Interactions Between RAMP2 And CRF Receptors: The Effect Of Receptor Subtypes, Splice Variants And Cell Context.

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ABSTRACT

Corticotrophin releasing factor (CRF) acts via two family B G-protein-coupled receptors, CRFR1 and CRFR2. Additional subtypes exist due to alternative splicing. CRFR1α is the most widely expressed subtype and lacks a 29-residue insert in the first intracellular loop that is present in CRFR1β. It has been shown previously that co-expression of CRFR1β with receptor activity modifying protein 2 (RAMP2) in HEK 293S cells increased the cell-surface expression of both proteins suggesting a physical interaction as seen with RAMPs and calcitonin receptor-like receptor (CLR). This study investigated the ability of CRFR1α, CRFR1β and CRFR2β to promote cell-surface expression of FLAG-tagged RAMP2. Four different cell-lines were utilised to investigate the effect of varying cellular context; COS-7, HEK 293T, HEK 293S and [ΔCTR]HEK 293 (which lacks endogenous calcitonin receptor). In all cell-lines, CRFR1α and CRFR1β enhanced RAMP2 cell-surface expression. The magnitude of the effect on RAMP2 was dependent on the cell-line ([ΔCTR]HEK 293>COS-7>HEK 293T>HEK 293S). RT-PCR indicated this variation may relate to differences in endogenous RAMP expression between cell types. Furthermore, pre-treatment with CRF resulted in a loss of cell-surface FLAG-RAMP2 when it was co-expressed with CRFR1 subtypes. CRFR2β co-expression had no effect on RAMP2 in any cell-line. Molecular modelling suggests that the potential contact interface between the extracellular domains of RAMP2 and CRF receptor subtypes is smaller than that of RAMP2 and CRL, the canonical receptor:RAMP pairing, assuming a physical interaction. Furthermore, a specific residue difference between CRFR1 subtypes (glutamate) and CRFR2β (histidine) in this interface region may impair CRFR2β:RAMP2 interaction by electrostatic repulsion.

Keywords: Receptor activity modifying protein (RAMP), corticotrophin releasing factor receptor (CRFR), family B GPCR, splice variants, translocation.

Abbreviations: ACTH, adrenocorticotropic hormone; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CRF, corticotrophin releasing factor; CRFR, corticotrophin releasing factor receptor; ECD, extracellular domain; ELISA, enzyme-linked

immunosorbant assay; FACS, fluorescence-activated cell sorting; GPCR, G-protein-coupled receptor; HA, haemagglutinin tag; RAMP, receptor activity modifying protein.

1. Introduction

Corticotrophin releasing factor (CRF) is produced by the hypothalamus and acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH), which in turn releases cortisol from the adrenal cortex. CRF also has numerous actions in the central nervous system related to stress and anxiety [1]. The hormone acts via two family B G-protein-coupled receptors (GPCRs), CRFR1 and CRFR2, and there are splice variants of both receptors. The CRFR1 is the best characterised, with high expression in the pituitary, adrenal gland and various brain regions. Furthermore, it is a target for anxiolytic drugs [1]. The full length sequence of the CRFR1 is that of the β-subtype. However, in humans the most widely expressed isoform is the α-subtype, which lacks exon 6 and consequentially has a 29-residue deletion (residues 146-174) in intracellular loop 1 compared to CRFR1β [1]. CRFR1β shows weaker coupling to Gs compared to the CRFR1α, typically resulting in >10-fold lower potency for CRF [1, 2]. For CRFR2, the β-isoform is the major splice variant as the CRFR2α lacks a signal peptide at its N-terminus, so tends to be retained intracellularly [3].

Many family B GPCRs can form complexes with receptor activity modifying proteins (RAMPs). In mammals, RAMPs form a family of three single-pass membrane proteins (RAMP1, RAMP2, RAMP3). They have a range of effects on GPCRs including promotion of cell-surface expression plus modulation of ligand binding profile and signal transduction pathways [4]. The archetypical RAMP:GPCR interaction is that between a RAMP and the calcitonin receptor-like receptor (CLR) where there is direct interaction between the two proteins [5, 6]. When assaying intracellular cAMP signalling, a calcitonin gene-related peptide (CGRP) receptor is created by a RAMP1:CLR complex whereas an adrenomedullin receptor is generated by RAMP2:CLR or RAMP3:CLR complexes [7].

It has been shown previously that co-expression of RAMP2 with CRFR1β results in increased cell-surface expression of both proteins [8]. However, the ability of RAMP2 to interact with the more widely expressed CRF receptor, the CRFR1α subtype, was not investigated. The purpose of the current study was to determine whether CRFR1α or CRFR2β could interact (either directly or indirectly) with RAMP2 and change cell-surface expression, as previously reported for CRFR1β. In addition, as the magnitude of the effect reported previously with RAMP2:CRFR1β complexes was cell-line dependent [8], the influence of cell context on RAMP2:CRFR interaction was also investigated. Finally, on the assumption of a direct interaction between the CRFR subtypes and RAMP2 (as seen with CLR and RAMPs, [5, 6]), molecular modelling was then employed to provide mechanistic insights into differences that had been observed experimentally between RAMP2:CRFR1 and RAMP2:CRFR2.

2. **Materials and methods**

2.1. Constructs

Both the α and β variants of haemagglutinin (HA)-tagged CRFR1 were constructed in the same way, with the HA-tag being inserted immediately after the signal peptide, between A23 (the end of the predicted signal peptide) and S24, the start of the mature protein. This replicates the construct design used previously [6]. These two constructs were custom-synthesised by GenScript (New Jersey, U.S.A.). The CRFR2 construct comprised the native signal peptide plus receptor sequence and was provided by Dr. Simon Dowell (GSK, Stevenage, UK). HA-tagged CLR was as previously described and characterised [9]. Briefly, the endogenous signal peptide was replaced by the 21-residue CD8A signal peptide, followed by a four-residue linker (DYAS), then the HA-tag plus a further linker (SLGGPSLEGSA), with the receptor sequence commencing at E23. FLAG-RAMP2 was as described previously [8], with the FLAG-tag inserted after the final residue of the signal peptide.

2.2. Cell culture

COS-7 cells, HEK 293T cells and HEK 293S cells (a gift from AstraZeneca), were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10 % heat-inactivated fetal bovine serum (FBS) plus 1% antibiotic/antimycotic (Sigma) and incubated at 37 °C in humidified 95 % air and 5 % CO_2. Cells were passaged 1 in 5 every 3 days when confluency was at ~80 %. HEK 293 cells lacking the calcitonin receptor ([ΔCTR]HEK 293 cells) were a gift from Drs. David Hornigold, Jacqueline Naylor and Alessandra Rossi (MedImmune, Cambridge, UK). [ΔCTR]HEK 293 cells were cultured in Minimal Essential Medium supplemented with 10 % heat-inactivated FBS plus 1 % non-essential amino acids and incubated at 37 °C in humidified 95 % air and 5 % CO_2 and passaged 1 in 5 every 5 days.

2.3. Flow cytometry

Fluorescence-activated cell sorting (FACS) was used to assess cell-surface expression of FLAG-RAMP2. HEK 293S, HEK 293T or [ΔCTR]HEK 293 cells were transfected using Fugene HD (Promega) in accordance with the manufacturer's instructions using a 1:3 (w:v) DNA:Fugene ratio. COS-7 cells were transfected with PEI, using a 1:3 (w:v) ratio of DNA:PEI. Co-transfections utilised 0.25 μg DNA per construct per well, a total of 0.5 μg DNA per well. The DNA/PEI mix was diluted in un-supplemented DMEM/F12 to a total volume of 25 μL and incubated at room temperature for 10 min before addition to cells cultured in complete media. Transfected cell lines were grown for 48 h prior to assaying. Mock-transfections were used as control. Transiently transfected cells were removed from the plate using trypsin and washed with PBS prior to counting. 5 x 10^5 cells were washed three times in FACS buffer (PBS supplemented with 1 % BSA plus 0.03% sodium azide). Cells were then resuspended in 50 μL FACS buffer containing APC-conjugated anti-FLAG monoclonal antibody (BioLegend, diluted 1:100 in FACS buffer) and incubated in the dark at room temperature for 1 h. The cells received a further three washes in FACS buffer and were re-suspended in 50 μL FACS buffer. To account for dead cells, 2.5 μL propidium iodide was added to each sample. Samples were analysed using BD Accuri C6 flow cytometer (BD Biosciences) with excitation and emission wavelengths of 633 nm and 660 nm, respectively. An increase in APC intensity indicated increased plasma membrane expression of FLAG-RAMP.

2.4. Enzyme-linked immunosorbant assay (ELISA)

ELISA was used to assess cell-surface expression of HA-CRFR1α and HA-CRFR1β. HEK 293S cells were seeded on poly D-lysine-coated 24-well plates at ~1.5 x 10^5 cells/well, transfected with PEI as described previously [8, 9] and incubated for 48 h post-transfection. Mock-transfected cells were used as control to determine the background value. ELISA was carried out as previously described [8]. The primary antibody (mouse anti-HA, Sigma H9658) was diluted 1:3500 in blocking solution and the secondary antibody (horse anti-mouse HRP-linked IgG, New England Biolabs, 7076S) was diluted 1:3500 in PBS.

2.5. Reverse-transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from COS-7, HEK 293T, HEK 293S or [ΔCTR]HEK 293 cells using Ambion RNAqueous™-4PCR kit (Life Technologies) as per the manufacturer's instructions. All RNA samples were treated with DNase I to remove any contaminating genomic DNA. Reverse transcription was performed using QuantiTect reverse transcription kit (Qiagen) as per the manufacturer's instructions. Minus Reverse Transcriptase negative controls were performed simultaneously and the PCR amplification was performed using the following conditions: 95 ˚C - 30 sec, 60 ˚C - 30 sec and 68 ˚C – 20 sec for 30 cycles then 68 ˚C – 5 minutes. Gene-specific primers to all RAMPs and CTR and CLR were used as follows: RAMP1, forward (5'-CTGCCAGGAGGCTAACTACG-3') and reverse (5'-GACCACGATGAAGGGGTAGA-3') [10]; RAMP2, forward (5'-GGGGGACGGTGAAGAACTAT-3') and reverse (5'- GTTGGCAAAGTGGATCTGGT-3') [10]; RAMP3, forward (5'- AACTTCTCCCGTTGCTGCT-3') and reverse (5'-GACGGGTATAACGATCAGCG-3') [10]; CTR forward (5'-CCTATCACCCAATAGAGCCCAAG-3') and reverse (5'-TGCATTCGGTCATAGCATTTGTA-3') (PrimerBank ID, 260064026c1); CLR forward (5'-ACCAGGCCTTAGTAGCCACA-3') and reverse (5'- ACAAATTGGGCCATGGATAA-3') [10]; and GAPDH forward (5'-TGCACCACCAACTGCTTAGC-3') and reverse (5'-GGCATGGACTGTGGTCATGAG-3'). All products were resolved on a 2 % agarose gel and imaged using a G:Box iChemi gel documentation system utilising GeneTools analysis software (Syngene) with densitometry performed using GeneTools. Expression of RAMP, CLR and CTR was standardised to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously [11].

2.6. cAMP accumulation assays

Assays were performed as described previously [9]. Briefly, HEK-293 cells transiently-transfected with an individual CRFR subtype were washed in PBS, resuspended in PBS containing 0.1% BSA plus 0.5 mM IBMX, and seeded at 2000 cells/well in 384-well white Optiplates. CRF was added at the concentrations indicated in the range of 1 pM to 1 μM and cAMP accumulation was measured after 8 min using a LANCE cAMP detection kit (PerkinElmer Life Sciences). Plates were read using a Mithras LB 940 multimode micro-plate reader (Berthold Technologies). Values were converted to concentration using a cAMP standard curve performed in parallel and standardised to cells stimulated with forskolin (100 μM) which provided the system maximum.

2.7. CRF-induced RAMP2 internalisation

FACS was used to determine RAMP2 internalisation induced by agonist stimulation of CRFR1α and CRFR1β. HEK293S cells were transfected as above. 48 h post-transfection, cells were removed from the plate using trypsin and washed with PBS. 5 x 10^5 cells were incubated in PBS supplemented with 0.1 % BSA ± 1 μM CRF for 1 h at room temperature, washed with ice-cold FACS buffer and placed on ice. Cells were then assayed as described above (Section 2.4) but kept at 4 °C throughout.

2.8. Molecular modelling

The sequences corresponding to the full length CRFR1 and CRFR2β receptor were obtained from UNIPROT [12] and submitted to the SignalP [13] server to determine the position of the cleavable signal peptides. A manual sequence alignment was generated between the CRF1R, CRF2R, CLR with the signal peptides removed and was used to generate homology models using the crystal structures of CLR-RAMP2 (PDB: 4RWF [6]) as templates. MODELLER [14] was used to generate 1000 models of each CRFR1:RAMP2 and CRFR2β:RAMP2 which were subsequently refined and scored using ROSETTA [15]. The best 50 ROSETTA scored structures were clustered and the middle structure from the largest cluster for each model was visually examined.

2.9. Data analysis

GraphPad Prism v7.0 was used for data and statistical analysis (GraphPad Software Inc, San Diego, CA). Flow cytometry and ELISA data were normalized to cell-surface expression (of RAMP or receptor) for cells co-transfected with calcitonin receptor-like receptor (HA-CLR) and FLAG-RAMP2 (100 %) and cells transfected with empty vector (0 %). CLR is the archetypical partner GPCR for RAMP2. Student's t-tests were performed on data where there was a comparison between the means of two data sets. A one-way ANOVA was performed when three or more means were compared, with post-hoc Dunnett's test or Tukey's test as appropriate. A result was determined to be statistically significant if the P-value was <0.05.

3. Results

3.1. The CRFR1α, CRFRβ and CRFR2β constructs are functional

It was important to establish that the CRFR1α, CRFR1β and CRFR2β constructs used in this study were expressed at the cell surface and were functional with respect to mediating CRF-induced intracellular signaling. Dose-response curves of CRF-stimulated cAMP production were generated for each CRFR subtype (Fig. 1). CRF had the expected potency order of CRFR1α> CRFR1β>CRFR2β [1].

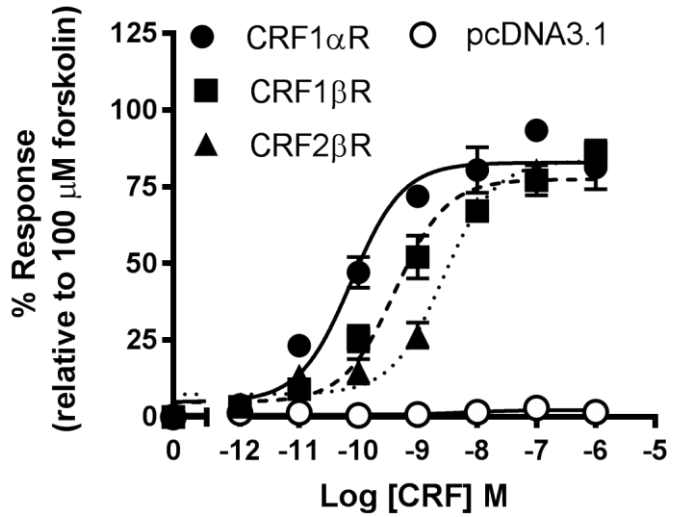


Fig. 1. CRF-induced cAMP production by individual CRFR subtypes.
The cAMP dose-response curves were constructed for CRFR1α (●), CRFR1β (■), CRFR2β (▲), or vector-only control (○) in response to CRF stimulation at the concentrations indicated using transiently-transfected HEK 293T cells. The cAMP values were normalised (100%) to the maximal cAMP concentration produced by 100 μM forskolin (range; 66.5 nM-69.5 nM per 2000 cells). Data are the mean ± s.e.m. of 4-6 independent experiments performed in triplicate.

3.2 Co-expression of RAMP2 and CRF receptors in COS-7 cells

CRF receptor (CRFR) subtypes were individually co-expressed with FLAG-tagged RAMP2 (FLAG-RAMP2) in COS-7 cells. When cell-surface expression of FLAG-RAMP2 was determined by flow cytometry, a significant increase was observed in the presence of CRFR1α (8.0-fold) or CRFR1β (7.5-fold). In marked contrast, CRFR2β co-expression did not increase RAMP2 cell-surface expression (Fig. 2).

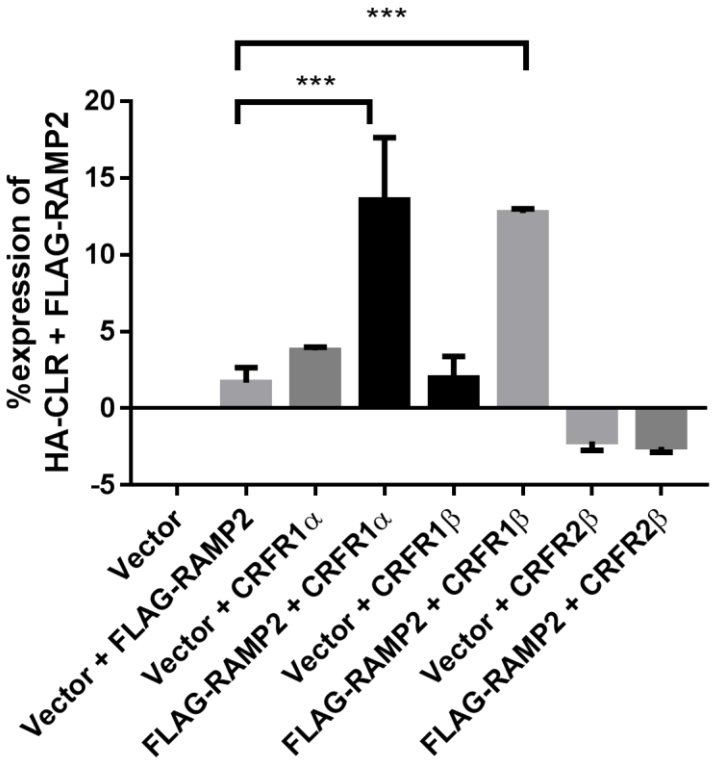


Fig. 2. The effect of co-expression of individual CRFR subtypes on cell-surface expression of FLAG-RAMP2 in COS-7 cells.
Expression was normalised (100%) to that seen when FLAG-RAMP2 was co-expressed with CLR, the archetypical GPCR partner for RAMP2. Data are the mean ± s.e.m. of three independent experiments performed in triplicate. ***$p<0.001$, one way ANOVA followed by Dunnett's test.

3.3. The effect of cell context on the interaction of CRFR subtypes with RAMP2

It has been reported previously that interaction of CRFR1β with RAMP2 was influenced by the cell-line employed, whereby the increase in cell-surface expression of RAMP2 was much greater in HEK 293S than CHO-K1 cells [8]. Consequently, the effect of the cell context in which the proteins were expressed was also investigated. When HEK 293T cells were employed, both of the CRFR1 subtypes increased FLAG-RAMP2 cell-surface expression (Fig. 3). This was the same effect observed in COS-7 cells (Fig. 2), however the extent of the increase in HEK 293T cells, for both CRFR1α (3.4-fold) and CRFR1β (4.6-fold), was smaller than that observed in COS-7 cells. As with COS-7 cells, there was no change in the cell-surface expression of FLAG-RAMP2 following co-expression with CRFR2β in HEK 293T cells (Fig.

3). A very similar effect was also observed using HEK 293S cells. There was a significantly increased cell-surface expression of FLAG-RAMP2 in the presence of CRFR1α (2.8-fold) or CRFR1β (3.4-fold) in HEK 293S cells but such an increase was completely absent in the presence of the CRFR2β (Fig. 4a).

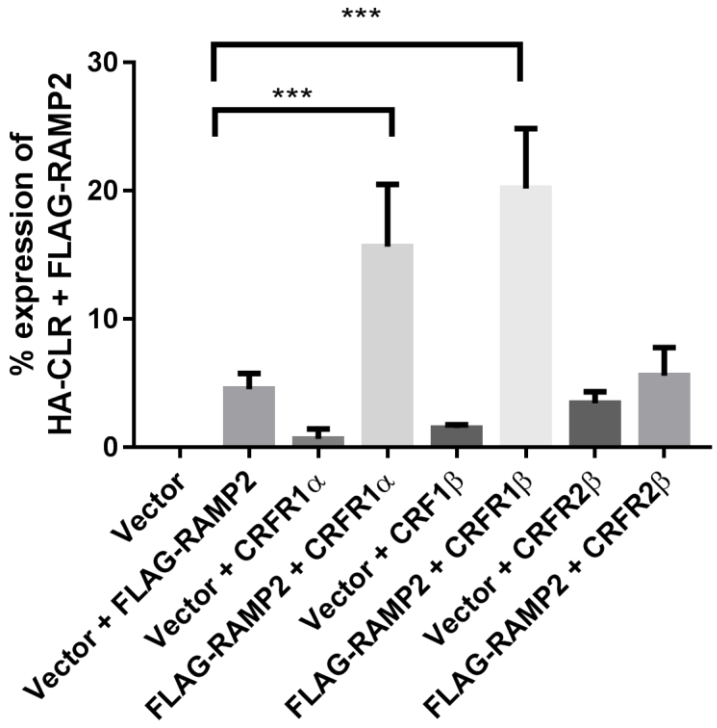
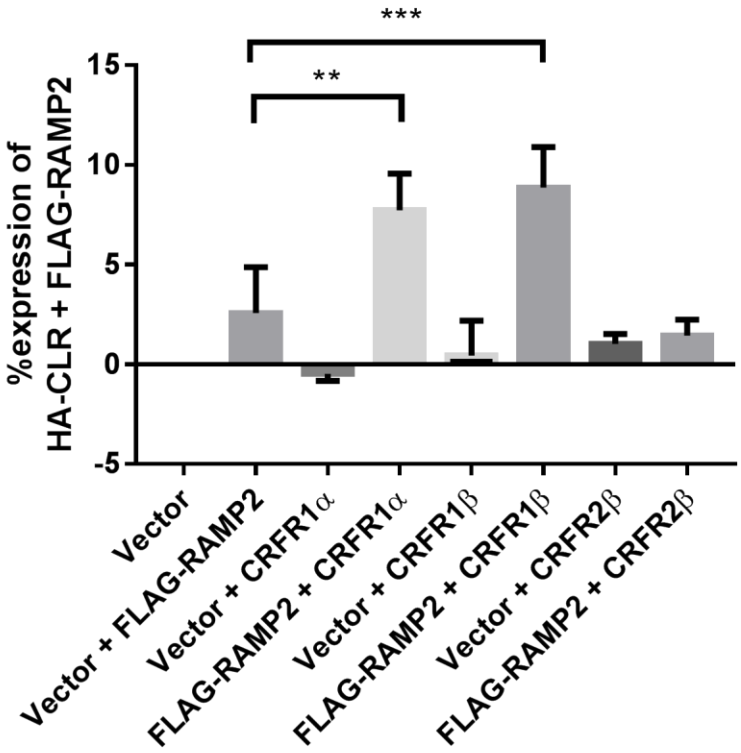


Fig. 3. The effect of co-expression of individual CRFR subtypes on cell-surface expression of FLAG-RAMP2 in HEK 293T cells.
Expression was normalised (100%) to that seen when FLAG-RAMP2 was co-expressed with CLR, the archetypical GPCR partner for RAMP2. Data are mean ± s.e.m. of three independent experiments performed in triplicate. ***$p<0.001$, one way ANOVA followed by Dunnett's test.

Wootten and co-workers [8] reported a reciprocal interaction between RAMP2 and CRFR1β in HEK 293S cells, whereby the receptor increased RAMP2 delivery to the cell surface and likewise, RAMP2 increased cell-surface expression of CRFR1β. Consequently, the ability of RAMP2 to traffic HA-CRFR1α and HA-CRFR1β to the cell surface was investigated in this cell line (Fig. 4b). Data presented in Fig. 4b establish that RAMP2 co-expression enhanced the delivery of both HA-CRFR1α (38.6%) and HA-CRFR1β (29.2%) to the cell surface. The

increase in HA-CRFR1β cell-surface expression observed in this study was consistent with the effect reported previously [8].

a)



b)

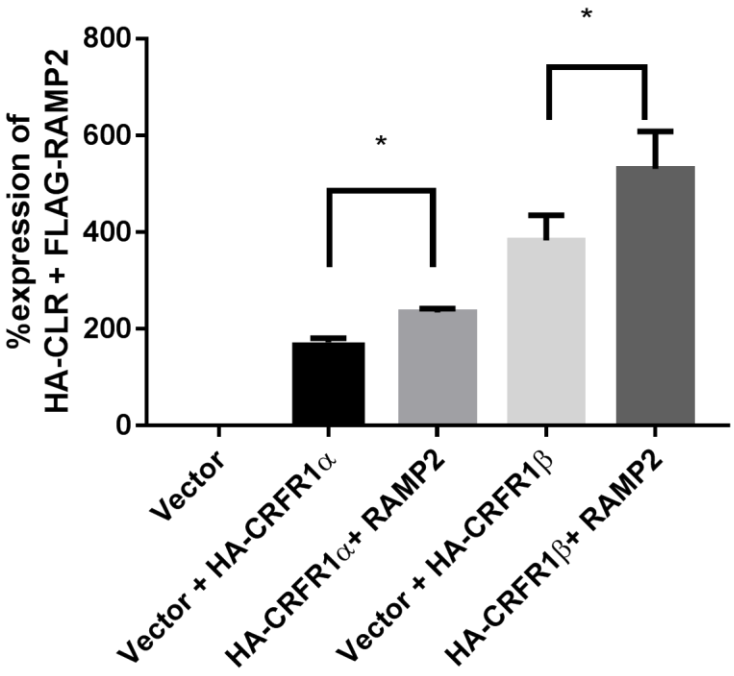


Fig. 4. Cell-surface expression of CRFR1 subtypes and RAMP2 in HEK 293S cells.
Panel a; The effect of co-expression of individual CRFR subtypes on cell-surface expression of FLAG-RAMP2. Panel b; The effect of RAMP2 co-expression on the cell-surface expression of HA-CRFR1α and HA-CRFR1β. Expression was normalised (100%) to that seen when FLAG-RAMP2 was co-expressed with HA-CLR, the archetypical GPCR partner for RAMP2. Data are the mean ± s.e.m of n>3 independent experiments performed in triplicate. *p<0.05, ** p< 0.01, ***p<0.001, one way ANOVA followed by Dunnett's test (panel a) or Tukey's test (panel b).

3.4. Co-expression of RAMP2 and CRFR subtypes in [ΔCTR]HEK 293 cells

It is well-established that the calcitonin receptor (CTR) can interact with all three RAMPs [7] and that HEK 293 cells usually express the CTR endogenously. It was therefore possible that endogenous CTR expression could influence RAMP2 interaction with co-transfected CRFRs by providing an alternative partner for RAMP2. This was investigated using a HEK 293 cell line completely lacking endogenous CTR ([ΔCTR]HEK 293 cells). RAMP2 delivery to the cell surface was markedly increased when co-transfected with CRFR1α (18.4-fold) or CRFR1β (14.6-fold). This was the largest increase in RAMP2 cell-surface expression observed with any cell line employed in this study. In marked contrast, but consistent with all the other cell lines tested, there was no increase in RAMP2 expression with CRFR2β (Fig. 5).

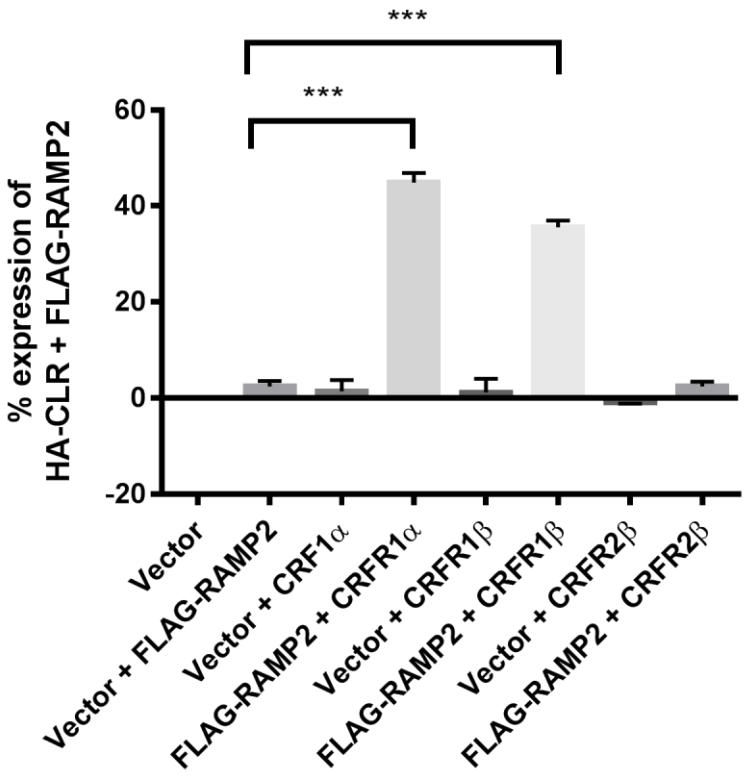


Fig. 5. The effect of co-expression of individual CRFR subtypes on cell-surface expression of FLAG-RAMP2 in [ΔCTR]HEK 293 cells.
Expression was normalised (100%) to that seen when FLAG-RAMP2 was co-expressed with CLR, the archetypical GPCR partner for RAMP2. Data are the mean ± s.e.m of three independent experiments performed in triplicate. ***$p<0.001$, one way ANOVA followed by Dunnett's test.

3.5. Endogenous expression of RAMPs and receptors in the cell lines employed in this study

The mRNA expression levels of all three RAMPs, relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was determined in all the cell lines (COS-7, HEK 293T, HEK 293S and [ΔCTR]HEK 293). mRNA expression was also determined for two GPCRs (CTR and CLR) that are well-established partner receptors for RAMPs (Fig. 6). In COS-7 cells, the RAMPs and the two GPCRs were either absent or detected at only very low levels. In contrast, there was appreciable expression of RAMP1, RAMP2 and CLR in both HEK 293T and HEK 293S cells. The low level expression of CTR detected in the HEK 293T/S cells was ablated in the [ΔCTR]HEK 293 cells as expected. In addition, the [ΔCTR]HEK 293 cells exhibited very low levels of expression of each RAMP and CLR (Fig. 6).

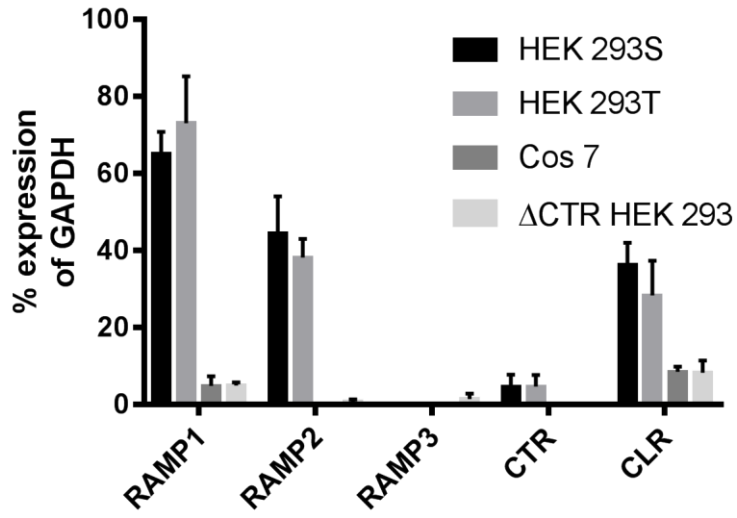


Fig. 6. Expression of mRNA for RAMPs, CTR and CLR.
Expression was measured by RT-PCR in the cell lines indicated and standardised to GAPDH. Data are mean ± s.e.m (n = 3).

3.6. Internalisation of RAMP2 following agonist challenge of co-transfected CRFR

It is well-established that agonist stimulation causes internalisation of GPCRs. To investigate whether there was likely to be a physical interaction between RAMP2 and the CRFR1 subtypes, it was determined whether CRF-stimulation of the CRFR1α or CRFR1β isoforms caused a decrease in cell-surface expression of FLAG-tagged RAMP2. As can be seen in Fig 7, following CRF challenge (1 μM for 1 h), a reduction in RAMP2 on the cell-surface was observed with both receptors.

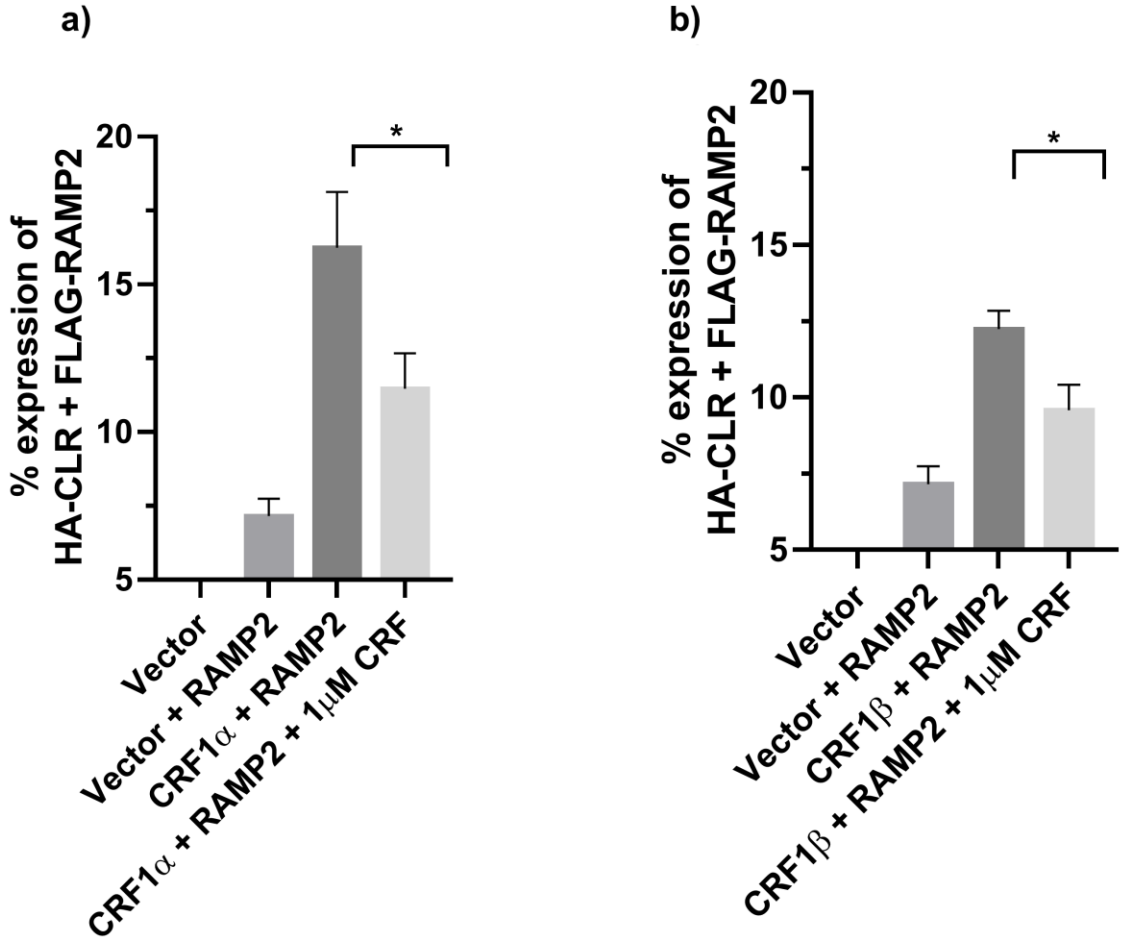
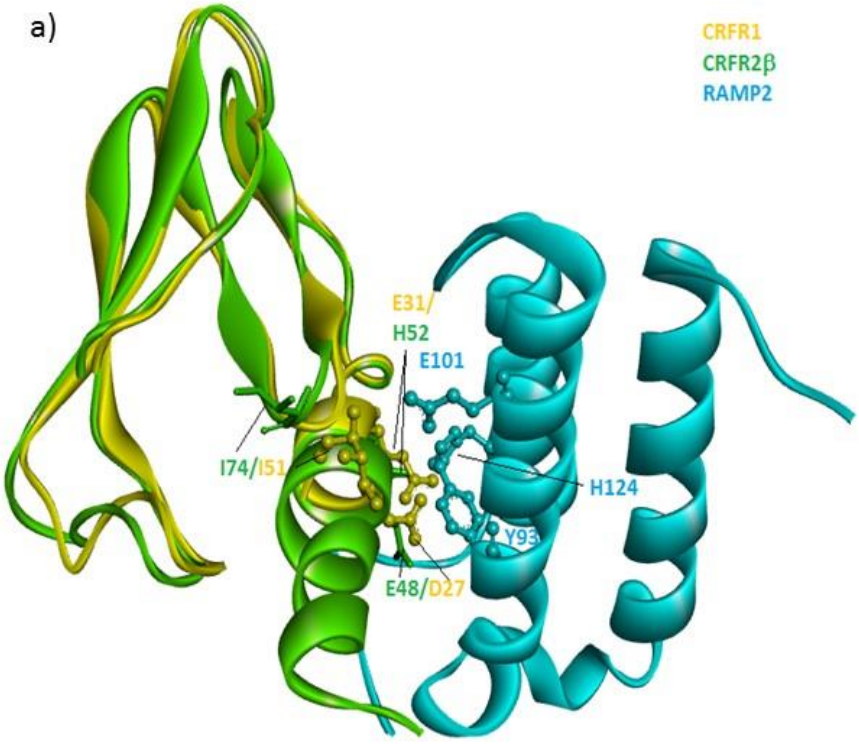


Fig 7. The effect of CRF stimulation of the a) CRFR1α and b) CRFR1β subtypes on cell-surface expression of FLAG-RAMP2 in HEK293S cells.
CRF treatment was for 1h. Expression was normalised (100%) to that seen when FLAG-RAMP2 was co-expressed with CLR. Data are the mean ± s.e.m. of three or four independent experiments. *p<0.01, one way ANOVA followed by Dunnett's test.

3.7. Molecular modelling of CRFR:RAMP2 complexes

Computational molecular modelling can provide insights into the molecular mechanisms underpinning empirical observations. From data presented above, it is clear that the CRFR1 variants exhibit interaction with RAMP2 whereas in marked contrast the CRFR2 does not. This subtype-specific difference was observed in all the cell lines utilised. In order to investigate why the CRFR1 splice variants might be better able to form a heterodimer with RAMP2

compared to CRFR2β, homology models of the ectodomains of CRFR1:RAMP2 and CRFR2β:RAMP2 were constructed based on the crystal structure of the CLR:RAMP2 ectodomain complex (3AQF; Kusano et al., 2011) and are directly compared in Fig. 8. Assuming a direct CRFR:RAMP2 interaction and that the complexes have a similar GPCR:RAMP interface to that seen in CLR:RAMP2, then residues Q26, D27, E31 and S35 of CRFR1 pack against RAMP2 (Fig. 8). Interestingly, this constitutes a smaller interface than seen with CLR where I41, M42, N45, Y46, Y49 and I52 all contribute to the association [6, 16]. With reference to Fig. 8, the two negatively-charged residues D27 and E31 of CRFR1 are positioned close to a histidine (H124) of RAMP2. However, in CRFR2β, the residue corresponding to E31 is replaced by a histidine (H52), which will undergo electrostatic repulsion away from H124 of RAMP2. Consequently, in CRFR2β, H52 moves away from the RAMP2 interface as does E48, the equivalent of D27 in CRFR1. There are also differences in loop 2 of the ECDs of the receptors, at the tip of the sheet formed by the β1 and β2 strands of these domains. In CRFR1 receptors, this is close to RAMP2, allowing I51 of CRFR1 to pack against the RAMP. In contrast, the equivalent loop region of CRFR2β is bent away from RAMP2 so that I74 (the equivalent of I51 in CRFR1) fails to make contact with the RAMP (Fig. 8).


a)
CRFR1
CRFR2β
RAMP2
E31/
H52
E101
I74/I51
H124
Y93
E48/D27

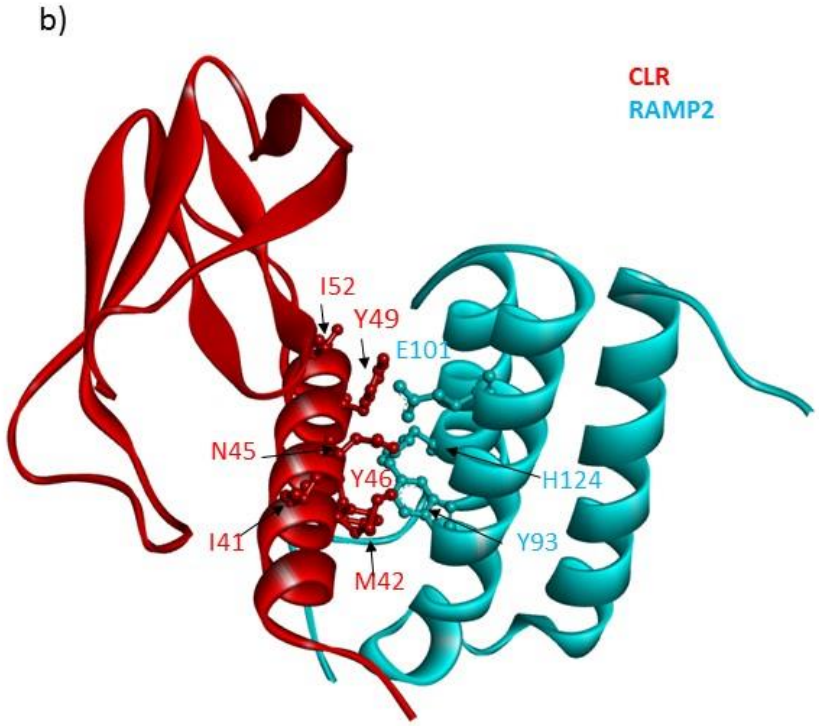

b)
CLR
RAMP2
I52
Y49
E101
N45
Y46
H124
I41
Y93
M42

Fig. 8. Molecular models of a) CRFR1:RAMP2 and CRFR2β:RAMP and b) CLR:RAMP2

Molecular models of CRFR1β:RAMP2 and CRFR2β:RAMP2 are shown superimposed, with CRFR1β (yellow), CRFR2β (green) and RAMP2 (blue). Individual key residues in the heterodimer interface, which are referred to in the text, are indicated for each CRFR subtype. CLR:RAMP2 is from PDB 3N7S [6]

4. Discussion

RAMPs have been shown to interact with a wide range of family B GPCRs and thereby affect fundamental characteristics of these receptors. These RAMP-mediated effects include alteration of cell-surface expression and changes to the receptor's ligand binding profile and intracellular signaling [4]. The best-characterized of these RAMP-mediated effects are with the receptors CLR and CTR [7]. The widespread occurrence of RAMP:GPCR interactions was evident following characterisation of a *RAMP2*$^{+/-}$ knockdown mouse strain, which revealed a broad spectrum of physiological changes, consistent with RAMP2 modulating an extensive and diverse range of receptors [17].

CRF stimulation of ACTH release from the pituitary gland is of pivotal importance to the physiological role of this hormone [3]. It is noteworthy therefore, that the *RAMP2*$^{+/-}$ mouse line exhibited a decrease (~20%) in CRF-stimulated release of ACTH into the blood compared to wild-type mice [8], thereby establishing the importance of RAMP2:CRFR interaction *in vivo.* Furthermore, the results with the *RAMP2*$^{+/-}$ mouse line probably under-represent the importance of RAMP2 to CRF function, as the mice are only heterozygote for RAMP2 and not a complete RAMP2 knockout strain (*RAMP2*$^{-/-}$ is lethal *in embryo*). The current study investigated the ability of RAMP2 to interact with human CRFR subtypes by measuring changes in FLAG-tagged RAMP2 cell-surface expression induced by co-expression of the individual CRF receptor subtypes CRFR1α, CRFR1β and CRFR2β. Both of the CRFR1 isoforms were shown to enhance delivery of RAMP2 to the cell surface. Our data for CRFR1β are in agreement with the findings of Wootten *et al.* [8] and furthermore, establish that RAMP2 interaction extends to the predominant CRFR1α isoform. Thus, RAMP modulation of either CRFR1 subtype should be considered in cells where these receptors can be shown to be co-

expressed with RAMP2. As the effects of RAMPs on GPCRs can be affected by the cell line, four different cell-lines were utilised to investigate the effect of varying cellular context *viz*. COS-7, HEK 293T, HEK 293S, plus [ΔCTR]HEK 293 (a HEK 293 cell-line lacking endogenous calcitonin receptor). In each case, the magnitude of the enhancement of RAMP2 cell-surface expression was similar for both CRFR1 isoforms, although the fold-increase varied between cell lines ([ΔCTR]HEK 293>COS-7>HEK 293T>HEK 293S). In addition, interaction of RAMP2 with either CRFR1α or CRFR1β resulted in an increase in the delivery of both the RAMP and the receptor to the cell surface. CRFR1α and CRFR1β share identical sequences, except for a 29-residue deletion in ICL1 of CRFR1α. It seems unlikely that ICL1 is in direct contact with the RAMP [5].

The largest enhancement of RAMP2 cell-surface expression was observed in [ΔCTR]HEK 293 cells which lack endogenous CTR (Fig. 5). This increase in RAMP2 delivery to the cell surface was notably higher in [ΔCTR]HEK 293 cells than that observed in HEK 293S cells (6.6-fold greater and 4.2-fold greater for CRFR1α and CRFR1β, respectively). Whilst the CTR can complex with RAMP2 (and so could act as a sink for RAMP2 in competition with the CRFR1 isoforms), this interaction has been reported to be weak [18]. Therefore it is not clear if the absence of CTR alone is sufficient to explain the increase in RAMP2 cell-surface abundance generated by CRFR1 isoforms in [ΔCTR]HEK 293 cells compared to HEK 293S cells. There may be other RAMP-interacting proteins differentially expressed by the cells, to dilute the RAMP pool. However, based on mRNA data presented in Fig. 6, there was a negative correlation between endogenous expression levels of RAMP2 in HEK-based cell lines and the extent to which FLAG-RAMP2 trafficking was affected by either CRFR1α or CRFR1β. This suggests that endogenous, untagged RAMPs may interact with the CRFR1, in effect competing with the transfected FLAG-RAMPs, thus diluting the cell-surface expression of FLAG-RAMPs. This reasoning indicates that receptor:RAMP stoichiometry is an important consideration but it is very rare for RAMP and receptor expression to be quantified in a single cell type [19]; we are unaware of any study where this has been done for every potential RAMP-interacting GPCR. Indications are that individual RAMPs and GPCRs are expressed at broadly similar levels [20], as seen in this study (Fig 6) but more work is needed to understand their interactions at a cellular level.

Throughout this work, the assumption has been that there is a physical association between RAMP2 and the CRF receptors. We cannot rule out an indirect effect of the RAMP on the receptor. However, robust physical interactions are seen with CLR and RAMPs [5, 6] and the simplest interpretation of the ability of prolonged CRF treatment to reduce cell surface RAMP2 expression (Fig 7) is that there is internalisation of a CRFR1-RAMP2 heterodimer. Furthermore, the effects of RAMP modulation on CRFR1β pharmacology [8] (also seen with the RAMP2 interaction with the glucagon receptor [21]), suggest that a physical interaction between the receptor and the RAMP is the most plausible mechanism. This may also provide a further explanation of cell line variability, if there are different degrees of compartmentalisation between RAMPs and GPCRs.

In marked contrast to the CRFR1 isoforms, there was no evidence that RAMP2 cell-surface expression was affected by CRFR2β in any of the cell lines employed, Thus if CRFR:RAMP heterodimerisation is the basis for this effect, it would seem that the CRFR2β does not form dimers with RAMP2. The CRFR2β shares ~70% amino acid sequence identity with CRFR1, with most of the divergence in sequence occurring in the ECD. Insights from computational molecular modelling suggested a feasible mechanism, arising from differences in the ECDs, to explain the subtype-specific interaction observed between CRFRs and RAMP2. These modelling studies indicated that there are charge differences between the residues of CRFR1 and CRFR2β that pack against a histidine (H124) of RAMP2, located in the GPCR:RAMP interface. Whilst CRFR1 isoforms form a favourable interaction via E31, electrostatic repulsion of the corresponding CRFR2β residue (H52) would result in failure of CRFR2β to complex with RAMP2. In addition, the smaller and more polar contact interface between CRFR1:RAMP2 compared to CLR:RAMP2 might explain why formation of CLR:RAMP2 complexes is more robust than CRFR1:RAMP2 complexes. We cannot exclude that differences in the transmembrane domains of the receptors may also be important.

In conclusion, the study demonstrates that both CRFR1α and CRFR1β can interact with RAMP2 but the magnitude of the effect was cell line-dependent. In contrast, the CRFR2β subtype did not complex with RAMP2 in any cell line examined. Molecular modelling studies suggested that this difference between CRFR1 and CRFR2 may be due to subtype-specific differences in individual residues in the ECDs which contributed to the CRFR contact interface in a RAMP2:CRFR complex.

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