A CRITICAL ROLE FOR THE SHORT INTRACELLULAR C-TERMINUS IN RECEPTOR ACTIVITY MODIFYING PROTEIN FUNCTION

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Non standard abbreviations: ∆, deletion mutant; AM, adrenomedullin; AMY, amylin receptor phenotype; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; h, human; HA, haemagglutinin epitope tag; hCT, human calcitonin; PDZ, Post synaptic density-95/Discs-large/ZO-1 homology; RAMP, receptor activity modifying protein; rAmy, rat amylin; RCP, receptor component protein; sCT, salmon calcitonin; TMD, transmembrane domain; WT, wild-type.
Abstract

Receptor activity modifying proteins (RAMPs) interact with and modify the behaviour of the calcitonin receptor (CTR) and calcitonin receptor like receptor (CLR). We have examined the contribution of the short intracellular C terminus, using constructs that delete the last 8 amino acids of each RAMP. C-terminal deletion of individual RAMPs had little effect on the signaling profile induced when complexed with CLR in COS-7 or HEK293 cells. Similarly, confocal microscopy revealed each of the mutant RAMPs translocated HA-tagged CLR to the cell surface. In contrast, a pronounced effect of RAMP C-terminal truncation was seen for RAMP/CTRa complexes, studied in COS-7 cells, with significant attenuation of amylin receptor phenotype induction that was stronger for RAMPs 1 and 2 than RAMP3. The loss of amylin binding upon C-terminal deletion could be partially recovered with overexpression of Gαs, suggesting an impact of the RAMP C-terminus on coupling of G proteins to the receptor complex. In HEK293 cells the c-Myc-RAMP1 C-terminal deletion mutant showed high receptor-independent cell surface expression, however, this construct showed low cell surface expression when expressed alone in COS-7 cells, indicating interaction of RAMPs with other cellular components via the C-terminus. This mutant also had reduced cell surface expression when co-expressed with CTR. Thus, this study reveals important functionality of the RAMP C-terminal domain and identifies key differences in the role of the RAMP C-terminus for CTR versus CLR-based receptors.
Introduction

The definition of G protein-coupled receptor (GPCR) phenotype has become increasingly complex with an array of receptor-protein interactions leading to altered pharmacology. The exemplar of this is the modulation of GPCRs by receptor activity modifying proteins (RAMPs) (Hay et al., 2006; Udawela et al., 2004; Poyner et al., 2002). RAMPs are a family of 3 type I transmembrane proteins that interact most commonly with Family B peptide GPCRs, most notably the calcitonin (CT) receptor (CTR) and calcitonin receptor-like receptor (CLR), to affect various aspects of their behaviour, which may include their cellular localization, signaling specificity, regulation and profile of ligand interaction (Hay et al., 2006). For the CTR and CLR, RAMP interaction determines receptor specificity with each individual RAMP forming a different receptor phenotype upon interaction with either GPCR. These GPCR/RAMP heterodimeric complexes are recognized as the molecular units comprising the distinct amylin (AMY1, AMY2 and AMY3), adrenomedullin (AM1 and AM2), and calcitonin gene-related peptide (CGRP1) phenotypes, while the CT receptor phenotype is defined by the independent expression of CTR (Poyner et al., 2002).

A number of studies have investigated the molecular and structural basis for RAMP function, most notably the N-terminal domain, and demonstrated that this domain is critical for interaction with CLR and also for the resultant phenotype of RAMP/CLR complexes (Fitzsimmons et al., 2003; Fraser et al., 1999; Kuwasako et al., 2001; Kuwasako et al., 2003). However, work with the CTR has revealed additional effects on phenotype that are cell background dependent where co-expression of RAMP2 and CTRa (the most common splice variant of the human receptor) in CHO-P but not COS-7 cells led to induction of an AMY receptor
phenotype (Tilakaratne et al., 2000). Phenotype differences were also seen between alternate splice variants of the CTR with a high level of Amy binding seen for RAMP2 complexes with the CTRb isoform, which has an additional 16 amino acids in intracellular loop 1 (Moore et al., 1995) in both CHO-P and COS-7 cells (Tilakaratne et al., 2000). These experiments indicated that RAMP/GPCR complexes functionally interacted with other cellular proteins and that the RAMP C-terminus may be an important domain for RAMP function.

RAMPs contain a short intracellular C-terminal tail of about 10 amino acids, although the role of this domain is largely unclear. Recent data from chimeras between RAMP1 and RAMP2 provided evidence for a significant role for the RAMP C-terminus in the signalling from RAMP/CTR heterodimers, with CGRP-induced accumulation of cAMP being strongly influenced by the C-terminal sequence in the chimeras (Udawela et al., 2006). This data suggested that the RAMP C-terminus could play a role in coupling of receptor complexes to G proteins. A general role for RAMPs in receptor-G protein interaction was also supported by other data from our laboratory where modulation of G_α subunit protein levels could “rescue” the poor induction of AMY_2 phenotype seen in COS-7 cells (Tilakaratne et. al., 2003; Christopoulos et al., 1999; Zumpe et al., 2000).

Deletion studies of the RAMP1 C-terminus have revealed that removal of most of the C-terminus (up to 9 amino acids) has relatively little impact on RAMP1 induction of the CGRP_1 receptor phenotype from CLR (Fitzsimmons et al., 2003; Steiner et al., 2002), with similar CGRP binding affinity and either no change in cAMP signalling in HEK-293 cells (Fitzsimmons et al., 2003) or a weak reduction in maximal agonist response and potency in COS-7 cells for the constructs truncated by 9 amino acids (Steiner et al., 2002). Consistent with this, translocation of CLR to the
cell surface was not altered (Fitzsimmons et al., 2003), however, deletion of 8, 9, 10 and 16, but not 4, amino acids resulted in high cell surface expression of the mutant in the absence of CLR in COS-7 cells (Steiner et al., 2002), suggesting that the C-terminus of RAMP1 contains a recognition sequence for intracellular retention in the absence of CLR.

More recently Bomberger et al (Bomberger et al., 2005a and 2005b) studied the role of the RAMP3 C-terminus in receptor trafficking. RAMP3 contains a PSD-95/Discs-large/ZO-1 homology (PDZ) motif (DTLL) at the C-terminus that is not present in RAMP1 or RAMP2 (McLatchie et al., 1998); in other GPCR systems interactions with PDZ domain proteins lead to altered receptor-targeting after agonist stimulation. RAMP3 interacts with N-ethylmaleimide-sensitive factor, via the PDZ domain, and promotes CLR/RAMP3 receptor recycling after AM stimulated internalization (Bomberger et al., 2005a). The RAMP3 PDZ motif could also interact with Na+/H+ Exchanger Regulatory Factor-1 to inhibit AM stimulated internalization of CLR/RAMP3, with Thr\textsuperscript{146} being crucial in this case (Bomberger et al., 2005b).

To date there are no data on the effect of loss of the RAMP C-terminus on AMY receptor function, and only limited information on the impact of RAMP2 or RAMP3 C-terminal deletion on AM receptor phenotypes (Kuwasako et al, 2006). To more broadly investigate the role of the RAMP intracellular C-terminus we created mutants of each of the RAMPs, deleting the last 8 amino acids (RAMP1\textdelta-C, RAMP2\textdelta-C, RAMP3\textdelta-C, respectively), and assessed the consequence of these deletions on functional interaction with both CLR and CTR. We show that RAMP truncation differentially affects interaction with CLR versus CTR, with RAMP1 or RAMP2 C-terminal deletion having a profound effect on interaction with CTR but little effect on CLR, while RAMP3 was the least detrimental to the modulation of
CTR phenotype. The loss of AMY phenotype was paralleled by a loss of CTR-dependent cell surface expression of the truncated RAMP (at least for RAMP1) and could be partially rescued by overexpression of G\textsubscript{as} protein. In contrast CLR-dependent cell surface expression of RAMPs was retained.

Materials and Methods

Human calcitonin (hCT), salmon calcitonin (sCT), human \(\alpha\)CGRP and rat amylin (rAmy) were purchased from Auspep (Parkville, VIC, Australia) and human adrenomedullin (AM) from Bachem (Bubendorf, Switzerland). Tissue culture reagents were from Invitrogen (Carlsbad, CA, USA). Oligonucleotide primers were synthesised by GeneWorks (Adelaide, SA, Australia). Rabbit anti-c-Myc antibody was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa 488- and Texas Red- conjugated goat anti-mouse and anti rabbit sera were from Molecular Probes (Eugene, OR, USA). \(^{125}\text{I}\)-labeled goat anti-mouse IgG was obtained from PerkinElmer (Boston, MA, USA). N-succinimidyl-3-(4-hydroxy-\(^{125}\text{I}\)-iodophenyl) propionate (Bolton-Hunter reagent; 2000Ci/mmol) was supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK). \(^{125}\text{I}\)-rAmy (specific activity 2000 Ci/mmole) was iodinated by the Bolton-Hunter method and purified by reverse phase high performance liquid chromatography (HPLC) as previously described (Bhogal et al., 1992).

cDNA constructs

Expression clones of hCLR, HA-CLR, wild-type hRAMPs, and chimeric RAMP1/2 and RAMP2/1 (all in pcDNA3) were provided by Dr S. M. Foord (Fraser et al., 1999). C-Myc-RAMP1 was provided by Dr K. Kuwasako (Kuwasako et al., 2000). Double HA epitope tagged human CTRa (HA-CTRa) was prepared as described previously.
This receptor is the Leu447 polymorphic variant of the receptor (Kuestner et al., 1994). EE tagged \( \text{G}_{\alpha_s} \) cDNA was purchased from the UMR cDNA resource center (www.cDNA.org).

A stop codon was introduced by site directed mutagenesis to delete the last eight amino acids of WT-RAMP1 (forward primer 5’-ctgtgcttggtctggcagtaaagcgcactgagggc-3’, reverse primer 5’-gcctcactgctttcactgcagcaccagc-3’), -RAMP2 (forward primer 5’-cctgtagttaggagtcgaaagacagtgaggcc-3’, reverse primer 5’-gccctcactgtctttcactccatacatacagc-3’) and -RAMP3 (forward primer 5’-ctgtgcttggtgcggcgactaagcgcaccagc-3’, reverse primer 5’-cgctgcttggtgcgcttcaccgccccacaccagc-3’) and c-Myc-RAMP1 (forward primer 5’-gtgtgcttggtgcgcttcaccgccccacaccagc-3’, reverse primer 5’-ctcagtgcgctttcactgcagcaccagc-3’), using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The resultant constructs are displayed schematically in Figure 1.

**Cell culture and transfections**

COS-7 and HEK293 cells were routinely maintained in 175 cm\(^2\) flasks at 37\(^\circ\) C in a humidified atmosphere with 5% CO\(_2\): 95% air, in complete DMEM supplemented with 5% heat inactivated FBS, 100 units/ml penicillin-G and 100 \(\mu\)g/ml streptomycin and 50 \(\mu\)g/ml fungizone. Transfections were carried out in serum and antibiotic free DMEM using lipofectamine (Invitrogen) or Metafectene (Scientifix; Cheltenham, VIC, Australia), when cells were ~70% confluent. Twenty four well plates or 4 well chamber slides were transfected with 100 ng receptor and 150 ng RAMP with 1\(\mu\)l lipid, 75 cm\(^2\) flasks with 4 \(\mu\)g receptor and 6 \(\mu\)g RAMP with 20 \(\mu\)l lipid and 25 cm\(^2\) flasks with 1 \(\mu\)g receptor and 1.5 \(\mu\)g RAMP with 8 \(\mu\)l lipid.
Receptor binding

Specific binding was determined as previously described (Christopoulos et al., 1999) 48 hours post transfection in 24-well plates. For competition binding COS-7 cells were transfected in 75 cm² flasks and grown for 48 hours, then harvested and resuspended in binding buffer (DMEM containing 1% BSA). Cells were added to 96-well plates (100,000 cells/well) with ~70 pM $^{125}$I-rAmy and competing unlabelled peptides. After incubating for 1 hour at $37^\circ$C, cells were harvested onto GF/C plates (coated with 0.5% polyvinylpyrolidone and 0.1% Tween20) using a Tomtec harvester. Plates were dried overnight and, after the addition of Micorscint 0⁻ (PerkinElmer), counted on a TopCount counter (PerkinElmer). Experiments were performed with triplicate repeats.

$^{125}$I-rat Amylin binding in the presence or absence of Gpp(NH)p

COS-7 cells were seeded to 90% confluency in 48 well plates. These were transfected with 50ng CTRa and 75ng RAMP1 per well, using 0.75 µL Metafectine. 48 hours post transfection, the cells were assayed for $^{125}$I-rAmy binding in competition with rat amylin and human αCGRP in the presence or absence of Gpp(NH)p (SIGMA) at a final concentration of $10^{-4}$M. Cells were permeabilised by pre-treating with PBST (Phosphate buffered saline, 0.3%Tween20) for 5 mins then washed once with PBS immediately prior to binding. Binding of $^{125}$I-rAmy (~100 pM) was performed at $37^\circ$C for 45 min. Cells were washed once with ice-cold PBS and solubilised with 0.5M NaOH. Cell lysates were counted on a Wizard γ-counter (PerkinElmer).

Cyclic AMP assays

Intracellular cAMP levels were determined using the AlphaScreen cAMP kit (PerkinElmer Life Sciences). Cells transfected in 25 cm² flasks were grown for 48
hours, and then serum starved overnight. Cells were subsequently harvested and assayed as previously described (Hay et al., 2005), at cell concentrations of 5,000 cells/well for COS-7 cells and 10,000 cells/well for HEK293 cells. Each assay point was done in triplicate.

**Measurement of cell surface expression by antibody binding**

Cell surface expression of HA-tagged CTR or c-Myc-tagged RAMP constructs were determined as previously described (Hay et al., 2005) 48 hours after transfection of COS-7 cells in 24-well plates, using anti-HA (12CA5) or anti-c-Myc (9E10) antibody.

**Confocal microscopic localisation of receptors and RAMPs**

Twenty-four hours post transfection, cells grown in 4 well chamber slides were fixed with 3.4% paraformaldehyde in PBS for 20 min at room temperature, then washed with PBS. Cells were permeabilised with 0.3% Tween20 in PBS for 5-10 mins then washed with PBS and then incubated for 30-60 min with 10% normal goat serum (NGS) in PBS at room temperature. Cells were incubated with rabbit or mouse anti-c-Myc (9E10) antibody for detection of tagged RAMP or mouse anti-HA (12CA5) antibody for detection of receptor, diluted 1/100 in PBS with 3% NGS, for 1 h at room temp. Cells were washed 3 times with PBS, then incubated with Alexa 488- or Texas Red- conjugated goat anti-mouse or anti rabbit antibody, diluted 1/200 in PBS, in the dark at room temp for 1 h. Cells were washed with PBS 3 times and coverslips were mounted with DAKO-fluorescent mounting media. Fluorescence was visualised on a Zeiss Acioplan-2 microscope (Zeiss, Jena, Germany) with a Bio-Rad MRC-1024 confocal microscopy system and Lasersharp 2000 software (Bio-Rad; Hercules, CA, USA).

**Data analysis**
A minimum of four independent repeats were performed for each of the above experiments and the results presented as mean ± standard error of means. Curve fitting was done using Prism 4 (Graphpad Software Inc, San Diego, CA, USA). $pIC_{50}$ and $pEC_{50}$ values were compared by two way $t$ tests or one-way ANOVA as appropriate, where $P<0.05$ was considered significant. Post-hoc testing was performed with Dunnet’s test (for comparison with vector) and Bonferroni’s test for comparison of wt and mutant RAMPs.
RESULTS

The effect of RAMP C-terminal deletion on induction of CGRP and AM receptors

The phenotype of CLR-based receptors was assessed in COS-7 and HEK293 cells. These cells do not respond significantly to CGRP and AM peptides when CLR is expressed alone (not shown). Unlike the CTR, functional CLRs are not expressed at the cell surface in the absence of RAMPs. As such, functional responses reflect CLR/RAMP interaction only and interpretation of experiments is not complicated by background phenotype of the free GPCR component (as is seen for CTR; Christopoulos et al., 1999; Hay et al., 2005; Muff et al., 1999).

To determine the role of the C-terminus of RAMPs in the induction of functional complexes from CLR, wild-type or deletion mutants of RAMPs 1, 2 and 3 were co-expressed with CLR in either COS-7 or HEK293 cells and cAMP production in response to hCGRP and hAM measured. At RAMP1 and c-Myc-RAMP1 induced phenotypes, hCGRP had high potency and hAM had a lower potency (Fig 2 A, B; Table 1), typical of classic CGRP₁ receptor pharmacology (Poyner et al., 2002). The potency and efficacy of hCGRP for cAMP production were unaffected by C-terminal deletion from either RAMP1 or c-Myc-RAMP1 (Fig 2 E, F; Table 1), although a reduction in AM potency was observed in COS-7 cells (Fig 2 E; Table 1). These data indicate that deletion of the RAMP1 C-terminus had little effect on the functional CGRP₁ receptor.

Co-expression of CLR with RAMP2 gave a receptor phenotype with higher potency for adrenomedullin than hCGRP, typical of classic AM₁ pharmacology
(Poyner et al., 2002; Fig 2 C; Table 1). Deletion of the RAMP2 C-terminus had minimal impact on the induced receptor phenotype, with no differences in AM or CGRP potency observed in COS-7 cells (Fig 2 G; Table 1) and only a small increase in AM potency observed in HEK-293 cells (Table 1). There was also a trend for the E_{max} to be higher with RAMP2 deletion in COS-7 cells and lower in the HEK-293 cells but this did not achieve statistical significance. When CLR was expressed with RAMP3 the resulting receptor phenotype had higher potency for AM than CGRP (Fig 2 D, Table 1). Deletion of the C-terminus of RAMP3 again had minimal effect on receptor phenotype, with no change in potency or efficacy of peptides seen in COS-7 cells (Fig 2 H; Table 1) and only a small decrease in CGRP potency observed in the HEK-293 cells (Table 1).

The effect of RAMP C-terminal deletion on the induction of AMY receptor phenotype with CTRs

Initial experiments on untransfected HEK293 cells revealed occasional low level expression of an endogenous CTR that was not readily attributable to cell passage number or confluence. As a consequence, experiments with CTRs were performed only in COS-7 cells where no background phenotype was found.

Induction of $^{125}$I-rAmy binding by wild-type and mutant RAMPs co-transfected with CTRs

Consistent with previous findings (Christopoulos et al., 1999; Zumpe et al., 2000; Muff et al., 1999), when expressed with CTRa in COS-7 cells RAMP1 and RAMP3 induced high levels of rat amylin binding, while RAMP2 induced a relatively low level of binding (Fig 3). c-Myc-RAMP1 also induced a high level of $^{125}$I-rAmy
binding. The deletion of the C-terminus resulted in a marked attenuation of $^{125}\text{I}\text{-rAmy}$ binding for all three RAMPs, although to a lesser extent with RAMP3 than RAMP1 and RAMP2. Deletion of the C-terminus of c-Myc-RAMP1 led to a similar loss of $^{125}\text{I}\text{-rAmy}$ binding to that seen with the untagged RAMP1 (Fig 3).

We have shown that host cell-environment contributes to the induction of AMY phenotype for CTR/RAMP and perhaps also CLR/RAMP complexes (Tilakaratne et al., 2000; Hay et al., 2005). Preliminary experiments performed in our laboratory have shown that overexpression of G$_{\alpha}s$ increases the low level of $^{125}\text{I}\text{-rAmy}$ binding to COS-7 cells co-transfected with CTRa and RAMP2 (Tilakaratne et al., 2003), suggesting that G$_{\alpha}s$ may be interacting with the RAMP, presumably via the C-terminus, to alter receptor behaviour. To investigate whether the loss in binding seen with the C-terminal deletion mutants was due to impaired coupling of RAMP/receptor complexes to G proteins, binding studies were performed in the presence of excess G$_{\alpha}s$ protein (Fig 4). Binding levels were normalised to HA-CTRa cell surface expression to minimise effects of variations in transfection efficiency. Co-transfection of G$_{\alpha}s$ with the deletion mutants of untagged and tagged RAMP1 led to only a partial recovery of binding, relative to levels seen with full length RAMP1 or c-Myc-RAMP1, either with or without G$_{\alpha}s$. Incubation of CTR/RAMP1 receptors with the GTP analogue GppNHp led to a marked reduction in the level of $^{125}\text{I}\text{-Amy}$ binding (Fig 5 A) with no change in the affinity of either rAmy (Fig 5 B) or hCGRP (Fig 5 C), consistent with a role for G protein-coupling on the level of functional AMY1 receptors. Co-expression of RAMP2$\Delta$-C with G$_{\alpha}s$ led to a pronounced increase in induced $^{125}\text{I}\text{-rAmy}$ binding to levels similar to those seen with the wild-type RAMP2, either with or without G$_{\alpha}s$. A similar effect was observed following, co-expression of RAMP3$\Delta$-C with G$_{\alpha}s$ (Fig 4).
Binding phenotype of AMY₃ receptors following deletion of RAMP3 C-terminus

Only very low ¹²⁵I-Amy binding was observed for cells co-transfected with CTRa and either RAMP2, or the RAMP1Δ-C or RAMP2Δ-C mutants, and rAmy and hCGRP competed poorly when binding was measurable (data not shown), consistent with low affinity binding of Amy to the CT receptor phenotype. To examine the nature of the RAMP3Δ-C induced phenotype, competition binding assays were performed in COS-7 cells expressing CTRa and either full length or C-terminally deleted RAMP3. Deletion of the C-terminus resulted in an apparent increase in affinity for human calcitonin but no change in affinity for other peptides tested. (Fig 6; Table 2)

The effect of C-terminal RAMP deletion on downstream signalling with CTRs

Unlike CLR, CTR expressed alone is efficiently transported to the cell surface and has a receptor phenotype distinct from that of CTR/RAMP heterodimers. This CT receptor phenotype is characterised by high affinity for mammalian CTs but only weak affinity for related peptides such as Amy and CGRP. Consistent with this, when CTRa was expressed in COS-7 cells in the absence of RAMPs, the phenotype showed highest potency for sCT, followed by hCT, and lower potency for hCGRP and rAmy (Fig 7 A; Table 3). When the CTRa was co-expressed with RAMP1, both hCGRP and rAmy displayed increased potency (Fig 7 B; Table 3). Upon deletion of its C-terminus RAMP1 failed to elicit changes in hCGRP and rAmy potency, rendering the phenotype similar to that of CTR alone (Fig 7 F; Table 3). Co-expression of CTRa with c-Myc-RAMP1 led to increased potency of rAmy and hCGRP and a decrease in hCT potency (Fig 7 C; Table 3). Like the wild-type RAMP1, deletion of the c-Myc-RAMP1 C-terminus reduced the extent of phenotype change seen with rAmy and hCGRP, although a small decrease in potency of hCT following c-Myc-RAMP1 co-transfection was also observed (Fig 7 G; Table 3).
In COS-7 cells, co-transfection of RAMP2 with CTRa only weakly induces an AMY phenotype (Christopoulos et al., 1999), although this can be delineated under appropriate experimental conditions (Zumpe et al., 2000). However, as a consequence of the “weak” response, the functional phenotype has not been widely investigated. In this study, co-expression of CTRa and RAMP2 did not lead to an overt change in the response to peptides (Fig 7 D; Table 3). Deletion of the RAMP2 C-terminus led to a significant decrease in hCT potency (Fig 7 H; Table 3).

Co-transfection of RAMP3 with the CTRa led to an increased potency of rAmy and hCGRP and a decreased potency of hCT (Fig 7 E; Table 3). Similar to the effect on RAMP1, deletion of the RAMP3 C-terminus led to a decreased potency of hCGRP and rAmy, however the hCT potency was increased, compared to the wild-type RAMP3 (Fig 7 I, Table 3). Whereas C-terminal deletion abolished the ability of RAMP1 to modify the rAmy response, RAMP3 C-terminal deletion led to an attenuation rather than abolition of phenotype induction with rAmy potency intermediate between CTRa alone and CTRa co-expressed with RAMP3 (Table 3).
The effect of RAMP C-terminal deletion on cell surface expression of proteins

Confocal microscopy studies were performed to examine the cellular distribution of the truncated c-Myc-RAMP1 mutant as well as the capacity of truncated RAMPs to translocate CLR to the cell surface. First the cell surface expression of full length and C-terminally truncated c-Myc-RAMP1 was investigated in HEK293 cells. In the absence of receptor, the deletion mutant showed high cell surface expression compared to the full length tagged RAMP1 (Fig 8 A). When co-transfected with HA-CLR both c-Myc-RAMP1 and the deletion mutant translocated to the cell surface (Fig 9 B).

To investigate whether truncation of the RAMPs modified their ability to translocate CLR to the cell surface, the cellular distribution of HA-CLR was monitored using anti-HA antibody detected via fluorescently labelled secondary antibodies. HA-CLR showed relatively low cell surface expression in the absence of RAMPs (Fig 9 C, bottom left panels). This was increased upon co-transfection of either c-Myc-RAMP1 or c-Myc-RAMP1Δ-C (Fig 9 C, bottom right hand panels). When visualised by double staining, both c-Myc-RAMP1 and c-Myc-RAMP1Δ-C demonstrated co-localisation with HA-CLR (data not shown). These results indicated that truncated c-Myc-RAMP1 was able to act as a chaperone for HA-CLR with similar efficiency to the full-length c-Myc-RAMP1, enabling translocation to the cell surface.

The HA-CLR was also co-expressed with the untagged deletion mutants. Both RAMP1Δ-C and RAMP3Δ-C led to marked increases in cell surface expression of the HA-CLR. The RAMP2Δ-C also caused a small increase in relative cell surface expression, but the total expression of HA-CLR tended to be lower than when transfected with the other RAMPs (data not shown).
The cell surface expression of c-Myc-RAMP1 and its truncation mutant was also examined in COS-7 cells. In the absence of receptor there was low cell surface expression of c-Myc-RAMP1 and also of the deletion mutant (Fig 8 B). This is in stark contrast to what was seen in the HEK293 cells, indicating that other components of the cellular background are playing a role in RAMP functionality, at least in part through interaction with the C-terminus. In the presence of HA-CTRa, c-Myc-RAMP1 showed high cell surface expression but cell surface expression of c-Myc-RAMP1Δ-C was low (Fig 9 A). In contrast, in the presence of CLR both full length and truncated c-Myc-RAMP1 translocated to the cell surface in these cells (Fig 9 B). This indicated that HA-CTRa did not facilitate the translocation of truncated c-Myc-RAMP1 as efficiently as CLR in COS-7 cells.

Both c-Myc-RAMP1 and its truncated mutant were able to translocate HA-CLR to the cell surface in COS-7 cells (Fig 9 C, upper panels). In these cells c-Myc-RAMP1Δ-C co-localized with HA-CLR at the cell surface (data not shown). HA-CTRa also exhibited co-localization with c-Myc-RAMP1Δ-C, however, this occurred with lower efficiency than seen with the full-length construct (data not shown) and was not correlated with a functional phenotype.

To determine the effect of RAMP C-terminal deletion on cell surface localization of CTR, ¹²⁵I-antibody binding to anti HA antibody was also measured in COS-7 cells expressing HA-CTRa and full length or truncated RAMPs. In the presence of c-Myc-RAMP1 there was reduced expression of HA-CTRa, but this was not further impaired by truncation of the C-terminus. The other constructs did not significantly modify the cell surface expression of CTRa when compared to the CTRa with vector control (data not shown). This suggests that deletion of the C-terminus of
RAMPs does not have a great impact on the intrinsic translocation of HA-CTRα to the cell surface.
DISCUSSION

This study explored the role of the short C-terminal domain of RAMPs through the analysis of deletion mutants generated by removing the last 8 amino acids of each protein. These mutants were initially examined for their effect on generation of a functional phenotype from the CLR. Both the deleted and full-length RAMP1 trafficked CLR to the cell surface with similar efficiency, where they remained colocalised. A similar observation was previously seen for RAMP1 truncated by 9 amino acids (Fitzsimmons et al., 2003). For RAMP1 and c-Myc-RAMP1, deletion of the C-terminus had minimal effect on the phenotype of the CGRP₁ receptor, as monitored by cAMP accumulation assay. These data were consistent with the previously published work of Steiner and colleagues (Steiner et al., 2002) and demonstrated that maintenance of function was essentially preserved across multiple cell types. However, further loss of amino acids may be significant, in a cell specific manner, with reports of a decrease in $E_{\text{max}}$ and potency of the receptor with deletion of 9 amino acids in COS-7 cells (Steiner et al., 2002), but no change in HEK293T cells (Fitzsimmons et al., 2003). Receptors in HEK293T cells are generally more efficiently coupled to $G_{\alpha}\$ signalling compared to those in COS-7 cells (Kuwasaki et al., 2004). The two cell lines also differ in their profile of regulatory protein expression (Purdue et al., 2004). Thus the differences between the two studies may reflect variance in the level or type of G proteins, or of regulatory proteins, expressed across the cell lines. Intriguingly, a reduction in AM potency was observed with C-terminal truncation in our COS-7 cells. A similar, peptide-dependent, effect was also seen in a recent publication of Kuwasako and colleagues (2006), where Tyr₀-CGRP but not CGRP exhibited reduced potency following deletion of 9 amino acids of the RAMP1 C-terminus.
Similar to RAMP1-based CGRP receptors, we observed only minor effects on receptor phenotype of AM receptors following truncation of RAMP2 or RAMP3 in each of the cell lines. The RAMP3 data is consistent with recent work with RAMP3 truncated at the C-terminus by 9 amino acids (Kuwasako et al., 2006). However, our data is in marked contrast to that seen for truncated RAMP2, where Kuwasako and colleagues observed a significant loss of AM binding and decreased $E_{\text{max}}$ following co-transfection with CLR into their HEK-293 cells. In those experiments, both CLR and RAMP2$\Delta$-C mutants (of 8 or 9 amino acids) were primarily retained in the ER. In our COS-7 cells expression of the CLR/ RAMP2$\Delta$-C complex was at least as efficient as that seen with the wild-type RAMP2. However, we did see a trend towards a reduction in $E_{\text{max}}$ in our HEK-293 cells, which may be related to the observations reported in the Kuwasako paper. The variation in data between the two studies likely relates to differences in cellular background of the HEK-293 cells of the Japanese laboratory and those of our HEK-293 and COS-7 cells, but may also be related, in part, to effects of either the GFP-fused to CLR or the epitope tagging of the RAMP2 as only tagged RAMPs were studied (Kuwasako et al., 2006). We have previously reported variations in the impact of N-terminal epitope tags for RAMP2 and RAMP3 (Christopoulos et al., 2003). Kuwasako and colleagues also report a marked loss of binding affinity for AM at the AM$_2$ receptor, but no change in AM potency; the latter is consistent with the current observations. However, inspection of the competition binding data presented suggests that the primary effect is on the level of non-specific binding rather than AM affinity.
In support of cellular background as the primary basis for the distinct phenotypes, significant differences in the impact of C-terminal truncation of the c-myc-RAMP1 were seen across the two cell lines used in the current study; strong receptor independent cell surface expression being seen in the HEK-293 cells but not in the COS-7 cells. Thus additional RAMP-protein interactions are likely to occur to modulate the cell surface delivery of both RAMP and complexes of RAMP-receptor, and these are differentially expressed across cell types. Indeed, analysis of the trafficking of AM receptors following C-terminal truncation indicates that this can be altered, and that the conserved Ser-Lys sequence may be important for the observed differences (Kuwasko et al., 2006). Collectively these data suggest that the RAMP C-terminus does not play a major role in the formation of functional CGRP or AM receptors, although this does not rule out an important role for the C-terminus in receptor regulation, as has been implicated by the work of Bomberger and colleagues (Bomberger et al., 2005a and 2005b).

In stark contrast to the minimal impact of RAMP C-terminal truncation on CLR-based receptor function, deletion of the C-terminal 8 amino acids of RAMP1, c-Myc-RAMP1 or RAMP2 resulted in almost complete abolition of their capacity to induce an AMY receptor phenotype from CTRa, in the equivalent cellular background. Furthermore, although less dramatic than the effects seen with RAMP1 or 2, RAMP3 C-terminal deletion also resulted in a marked attenuation of binding and signalling phenotypes. The lack of functional high affinity AMY receptor phenotype, however, was not due to destabilization of the CTR nor its capacity to be expressed at the cell surface, as direct assay of the receptor via the N-terminal HA-epitope revealed little impact of RAMP truncation on the level of cell surface expressed receptor. In this light, the strong reduction in hCT potency seen in cells co-transfected with CTRa
and the RAMP2Δ-C mutant, or other RAMP mutants, is likely to reflect a decrease in the level of free CTRa at the cell surface. The data also imply that the RAMP2Δ-C/CTRa complex is still translocated to the cell surface but that the receptor is still only poorly able to interact with endogenous G proteins, leading to low affinity of the complex (and hence low $^{125}$I-Amy binding). Furthermore, it suggests that the RAMP2Δ-C forms a functional interaction with CTRa more efficiently than does the full length RAMP2 in this cell background, or potentially that the RAMP2Δ-C is more stable than RAMP2.

Preliminary work in our laboratory has provided evidence that the level and type of G protein can modify the formation of functional RAMP2/CTRa complexes in COS-7 cells. In particular, $G_\alpha_s$ over-expression caused a marked increase in the level of induced $^{125}$I-Amy binding with RAMP2 (Tilakaratne et al., 2003). In the current experiments, there was a relatively high level of induced $^{125}$I-Amy binding with RAMP2 in the absence of excess G protein, and this likely reflects cell culture related differences in the background expression of cellular proteins between experiments. The effect of G protein on $^{125}$I-Amy binding led to speculation that loss of high affinity binding upon RAMP truncation may be due, at least in part, to a decrease in the efficiency of G protein coupling to the RAMP/receptor complex. Consistent with this hypothesis, increasing the level of $G_\alpha_s$ protein led to a recovery of RAMP-induced binding for all 3 deletion mutants, being almost equivalent to wild-type levels for RAMP2 and RAMP3. The importance of G protein interaction for formation of high affinity functional complexes is further supported by the effects of guanine nucleotides on $^{125}$I-Amy binding, where uncoupling of the G protein leads to loss of binding. Thus, these data indicated that the RAMP C-terminus was playing a direct role in the efficiency of G protein coupling. This contrasts strongly with the results for
CLR and suggests that there are significant differences in how CLR and CTR based receptors signal. A potential basis for this difference is the role of receptor component protein (RCP) in CLR-based receptor function. RCP plays a key role in the efficiency of CGRP and AM receptor signalling, presumably via contributing to receptor-G protein interaction as knock down of RCP expression leads to marked attenuation of cAMP signalling (Evans et al., 2000; Prado et al., 2001; Prado et al., 2002). More recent data indicates that RCP stabilizes the interaction between RAMP and CLR, with knock down of RCP preventing co-immunoprecipitation of RAMP and CLR (Dickerson and Louiseau, 2004). There is no evidence to date that RCP is required for RAMP/CTR function. This provides one potential rationale for the differences in the outcome of RAMP truncation for the 2 receptors. For CLR-based receptors, the RAMP C-terminus does not play a strong role in the efficiency of G protein coupling due to the additional complexing of the RAMP/receptor dimer with RCP and, therefore, there is a relatively low impact of deletion on receptor phenotype. In contrast, for CTR/RAMP dimers, the RAMP C-terminus appears to be directly involved in G protein coupling and removal of this domain has a profound effect on phenotype. However, as discussed above, there may be additional cell-background dependent factors that influence the behaviour of the distinct RAMP/receptor complexes.

The effect of RAMP3 C-terminal deletion on AMY phenotype was less marked than that seen for the other RAMPs. The full length RAMP3 sequence contains a PDZ binding domain that is not present in the other 2 RAMPs (see Fig 1). It is possible that RAMP3 may physiologically interact with other proteins via this domain and, as a consequence, it may play a lesser role in G protein coupling, and therefore loss of the C-terminus has less impact on the receptor phenotype.
Analysis of the cellular localization of c-Myc tagged RAMP1 and RAMP1Δ-C revealed that CLR efficiently translocated both proteins to the cell surface trafficking by CTRa was attenuated by C-terminal truncation. This suggests, at least for RAMP1, that the absence of the C-terminus decreases the stability of the complex with CTRa, leading to reduced cell surface translocation. Furthermore, as the level of 125I-Amy binding in RAMP1Δ-C/CTRa co-transfectants was increased with over-expression of Gαs, the data suggests that G protein interaction may contribute to stabilization of RAMP/CTR complexes. Thus, the prerequisite interactions for stability of functional RAMP-receptor complexes that translocate to the cell surface are clearly different for CLR and CTR. For CLR, loss of the C-terminus does not prevent functional interaction; indeed the expression of the N-terminal domain of RAMP1 alone can be sufficient for interaction with CLR and their co-translocation together through ER-Golgi-plasma membrane, albeit that the overall stability of the complex is impaired as soluble N-terminal domain could be recovered from the supernatant of cells transfected with this construct and CLR (Fitzsimmons et al., 2003). This latter finding is consistent with a potential role for RCP in stabilising CLR/RAMP complexes.

In conclusion this study provides insight into the role of the RAMP C-terminus in modulation of receptor function. The data suggest that this function varies for different GPCR partners and that for the CTR the C-terminus may provide a direct interaction with G proteins to stabilize the RAMP-receptor heterodimer. This may have implications for signaling pathways activated by different RAMP-interacting receptors.
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REFERENCES


**FOOTNOTES**

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FIGURE LEGENDS

Figure 1: Schematic representation of RAMP C-terminal deletion mutants, showing the extracellular N-terminal domain, including the signal peptide and its predicted cleavage site, the transmembrane domain (TMD), and the position of the introduced stop codon (asterisk) preventing translation of the last 8 amino acids of the C-terminal domain. Tagged RAMP1 construct contains an artificial signal sequence (from Influenza HA) at the N-terminus, and the c-Myc-tag immediately downstream of a cleavage site.

Figure 2: Effect of RAMP C-terminal deletion on induction of cAMP accumulation at CLR/RAMP receptors. COS-7 cells were co-transfected with 1 µg CLR and 1.5 µg (A) RAMP1, (E) RAMP1Δ-C, (B) c-Myc-RAMP1, (F) c-Myc RAMP1Δ-C, (C) RAMP2 (G) RAMP2Δ-C, (D) RAMP3 or (H) RAMP3Δ-C and stimulated with hAM (O) and hCGRPα (●). Data are mean ± S.E.M of 4 - 9 separate experiments, normalised to maximal peptide response. pEC₅₀ values are given in Table 1. E₅₀ values for RAMPΔ-C co-transfected cells tended to be higher than observed for full-length RAMP co-transfected cells (RAMP1Δ-C, 134 ± 25%; RAMP2Δ-C, 152 ± 24%; RAMP3Δ-C, 123 ± 14%) although none of these achieved statistical significance.

Figure 3: Effect of RAMP C-terminal deletion on specific ¹²⁵I-rAmy binding to CTRa/RAMP receptors. COS-7 cells were transfected with 100 ng CTRa and 150 ng RAMP WT or C-terminal deletion mutants. Data are mean ± S.E.M. of six separate experiments, expressed as a percentage of RAMP1 induced binding. (*, P<0.05; paired t test).
**Figure 4:** Effect of Gαs co-transfection on specific 125I-rAmy binding to CTRα/truncated RAMPs. COS-7 cells were transfected with 100 ng HA-CTRa and 150 ng RAMP WT or C-terminal deletion mutants with (■) or without (■) 150 ng Gαs protein. Data are mean ± S.E.M. of seven separate experiments expressed as percentage of the level or binding induced by RAMP1, normalised to HA-CTRa cell surface expression measured in parallel. (*, P<0.05; paired t test).

**Figure 5:** Effect of GppNHp on binding of 125I-Amy to lysed COS-7 cells co-expressing CTRα and RAMP1. Cells in 48-well plates were co-transfected with 50 ng HA-CTRa and 75 ng RAMP1. Prior to assay cells were lysed and incubated with buffer or 10⁻⁴M GppNHp. (A) Specific 125I-Amy binding, (B) Competition of 125I-Amy binding by rAmy, (C) Competition of 125I-Amy binding by hCGRP.

**Figure 6:** Effect of C-terminal deletion on peptide competition for 125I-rAmy binding to CTRα/RAMP3. COS-7 cells were co-transfected with 4 µg CTRα and 6 µg (A) RAMP3 or (B) RAMP3∆-C. hCGRPα (⚫), rAmy (▲), hCT (▼) and sCT (■) were used to compete for 125I-rAmy binding. Data are mean ± S.E.M. of four or more separate experiments. B, 125I-rAmy bound; B₀, total binding in the absence of competing peptide; N, nonspecific binding (measured in the presence of 10⁻⁶M peptide). pIC₅₀ values are given in Table 2.

**Figure 7:** Effect of RAMP C-terminal deletion on induction of cAMP accumulation at CTR/RAMP receptors. COS-7 cells were co-transfected with hCTRa and (A) empty vector, (B) RAMP1, (F) RAMP1∆-C, (C) c-Myc-RAMP1, (G) c-Myc-
RAMP1Δ-C, (D) RAMP2, (H) RAMP2Δ-C, (E) RAMP3 or (I) RAMP3Δ-C and stimulated with hCGRPα (●), rAmy (▲), hCT (▼) and sCT (■). Data are mean ± S.E.M. of four or more separate experiments, normalised to the maximal sCT response. pEC_{50} values are given in Table 3.

**Figure 8:** Expression of c-Myc epitope in HEK293 cells (A) or COS-7 cells (B) transfected with 150ng c-Myc-RAMP1 (upper panels) or c-Myc-RAMP1Δ-C (lower panels). The left hand column represents cell surface binding (non-permeabilised) and the right hand column represents total binding (permeabilised with 0.3% Tween 20) to anti c-Myc antibody. The figure is representative of at least three independent experiments.

**Figure 9:** (A) Expression of c-Myc epitope in COS-7 cells co-transfected with 100ng HA-CTRα and 150ng c-Myc-RAMP1 (upper panels) or c-Myc-RAMP1Δ-C (lower panels). The left column represents cell surface binding (non-permeabilised) and the right column represents total binding (permeabilised with 0.3% Tween 20) to anti c-Myc antibody. (B) Expression of c-Myc epitope in COS-7 cells co-transfected with 100ng HA-CLR and 150ng c-Myc-RAMP1 (upper panels) or c-Myc-RAMP1Δ-C (lower panels). The left column represents cell surface binding (non-permeabilised) and the right column represents total binding (permeabilised with 0.3% Tween 20) to anti c-Myc antibody. (C) Expression of HA epitope in COS-7 (upper panels) or HEK-293 (lower panels) cells transfected with 100ng HA-CLR in absence of RAMPs (left hand panels) or in presence of 150ng c-Myc-RAMP1 or c-Myc-RAMP1Δ-C (right hand panels). The figure is representative of at least three independent experiments.
### Table 1: pEC<sub>50</sub> values for peptide-induced cAMP production in COS-7 or HEK293 cells co-transfected with hCLR and RAMPs.

Data are represented as mean ± S.E.M. (n≥4).

<table>
<thead>
<tr>
<th>COS-7 cells</th>
<th>hAM</th>
<th>hCGRPα</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLR + RAMP1</td>
<td>8.51 ± 0.32</td>
<td>9.60 ± 0.14</td>
</tr>
<tr>
<td>CLR + RAMP1Δ-C</td>
<td>7.18 ± 0.30*</td>
<td>9.43 ± 0.14</td>
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<tr>
<td>CLR + c-Myc-RAMP1</td>
<td>7.78 ± 0.27</td>
<td>9.63 ± 0.13</td>
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<td>CLR + c-Myc-RAMP1Δ-C</td>
<td>7.20 ± 0.17</td>
<td>9.32 ± 0.16</td>
</tr>
<tr>
<td>CLR + RAMP2</td>
<td>9.39 ± 0.16</td>
<td>6.97 ± 0.16</td>
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<tr>
<td>CLR + RAMP2Δ-C</td>
<td>9.32 ± 0.14</td>
<td>7.09 ± 0.14</td>
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<tr>
<td>CLR + RAMP3</td>
<td>9.48 ± 0.17</td>
<td>6.93 ± 0.16</td>
</tr>
<tr>
<td>CLR + RAMP3Δ-C</td>
<td>9.07 ± 0.17</td>
<td>6.94 ± 0.16</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>HEK-293 cells</th>
<th>hAM</th>
<th>hCGRPα</th>
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<tbody>
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<td>CLR + RAMP1</td>
<td>7.07 ± 0.56</td>
<td>8.49 ± 0.17</td>
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<td>CLR + RAMP1Δ-C</td>
<td>6.57 ± 0.11</td>
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<td>6.50 ± 0.06</td>
<td>8.99 ± 0.07</td>
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<tr>
<td>CLR + c-Myc-RAMP1Δ-C</td>
<td>6.39 ± 0.15</td>
<td>8.78 ± 0.12</td>
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<tr>
<td>CLR + RAMP2</td>
<td>7.63 ± 0.10</td>
<td>6.80 ± 0.08</td>
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<tr>
<td>CLR + RAMP2Δ-C</td>
<td>8.47 ± 0.14*</td>
<td>6.98 ± 0.08</td>
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<tr>
<td>CLR + RAMP3</td>
<td>8.29 ± 0.14</td>
<td>7.32 ± 0.04</td>
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<tr>
<td>CLR + RAMP3Δ-C</td>
<td>8.14 ± 0.14</td>
<td>6.76 ± 0.12*</td>
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</tbody>
</table>

*significantly different from full length RAMP (P<0.05; unpaired t test)
Table 2: $p$IC$_{50}$ values for peptides in competition for $^{125}$I-rAmy binding to COS-7 cells co-transfected with hCTRa and RAMPs.

Data are represented as mean ± S.E.M. ($n$≥4).

<table>
<thead>
<tr>
<th></th>
<th>hCGRPα</th>
<th>rAmy</th>
<th>hCT</th>
<th>sCT</th>
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<tbody>
<tr>
<td>CTRa + RAMP3</td>
<td>7.00 ± 0.13</td>
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<td>CTRa + RAMP3Δ-C</td>
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<td>8.50 ± 0.16</td>
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*significantly different from RAMP3 ($P<0.05$; unpaired t test)
Table 3: \( p\text{EC}_{50} \) values for peptide-induced cAMP production in COS-7 cells co-transfected with hCTRa and RAMPs.

Data are represented as mean ± S.E.M. (n≥7).

<table>
<thead>
<tr>
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<th>hCGRP( \alpha )</th>
<th>rAmy</th>
<th>hCT</th>
<th>sCT</th>
</tr>
</thead>
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<td>CTRa + vector</td>
<td>6.88 ± 0.10</td>
<td>7.13 ± 0.19</td>
<td>9.43 ± 0.17</td>
<td>10.10 ± 0.22</td>
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<tr>
<td>CTRa + RAMP1</td>
<td>8.45 ± 0.15(^{^\wedge})</td>
<td>8.47 ± 0.16(^{^\wedge})</td>
<td>9.00 ± 0.14</td>
<td>10.12 ± 0.23</td>
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<tr>
<td>CTRa + RAMP1( \Delta )-C</td>
<td>7.18 ± 0.13(^{*})</td>
<td>6.95 ± 0.18(^{*})</td>
<td>8.92 ± 0.13</td>
<td>10.16 ± 0.21</td>
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<tr>
<td>CTRa + c-Myc-RAMP1</td>
<td>8.50 ± 0.36(^{^\wedge})</td>
<td>8.44 ± 0.29(^{^\wedge})</td>
<td>8.72 ± 0.15</td>
<td>9.77 ± 0.21</td>
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<td>CTRa + c-Myc-RAMP1( \Delta )-C</td>
<td>6.78 ± 0.38(^{*})</td>
<td>6.89 ± 0.32(^{*})</td>
<td>8.42 ± 0.23(^^{^\wedge})</td>
<td>9.35 ± 0.29</td>
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<tr>
<td>CTRa + RAMP2</td>
<td>7.11 ± 0.17</td>
<td>7.16 ± 0.18</td>
<td>9.39 ± 0.19</td>
<td>9.70 ± 0.25</td>
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<tr>
<td>CTRa + RAMP2( \Delta )-C</td>
<td>6.90 ± 0.24</td>
<td>7.40 ± 0.20</td>
<td>8.32 ± 0.17(^{^*^{^\wedge}})</td>
<td>10.37 ± 0.22</td>
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<tr>
<td>CTRa + RAMP3</td>
<td>7.62 ± 0.19</td>
<td>8.61 ± 0.15(^{^\wedge})</td>
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<td>9.58 ± 0.27</td>
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<td>CTRa + RAMP3( \Delta )-C</td>
<td>6.57 ± 0.26(^{*})</td>
<td>7.88 ± 0.17</td>
<td>9.21 ± 0.18(^{*})</td>
<td>9.94 ± 0.23</td>
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</table>

\(^{^\wedge}\)significantly different from vector control

\(^{*}\)significantly different from full length RAMP

\((P<0.05; \text{one-way ANOVA})\)
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6