in Fig. 6*A*.

The above data suggested that syndecan-2 is the predominant cell surface receptor that can bind and mediate FN fibril formation on TG-FN matrix in the presence of RGD peptide

FIGURE 4. **The importance of α5β1 integrin in RGD-independent cell adhesion and FN fibril formation mediated by TG-FN.** *A*, pretreated MEF cell suspensions (2.5 × 10⁵/ml) with 25 μg/ml HMβ1-1 β1 integrin functional blocking antibody or its isotype control antibody (*CNTL*) were seeded on FN or TG-FN matrix with RAD or RGD peptide as described under "Experimental Procedures." *B*, MEF cells treated as above were seeded on FN and TG-FN. The presence of FAK-Y397 and FAK-Y861 was detected via Western blotting. α-Tubulin was used to ensure equal loading. *C*, EA5 (α5 integrin knock-out embryo cells) and EA5/α5 (EA5 cells transfected with human α5 integrin cDNA) were used in the TG-FN cell adhesion assay. Mean cell attachment and spreading are expressed as a percentage of control values of non-treated EA5/α5 cells on FN (which represents 100%) ± S.D. *D*, MEF cells were pretreated with various concentrations of α5β1 integrin-targeted peptide A5-1 for 30 min, and cell adhesion was performed, whereas RAD and RGD peptides were used as controls. Mean cell attachment and spreading are expressed as the percentage of control values of non-treated MEF cells on FN (which represents 100%) ± S.D. in the presence of RAD peptide. *E*, TG-FN does not compensate for the loss of FN deposition caused by the absence of α5 and β1 integrin. Following a 16-h incubation, biotinylated FN fibril formation by α5 (*EA5*) or β1 integrin null cells (*β1 INT ko*) seeded on FN or TG-FN matrix was detected via fluorescence staining. *Bar*, 20 μm.

A

Syndecan-2

Syndecan-4

β1 integrin

α-Tubulin

CNTL   NS   siRNA1   siRNA2   Scr-   Scr-
        siRNA   siRNA1   siRNA2

B

NS siRNA   siRNA1   siRNA2

C

D

NS siRNA   siRNA1   siRNA2

RAD-FN

RGD-FN

RGD-TG-FN

A

NS siRNA

siRNA1

siRNA2

FN   TG-FN   FN   TG-FN

B

b-FN→

α-Tubulin→

C

RAD   RGD   RGD + Heparin
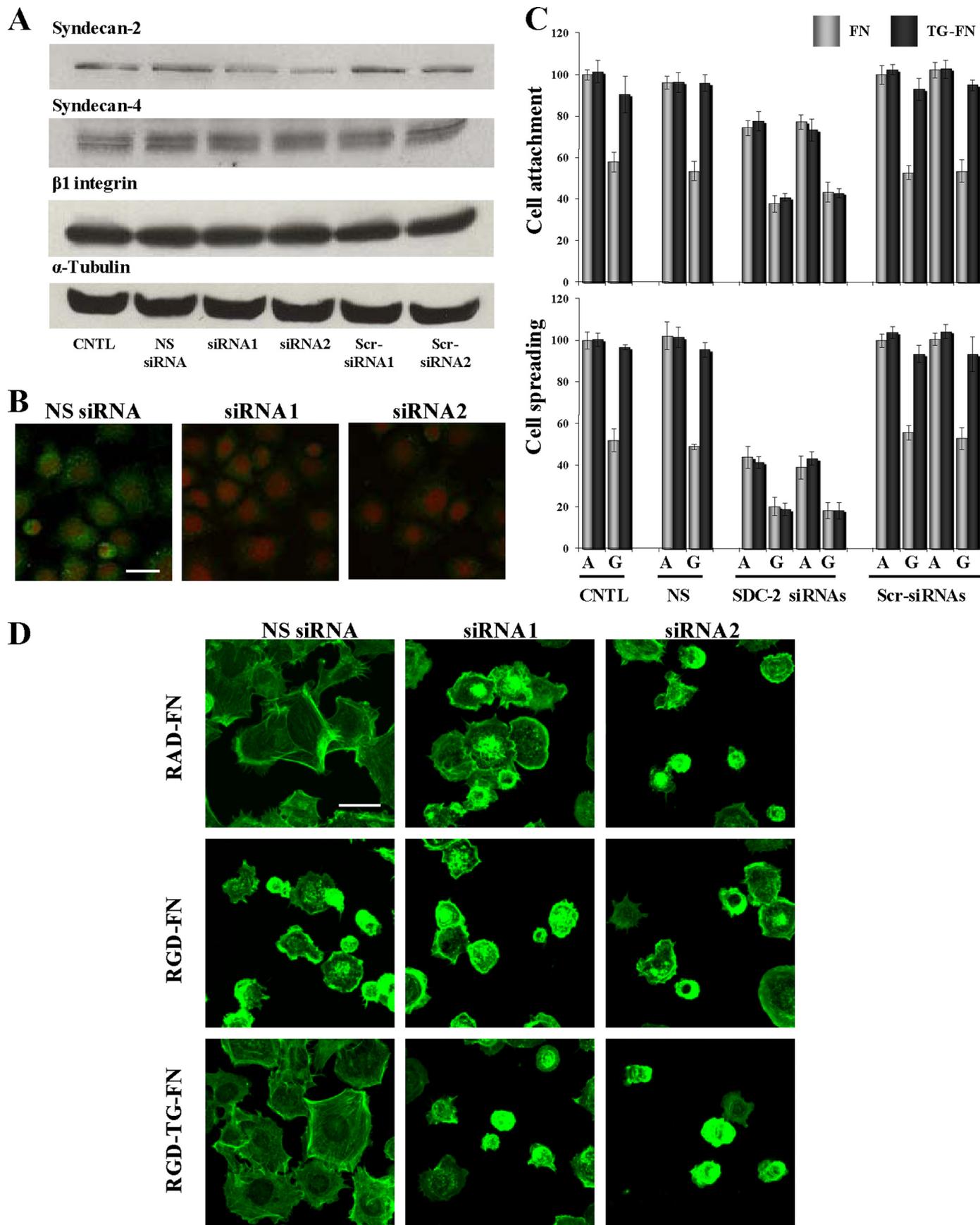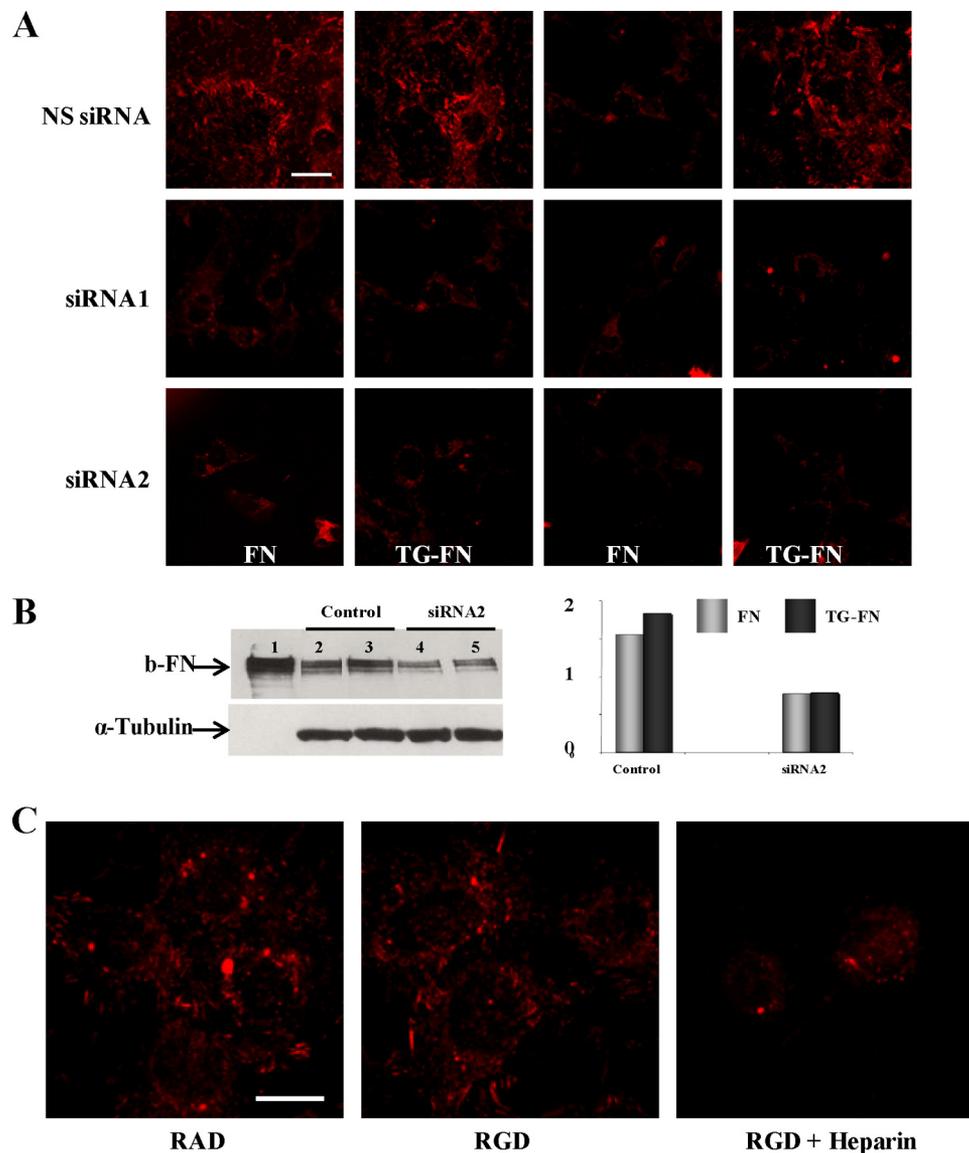
FIGURE 6. **Syndecan-2 is required by TG-FN in mediating FN fibril formation.** *A*, the syndecan-2 siRNAs (*siRNA1* and *siRNA2*) and the negative control siRNA-treated (*NS siRNA*) MEF monolayer cells were incubated with biotin-labeled FN for 6 h in the presence of RAD or RGD peptides, and fluorescence was staining performed as described above. *B*, Western blotting was used to detect the biotin-labeled FN from the syndecan-2 siRNA2-treated MEF cells as described under "Experimental Procedures." *Lane 1*, biotin-labeled FN marker; *lanes 2* and *3*, cell lysates from the negative control siRNA-treated cells seeded on FN or TG-FN matrix, respectively; *lane 4*, the sample from syndecan-2 siRNA2-treated cells plated on FN or TG-FN (*lane 5*). The histogram shows representative densitometric values (*n* = 2) obtained from the bands when normalized using the densitometric values of the α-tubulin bands. *C*, FN fibril formation on TG-FN can be blocked by heparin. After adhering to the TG-FN matrix for 1 h to form the cell monolayer, the RGD or RAD peptide-treated MEF cells were incubated with biotin-labeled FN with or without the presence or absence of 1 mg/ml heparin (to block the cell surface heparan sulfates). Cy5-conjugated streptavidin was used to detect the FN, and the signals were detected via confocal microscopy. *Bar*, 20 μm.

because RGD peptides block the integrins and matrix TG2 keeps syndecan-4 occupied via their direct interactions. Thus, the blocking of syndecan-2 should further affect the FN depo-

sition by the cells seeded on TG-FN matrix in the presence of RGD peptide. To test the theory, heparin was used to block the available cell surface heparan sulfates during the incuba-

FIGURE 5. **The role of syndecan-2 in regulating RGD-independent cell adhesion on TG-FN.** *A*, WT MEF cells were treated with specific syndecan-2-targeting siRNAs (*siRNA1* or *-2*), whereas the scrambled siRNAs (*scr-siRNA1* and *-2*) and the negative non-silencing siRNA were used as the control treatments. After incubation with siRNAs for 30 h, the samples were collected in cell lysis buffer to detect the presence of syndecan-2 via Western blotting by using anti-syndecan-2 antibody. The membranes were stripped and reprobed with anti-syndecan-4 or β1 integrin antibody, whereas tubulin was used as the equal loading standard. *B*, to visualize the presence of syndecan-2 in the syndecan-2 siRNA-treated MEF cells, the treated cells were seeded into 8-well chambers and incubated with anti-syndecan-2 antibody (recognizing the intracellular domain of the syndecan-2 core protein) and anti-rabbit secondary antibody after fixation and permeabilization as described under "Experimental Procedures." The nuclei were detected with propidium iodide, and the signals were detected via confocal microscopy. *Bar*, 20 μm. *C*, MEF cell monolayers were treated with syndecan-2 siRNAs and control siRNAs as described under "Experimental Procedures." Cells were then seeded on either FN or TG-FN with 100 μg/ml RGD (*G*) or RAD (*A*) synthetic peptide. The mean percentage value of non-treated attached and spread cells on FN in the presence of RAD ± S.D. (*error bars*) (control) was used as 100%. *D*, actin stress fiber formation was detected in syndecan-2 siRNAs (*SDC-2 siRNAs*) or control siRNA-treated (non-silencing (*NS*) siRNA) MEF cells with RAD or RGD peptide. Actin stress fibers were stained by using FITC-labeled phalloidin. *Bar*, 20 μm.
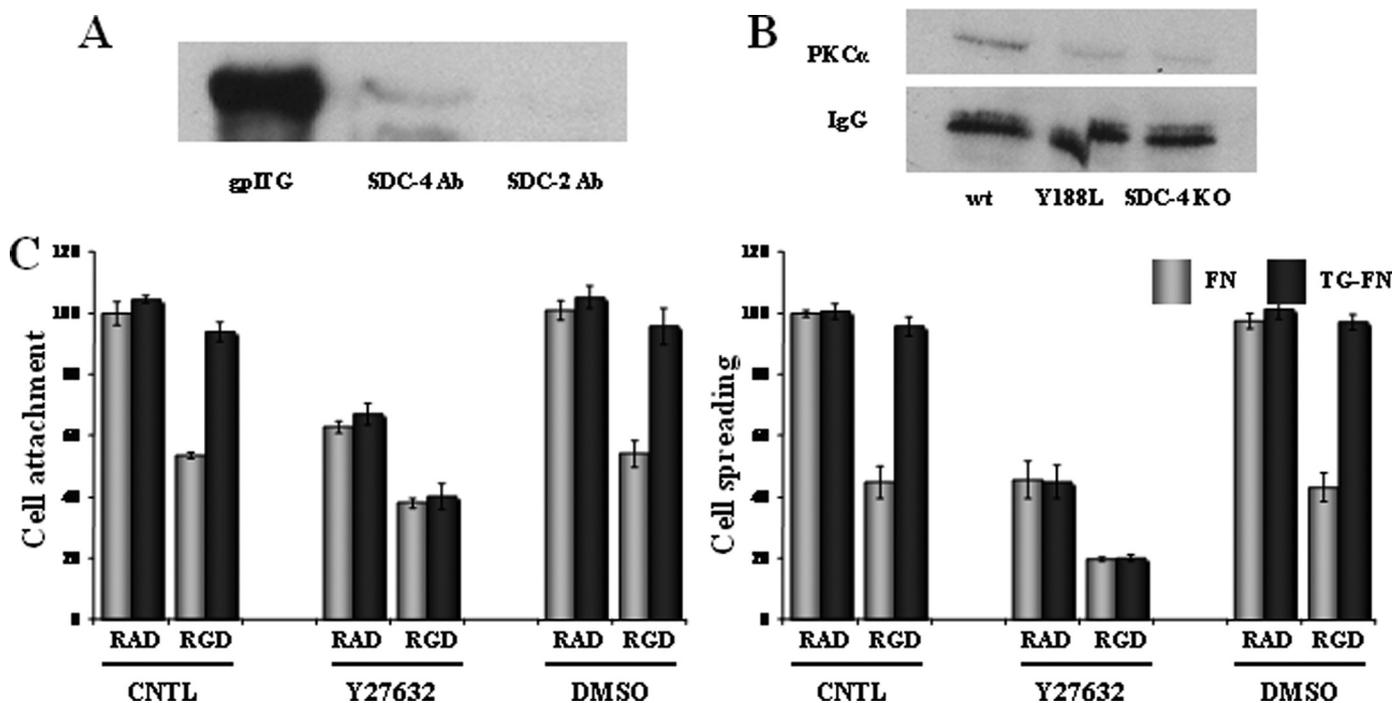
FIGURE 7. **Syndecan-2 does not interact with TG2 directly but requires activation by PKCα and can be blocked by ROCK inhibitor.** *A*, co-immunoprecipitation of syndecan-2 and TG2 was performed as described under "Experimental Procedures," whereas syndecan-4 antibody was used as the positive control antibody for the pull-down procedure. gpITG was loaded as the internal standard. *B*, co-immunoprecipitation of PKCα and syndecan-2 using syndecan-2 antibody and detection of PKCα by Western blotting. Samples from wild type (*wt*), syndecan-4 Y188L mutant (*Y188L*), and syndecan-4 null (*SDC-4 ko*) MEF cells were prepared as described under "Experimental Procedures." *C*, after treatment with ROCK inhibitor Y27632 (10 mM) or DMSO treatment (0.1%), MEF cells were seeded on FN or TG-FN with RAD or RGD peptide. The percentage value of non-treated attached and spread cells on FN was used as 100%.

tion of biotin-labeled FN. As shown in Fig. 6*C*, as expected, the presence of heparin completely blocked the FN fibril formation when cells were seeded on FN-TG in the presence of RGD peptide (35) after a 3-h incubation with the cells.

In order to further investigate the role of syndecan-2 in this signaling pathway of TG-FN, we used immunoprecipitation assays to detect the direct interaction of syndecan-2 with TG2 in the initial cell adhesion event. Unlike cell surface syndecan-4, we could not demonstrate any direct interaction with syndecan-2, confirming that syndecan-2 is acting downstream of syndecan-4 (Fig. 7*A*). Given this downstream effector role for syndecan-2 in cell adhesion and fibronectin fibril formation mediated by the TG-FN heterocomplex, we further explored whether the cross-talk between syndecan-4 and -2 was mediated by PKCα. Immunoprecipitation assays were performed by using the anti-syndecan-2 antibody to pull down the syndecan-2-associated proteins in wild type, syndecan-4 null cells and syndecan-4 mutant MEF cells (mutant Y118L, without the PKCα binding site in the syndecan-4 cytoplasmic domain). As shown in Fig. 7*B*, by using a specific anti-PKCα antibody in Western blotting, the presence of this antigen was detected in all three cell types, whereas interestingly, as might be expected without the binding between PKCα and syndecan-4 mutant cell) or the presence of syndecan-4 (null cells), the interaction between PKCα and syndecan-2 was significantly decreased.

Because it has been reported that Rho and ROCK are the downstream signaling molecules in the syndecan-2 signaling pathway (32), cell adhesion assays were further performed in MEF cells by using the ROCK inhibitor Y27632, previously

shown to block the syndecan-2-mediated cell spreading and focal adhesion formation in cells seeded on FN matrix (32). As shown in Fig. 5*C*, the treatment of cells with Y27632 inhibited more than 30% ($p < 0.05$) cell attachment and spreading on FN, compared with the control cells treated with DMSO. Unlike the observed compensatory effect on the control cells, TG-FN did not rescue the loss of cell attachment and spreading in the Y27632-treated MEF cells (Fig. 7*C*), thus confirming the importance of ROCK.

Overexpression of either Syndecan-2 or Syndecan-4 Can Enhance the Compensatory Effect of TG-FN in the RGD-independent Cell Adhesion—To further confirm the wider importance of both syndecan-2 and 4 in FN-TG-mediated RGD-independent cell adhesion, investigations were also undertaken in transfected human glioblastoma cells T98G overexpressing either syndecan-2 or syndecan-4 (29). TG-FN compensated for the effect of RGD peptide and restored the cell adhesion and spreading back to the levels in RAD peptide-treated cells on FN in wild type T98G cells. The study was further performed using glioblastoma cells overexpressing syndecan-2 or syndecan-4 (29) (Fig. 8, *A* and *C*, respectively). In the FN control groups, overexpressed syndecan-2 or syndecan-4 did not support any increased cell attachment and spreading in the presence of RGD peptide, suggesting the associated roles of these receptors in β1 integrin-mediated cell adhesion. However, when seeded on TG-FN, over 30% more cell attachment and around 40% more cell spreading were achieved in both syndecan-2- and syndecan-4-overexpressing T98G cells in the RGD peptide-treated cells (Fig. 8, *B* and *D*, respectively).
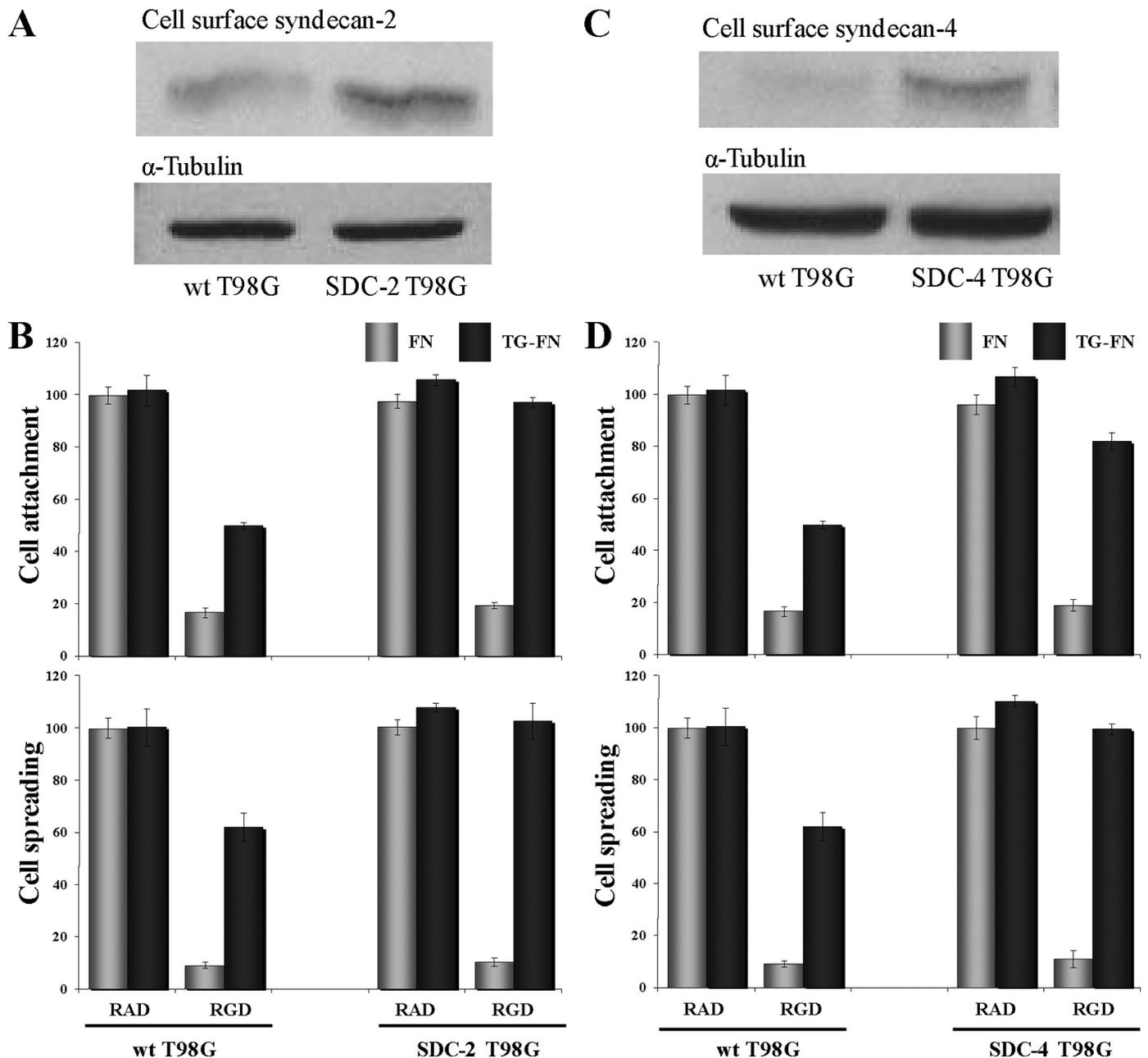
FIGURE 8. **Overexpression of syndecan-2 or syndecan-4 further enhances the compensatory effect of TG-FN complex.** *A* and *C*, Western blotting of syndecan-2 or syndecan-4 in wild type and syndecan-2- or syndecan-4-overexpressing T98G glioblastoma cells. Following cell surface biotinylation as described under "Experimental Procedures," α-tubulin is used to control for equal loading of proteins. *B* and *D*, cell adhesion assay for syndecan-2 (SDC-2 T98G) or syndecan-4 T98G (SDC-4 T98G) cells was undertaken in the presence of RAD or RGD peptide. Mean cell attachment and spreading are expressed as the percentage of control values of wild type T98G (*wt T98G*) or FN (which represents 100%) $\pm$ S.D. in the presence of RAD peptide.

TG-FN-mediated Cell Adhesion in the Presence of RGD Peptides Does Not Depend on either $\alpha4$ Integrin- or $\beta3$ Integrin-activated Pathways—Because TG2 is also known to be a ligand for $\alpha4\beta1$ integrin (39), which has been reported to be expressed in mouse fibroblasts (40), we explored whether the RGD-independent cell adhesion in response to TG-FN involves $\alpha4\beta1$ integrins in fibroblasts (41). The cell attachment and spreading of MEF cells on FN and/or TG-FN was not significantly ($p > 0.05$) affected with either control mouse IgG treatment or function-blocking anti-integrin $\alpha4$ antibody 9C10 (42) treatments at 30 μg/ml (Fig. 9*A*) in the presence of RAD peptide. This antibody did not induce further loss of cell

attachment and spreading caused by the addition of RGD peptides, agreeing with previous reports that this integrin seems to only affect cell adhesion after incubation times longer than those used here (42). In both mouse IgG- and 9C10 antibody-treated cells, TG-FN rescued significantly the loss of cell adhesion mediate by RGD peptides, suggesting that the compensatory effect of this complex is independent of the presence of cell surface $\alpha4\beta1$ integrins.

Because of its reported involvement with TG2 (43), another cell surface integrin, $\beta3$ integrin was also studied in RGD-independent cell adhesion mediated by the TG-FN hetero-complex (supplemental Fig. 1*C*). The absence of $\beta3$ integrins
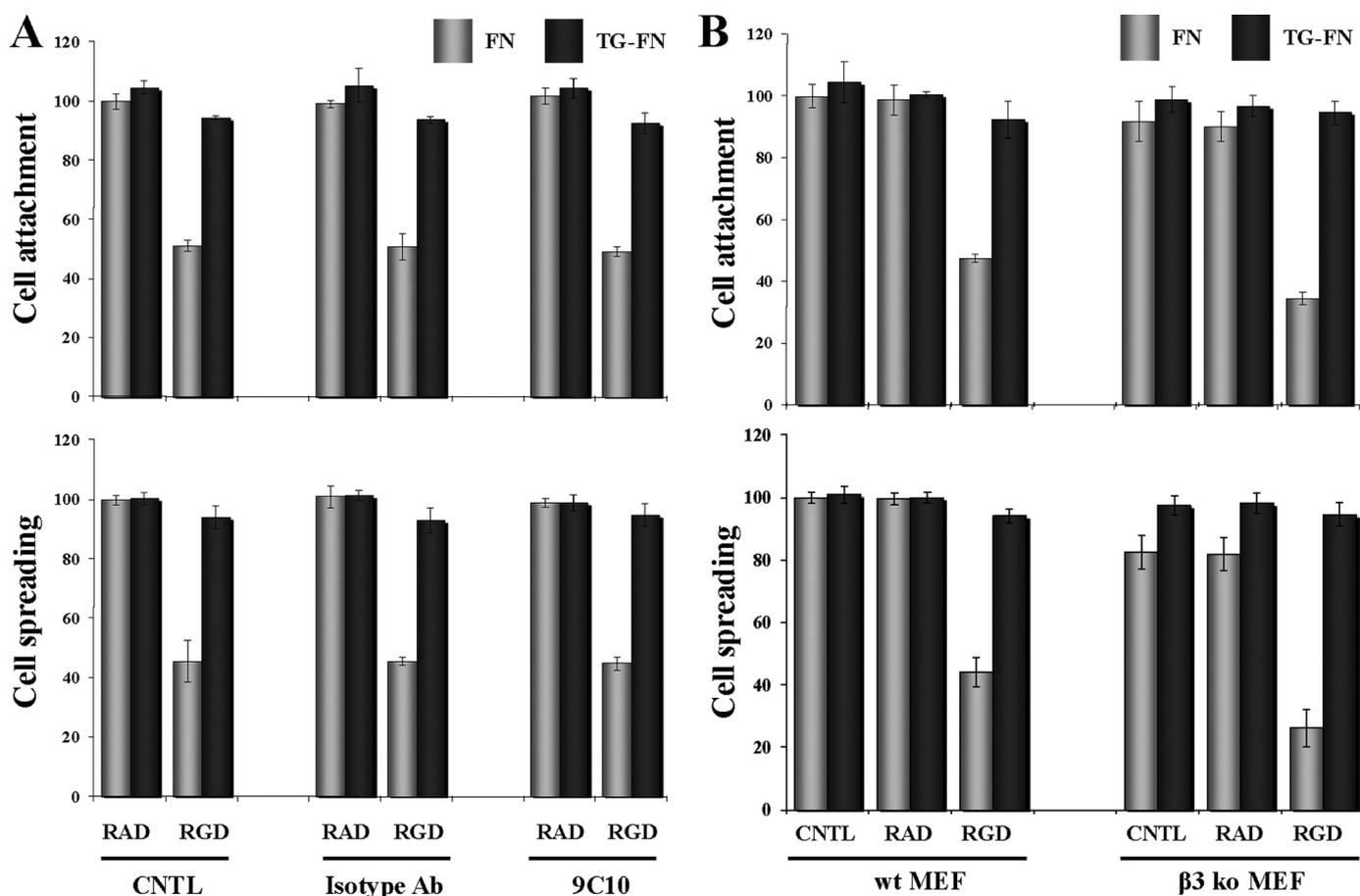
FIGURE 9. **The roles of $\alpha 4\beta 1$ and $\beta 3$ integrins in cell adhesion on TG-FN.** *A*, MEF cells in suspension (2.5×10^5) were pretreated with 30 μg/ml anti-$\alpha 4$ integrin antibody (9C10) or control mouse IgGs (isotype Ab) with RGD or RAD (100 μg/ml) peptides before seeding on either FN or TG-FN. *B*, $\beta 3$ integrin wild type (*wt MEF*) and null (*$\beta 3$ ko MEF*) MEF cells were used in a cell adhesion experiment in the presence of RAD or RGD peptide. The mean cell attachment and spreading values are expressed as the percentage of control values of non-treated wild type MEF on FN (which represents 100%) $\pm$ S.D.

in $\beta 3$ null MEF cells (Fig. 9*B*) did not affect cell attachment significantly ($p > 0.05$) compared with the wild type cells, whereas the influence of this integrin on cell spreading was very apparent in control and RAD groups seeded on FN matrix. Without the presence of $\beta 3$ integrins, the cell lost $\sim$20% of cell spreading. Importantly, the loss of $\beta 3$ integrins did not influence the effect of the TG-FN heterocomplex in compensating for RGD peptide-induced loss of cell attachment and spreading.

DISCUSSION

TG2 is now recognized as a wound response enzyme, which, under stress conditions, is up-regulated, leading to its secretion into the extracellular matrix (23, 24). In TG2 KO animals, wound healing is delayed (23), whereas overexpression of the enzyme can lead to fibrosis (44). Once secreted into the extracellular matrix, TG2 forms a heterocomplex with its high affinity binding partner FN, which is thought to mediate down-regulation of its transamidating activity and make it more resistant to proteolytic degradation (7). We previously reported that this TG-FN complex could by virtue of its binding to syndecan-4 and by activation of $\beta 1$ integrin cosignaling maintain cell adhesion and facilitate cell survival in the presence of the RGD-containing peptides released during matrix turnover. Interestingly, like TG2, syndecan-4 KO mice

also show delayed wound healing (45). In this paper, we extend our previous work first by investigating the role of this RGD-independent cell adhesion process on fibronectin fibril formation, an event key to wound healing; second, we also explore the involvement of other cell surface receptor(s) and signaling pathway(s) that are central to this event. Cell surface TG2 involvement in fibronectin deposition in an integrin-dependent but transamidating-independent manner has been reported previously (46). In contrast, in other reports (47, 48), the cross-linking activity of TG2 was also reported to be required in FN assembly and deposition. We therefore first ruled out that the mechanism used by fibronectin matrix-bound TG2 for both cell adhesion and FN fibril formation was different from that used by cell surface TG2 (46) by demonstrating that MEF cells overexpressing cell surface TG2 were unable to compensate for RGD-mediated loss of cell adhesion. By monitoring FN fibril formation through the addition of soluble biotinylated FN, we also ruled out the involvement of TG2 transamidating activity in this event because the addition of a site-directed TG irreversible inhibitor R283 had no effect on the process. We show that the TG-FN complex can increase the rate of fibril formation by wild type MEF cells when compared with equal numbers of cells adhered on FN alone in the early stages of FN deposition (1–6 h). Impor-

tantly, in keeping with the role of this stress-induced hetero-complex in wound healing and matrix turnover, we demonstrate that even in the presence of the RGD-containing peptides, FN fibril formation was still maintained. This was in direct contrast to cells seeded on FN alone, where the RGD peptide treatment led to rounded cells with a lack of focal adhesion assembly and a marked inhibition of FN fibril assembly. This fits with previous observations in that focal adhesion point assembly, actin cytoskeleton formation, and intracellular signaling via RhoA are required for FN fibril formation to occur (16), all of which are present in cells seeded on TG-FN in the presence of RGD peptides.

Interestingly, it has been reported in longer term incubations (20 h) that no difference in FN fibril formation was observed between syndecan-4 siRNA-treated cells and control cells (15). Given our hypothesis that syndecan-4 may be important in the early stages of fibril formation following cell spreading, we further investigated fibril formation in syndecan-4 KO cells, the syndecan-4 Y188L mutant (which is unable to bind PKCα), and cells treated with GK21 peptide (which blocks binding of PKCα to the cytoplasmic domain of β1 integrins (22)). The results of these experiments clearly indicated that syndecan-4 and its downstream signaling molecule PKCα is required for FN bound TG2 to mediate FN fibril formation. Given that there are two major sources of globular FN present in any physiological system, the extracellular FN from serum that is both in solution and surface-bound and the cellular secreted FN from fibroblasts, we undertook to investigate the effect of TG-FN matrix on cellular FN deposition. Our results confirmed that the FN-TG complex can also deposit cellular FN in the presence of RGD peptide, indicating that the two major sources of globular FN available to the cell are deposited, not fibrillar, FN by this mechanism.

Our next step was to determine whether the TG2 bound to the globular FN present on the original matrix remained detectable and hence available to cells after FN fibril deposition had occurred. To do this, the cells were removed by EDTA, TG2 antibody was added to the remaining matrix, and the immunocomplex was then detected by ELISA. Our data indicated no change in the detectable levels of FN-bound TG2, strongly suggesting the availability of this reservoir of TG2 to the adherent cells for up to 16 h when RGD peptides are present, thus confirming the ability of the TG-FN complex to maintain cell spreading and facilitate FN fibril formation over sustained periods of time.

Our next task was to determine which other cell surface receptors were involved in TG-FN-induced cell adhesion in the presence of RGD peptides and to assess their importance in FN fibril formation. The important role of β1 integrin signaling in regulating RGD-independent fibroblast cell adhesion has been proven by our previous data (22). By using a β1 functional blocking antibody HMβ1-1, we confirmed that TG-FN regulates signaling transduction through the inside-out signaling pathway of β1 integrins because HMβ1-1 inhibited cell adhesion on FN via blocking the phosphorylation of FAK[397], whereas with the phosphorylation of FAK[861], the ligand-binding independent activation of FAK was maintained. We then confirmed by using KO cells, specific inhibitory pep-

tides, and function blocking antibodies that α5 integrin, the closest partner for β1 integrins, is involved in the RGD-independent cell adhesion mediated by TG-FN, whereas the involvement of other TG2-associating integrins, α4β1 and β3 integrins (39, 43), was ruled out. The poorly formed FN fibrils by β1 or α5 integrin KO fibroblasts also confirmed that these two receptors are required by TG-FN to enhance both cell adhesion and FN fibril formation when fibroblasts are seeded on it.

Syndecan-2 has been implicated as a downstream effector in both β1 integrin and syndecan-4 signaling via facilitating the formation of actin stress fiber formation (14). Because RGD-dependent integrins are blocked by the presence of RGD peptides and FN-bound TG2 is occupying heparan sulfate binding sites on syndecan-4, since we ruled out TG2 binding to the syndecan-2 core protein, other cell surface receptors would need to be involved to facilitate soluble FN capture and fibril formation at the efficiency we observe. By using syndecan-2 siRNA silencing, we first demonstrate a role for this other important syndecan in the ability of TG-FN to restore RGD-induced loss of cell adhesion. Importantly, by using both siRNA silencing and the ROCK inhibitor Y27632, we showed that syndecan-2 was key to formation of the actin cytoskeleton and hence cell contraction, but unlike syndecan-4, this role for syndecan-2 was independent of its binding to TG2, leaving it free to bind to and assemble the biotinylated FN.

Indeed, when cells were seeded on TG-FN and treated with heparin prior to and during FN assembly in the presence of RGD peptide, it considerably reduced the amount of FN deposited, confirming the importance of the syndecan-2 in FN deposition. Previous reports have demonstrated a role for RGD-independent mechanisms involving heparan sulfate proteoglycans in FN polymerization (35), and syndecan-2 has been implicated as having a regulatory role in this mechanism (49). The observation that siRNA silencing of syndecan-2 considerably reduced FN fibril formation when cells were bound to FN-TG in the presence of RGD peptides strongly suggests the importance of syndecan-2 in TG-FN-mediated cell adhesion, cell contractility, and FN fibril formation.

Moreover, our data showed that β3 integrin is not required for TG-FN-mediated cell adhesion, suggesting that the involvement of β3 integrin, which can also assemble FN (50, 51), is not required in the RGD-independent mechanism of FN fibril assembly mediated by the TG-FN heterocomplex. Because phosphorylation of the V region of syndecan-2, like that of syndecan-4, has been implicated in the role of syndecan-2 (49) in regulating FN assembly, we investigated whether PKCα could be the potential link between syndecan-4 and syndecan-2 as well as that between syndecan-4 and α5β1 integrin, thus facilitating both cell adhesion and FN fibril assembly. Although it has been reported that syndecan-2 is involved in the syndecan-4 signaling pathway, there has not been any confirmed mechanism indicating how these two receptors are linked. Regarding the fact that several members, including PKCδ (52) and PKCγ (53), have been reported to be involved in syndecan-2 signaling, we hypothesized that PKCα could be the potential link to connect these receptors. Our
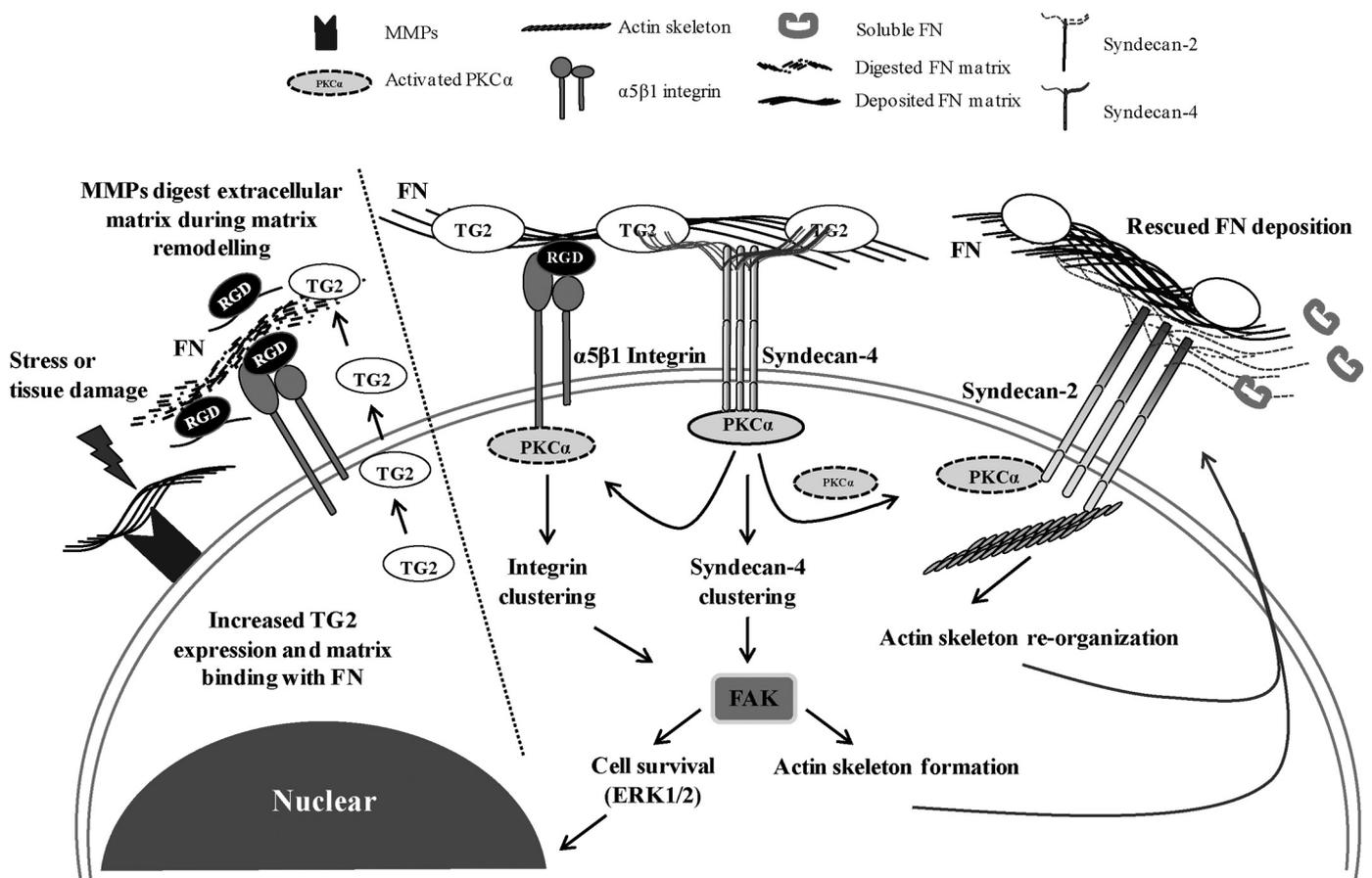
FIGURE 10. **Summary of TG-FN-mediated cell adhesion and FN fibril formation pathway.** As a response to stress induction/tissue injury, the increased expression of TG2 leads to its externalization and deposition into extracellular matrix and formation of TG-FN complex; meanwhile, the up-regulation and secretion of proteases, such as the matrix metalloproteinases (*MMPs*), start to degrade the matrix protein during matrix turnover, leading to release of the RGD motif-containing fragments, which block integrin-mediated cell adhesion, inducing the loss of cell adhesion and cell death. The direct interaction between matrix FN-bound TG2 and the heparan sulfate chains of syndecan-4 leads to activation of the inside-out signaling of β1 integrins by the binding between PKCα and the intracellular domain of β1 integrins, promoting α5β1 integrin-related cell adhesion and focal adhesion formation. As a downstream molecule of syndecan-4, syndecan-2 is activated by intracellular PKCα, without the direct interaction with the matrix TG2; this mediates cytoskeletal formation and cell contraction via activation of ROCK, whereas its extracellular domain together with any α5β1 integrins not blocked by RGD peptides capture the soluble FN molecules and mediate the FN fibril deposition process, thus maintaining cell homeostasis.

observation that PKCα and syndecan-2 could be immunoprecipitated together and that this association was reduced in syndecan-4 null cells and in syndecan-4 mutant Y188L cells (without the PKCα binding site in the syndecan-4 cytoplasmic domain) seemed to confirm our hypothesis that PKCα could be the important link between these different syndecan receptors. Moreover, the importance of both syndecan-2 and syndecan-4 in TG-FN-mediated cell adhesion in the presence of RGD was confirmed using T98G glioblastoma cells overexpressing either syndecan-2 or syndecan-4 (29), which further promoted the compensatory effect of TG-FN in an RGD-independent manner.

To conclude, our work demonstrates a number of important cellular functions of TG2 not related to its cross-linking activity when it is released into the extracellular matrix under stress conditions such as those following tissue insult/injury. Once bound to its high affinity binding partner FN, which is also prevalent following tissue injury, it can maintain cell adhesion and cell viability in the presence of RGD-containing peptides released during matrix turnover. Importantly, while maintaining cell adhesion and promoting cell spreading

through a PKCα-mediated signaling cascade involving the activation of syndecan-4, syndecan-2, and α5β1, the TG-FN complex can also mediate FN fibril deposition (see Fig. 10), a process that is central to a multitude of physiological and pathological events.

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RGD-independent Cell Adhesion via a Tissue Transglutaminase-Fibronectin Matrix Promotes Fibronectin Fibril Deposition and Requires Syndecan-4/2 and α5 β1 Integrin Co-signaling

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