0026-895X/06/7005-1750–1760$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 70:1750–1760, 2006

Vol. 70, No. 5
24257/3148685
Printed in U.S.A.

A Critical Role for the Short Intracellular C Terminus in Receptor Activity-Modifying Protein Function

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Received March 9, 2006; accepted August 15, 2006

ABSTRACT

Receptor activity-modifying proteins (RAMPs) interact with and modify the behavior 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 eight 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 human embryonic kidney (HEK)293 cells. Likewise, confocal microscopy revealed each of the mutant RAMPs translocated hemagglutinin-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 RAMP1 and -2 than RAMP3. The loss of amylin binding upon C-terminal deletion could be partially recovered with overexpression of $G\alpha_s$, 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 coexpressed 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.

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) (Poyner et al., 2002; Udawela et al., 2004; Hay et al., 2006). RAMPs are a family of three 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 behavior, 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 (AMY_1, AMY_2, and AMY_3), adrenomedullin (AM_1 and AM_2), and calcitonin gene-related peptide $(CGRP)_1$ receptor phenotypes, whereas the CT receptor phenotype is defined by the independent expression of CTR (Poyner et al., 2002).

Many 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 (Fraser et al., 1999; Kuwasako et al., 2001, 2003; Fitzsimmons et al., 2003). However, work with

This work was supported by National Health and Medical Research Council (NHMRC) grant 299810 and the Ian Potter Neuropeptide laboratory. P.M.S. is a Principal Research Fellow of the NHMRC of Australia. A.C. is a Senior Research Fellow of the NHMRC.

M.U., G.C., and M.M. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.024257.

ABBREVIATIONS: Δ, deletion mutant; GPCR, G protein-coupled receptor; RAMP, receptor activity-modifying protein; CT, calcitonin; CTR, calcitonin receptor; CLR, calcitonin receptor-like receptor; AMY, amylin receptor phenotype; AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; PDZ, postsynaptic density-95/Discs-large/ZO-1 homology; h, human; sCT, salmon calcitonin; rAmy, rat amylin; HA, hemagglutinin; WT, wild-type; DMEM, Dulbecco's modified Eagle's medium; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; PBS, phosphate-buffered saline; RCP, receptor component protein.

the CTR has revealed additional effects on phenotype that are cell background-dependent where coexpression 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 approximately 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 signaling 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). These 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 Gα subunit protein levels could "rescue" the poor induction of AMY_2 phenotype seen in COS-7 cells (Christopoulos et al., 1999; Zumpe et al., 2000; Tilakaratne et., 2003).

Deletion studies of the RAMP1 C terminus have revealed that removal of most of the C terminus (up to nine amino acids) has relatively little impact on RAMP1 induction of the $CGRP_1$ receptor phenotype from CLR (Steiner et al., 2002; Fitzsimmons et al., 2003), with similar CGRP binding affinity and either no change in cAMP signaling in HEK293 cells (Fitzsimmons et al., 2003) or a weak reduction in maximal agonist response and potency in COS-7 cells for the constructs truncated by nine 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. (2005a,b) 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^{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 eight amino acids (RAMP1Δ-C, RAMP2Δ-C, RAMP3Δ-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, whereas 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 $G\alpha_s$ protein. In contrast CLR-dependent cell surface expression of RAMPs was retained.

Materials and Methods

Human calcitonin (hCT), salmon calcitonin (sCT), human αCGRP, and rat amylin (rAmy) were purchased from Auspep (Parkville, VIC, Australia), and human AM was from Bachem (Bubendorf, Switzerland). Tissue culture reagents were from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by GeneWorks (Adelaide, SA, Australia). Rabbit anti-c-Myc antibody was supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa 488- and Texas Red-conjugated goat anti-mouse and anti rabbit sera were from Invitrogen. ^{125}I-labeled goat anti-mouse IgG was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). *N*-Succinimidyl-3-(4-hydroxy-[^{125}I]iodophenyl) propionate (Bolton-Hunter reagent; 2000 Ci/mmol) was supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK). ^{125}I-rAmy (specific activity, 2000 Ci/mmol) was iodinated by the Bolton-Hunter method and purified by reversed phase–high-performance liquid chromatography as described previously (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 (GlaxoSmithKline, Stevenage, UK) (Fraser et al., 1999). C-Myc-RAMP1 was provided by Dr. K. Kuwasako (University of Miyazaki, Miyazaki, Japan) (Kuwasako et al., 2000). Double HA epitope-tagged human CTRa (HA-CTRa) was prepared as described previously (Pham et al., 2004). This receptor is the Leu^{447} polymorphic variant of the receptor (Kuestner et al., 1994). EE-tagged $G\alpha_s$ cDNA was purchased from the UMR cDNA Resource Center (University of Missouri, Rolla, MO) (http://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′-ctggtggtctggcagtgaaagcgcactgagggc-3′, reverse primer 5′-gccctcactgcgctttcactgccagaccaccag-3′), -RAMP2 (forward primer 5′-cctgtagtatggaggtgaaaagacagtgaggcc-3′, reverse primer 5′-ggcctcactgtcttttcacctccatactacaag-3′), and -RAMP3 (forward primer 5′-ctggtggtgtggcgctgaaaacgcaccgacacg-3′, reverse primer 5′-cgtgtcggtgcgttttcagcgccacaccaccag-3′) and c-Myc-RAMP1 (forward primer 5′-ggtctggcagtgaaagcgcactgagggc-3′, reverse primer 5′-ctcagtgcgctttcactgccagaccacc-3′), using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resultant constructs are displayed schematically in Fig. 1.

Cell Culture and Transfections. COS-7 and HEK293 cells were routinely maintained in 175-cm^2 flasks at 37°C in a humidified atmosphere with 5% CO_2/95% air, in complete DMEM supplemented with 5% heat inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 50 μg/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

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or four-well chamber slides were transfected with 100 ng of receptor and 150 ng of RAMP with 1 μl of lipid, 75-cm^2 flasks with 4 μg of receptor and 6 μg of RAMP with 20 μl of lipid, and 25-cm^2 flasks with 1 μg of receptor and 1.5 μg of RAMP with 8 μl of lipid.

Receptor Binding. Specific binding was determined as described previously (Christopoulos et al., 1999) 48 h posttransfection in 24-well plates. For competition binding, COS-7 cells were transfected in 75-cm^2 flasks and grown for 48 h, and then they were harvested and resuspended in binding buffer (DMEM containing 1% bovine serum albumin). Cells were added to 96-well plates (100,000 cells/well) with ~70 pM ^{125}I-rAmy and competing unlabeled peptides. After incubating for 1 h at 37°C, cells were harvested onto GF/C plates (coated with 0.5% polyvinylpyrolidone and 0.1% Tween 20) using a harvester (Tomtec, Orange, CT). Plates were dried overnight, and after the addition of Micorscint 0$^-$ (PerkinElmer Life and Analytical Sciences, Boston, MA), they were counted on a TopCount counter (PerkinElmer Life and Analytical Sciences). Experiments were performed with triplicate repeats.

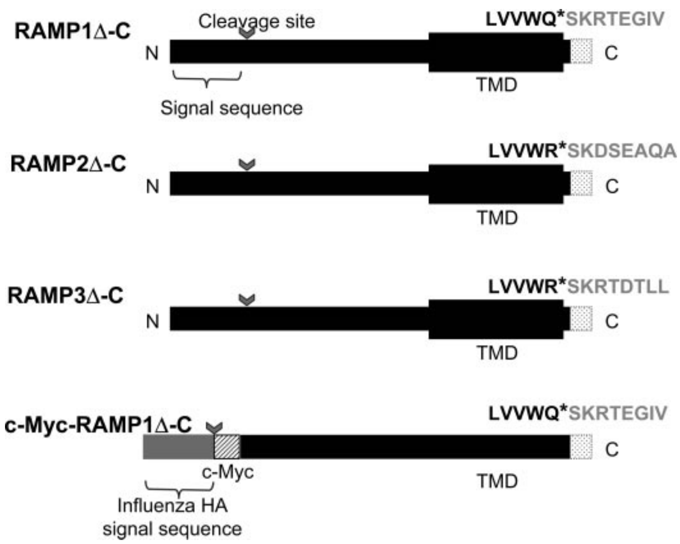

Fig. 1. Schematic representation of RAMP C-terminal deletion mutants, showing the extracellular N-terminal domain, including the signal peptide and its predicted cleavage site, the transmembrane domain (TMD), and the position of the introduced stop codon (asterisk) preventing translation of the last eight 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.

^{125}I-Rat Amylin Binding in the Presence or Absence of Gpp(NH)p. COS-7 cells were seeded to 90% confluence in 48-well plates. These were transfected with 50 ng of CTRa and 75 ng of RAMP1 per well, using 0.75 μl of Metafectine. Forty-eight hours after 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-Aldrich, St. Louis, MO) at a final concentration of 10^{-4} M. Cells were permeabilized by pretreating with phosphate-buffered saline (PBS) and 0.3% Tween 20 for 5 min and then washed once with PBS immediately before binding. Binding of ^{125}I-rAmy (~100 pM) was performed at 37°C for 45 min. Cells were washed once with ice-cold PBS and solubilized with 0.5 M NaOH. Cell lysates were counted on a Wizard gamma-counter (PerkinElmer Life and Analytical Sciences).

Cyclic AMP Assays. Intracellular cAMP levels were determined using the AlphaScreen cAMP kit (PerkinElmer Life and Analytical Sciences). Cells transfected in 25-cm^2 flasks were grown for 48 h and then serum-starved overnight. Cells were subsequently harvested and assayed as described previously (Hay et al., 2005), at cell concentrations of 5000 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 described previously (Hay et al., 2005) 48 h after transfection of COS-7 cells in 24-well plates, using anti-HA (12CA5) or anti-c-Myc (9E10) antibody.

Confocal Microscopic Localization of Receptors and RAMPs. Twenty-four hours after transfection, cells grown in four-well chamber slides were fixed with 3.4% paraformaldehyde in PBS for 20 min at room temperature and then washed with PBS. Cells

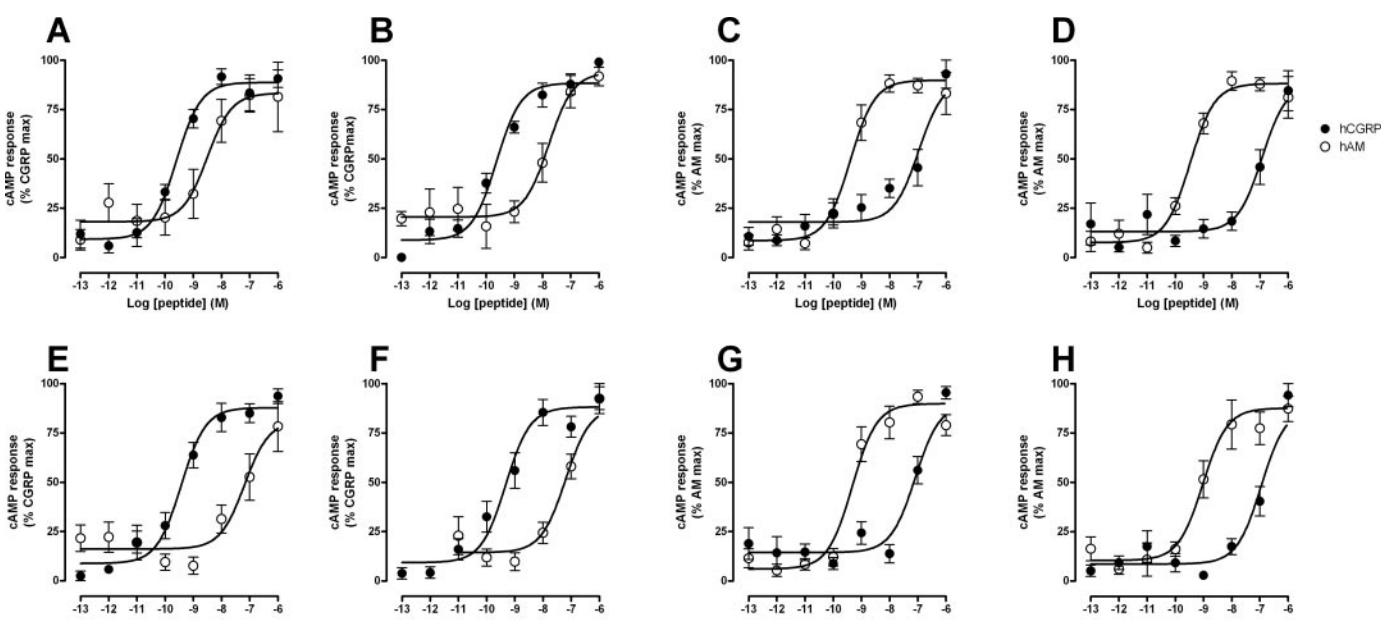

Fig. 2. Effect of RAMP C-terminal deletion on induction of cAMP accumulation at CLR/RAMP receptors. COS-7 cells were cotransfected with 1 μg of CLR and 1.5 μg of RAMP1 (A), RAMP1Δ-C (E), c-Myc-RAMP1 (B), c-Myc RAMP1Δ-C (F), RAMP2 (C), RAMP2Δ-C (G), RAMP3 (D), or RAMP3Δ-C (H) and stimulated with hAM (○) and hCGRPα (●). Data are mean ± S.E.M. of four to nine separate experiments, normalized to maximal peptide response. pEC$_{50}$ values are given in Table 1. E_{max} values for RAMPΔ-C cotransfected cells tended to be higher than observed for full-length RAMP cotransfected cells (RAMP1Δ-C, 134 ± 25%; RAMP2Δ-C, 152 ± 24%; and RAMP3Δ-C, 123 ± 14%), although none of these achieved statistical significance.

were permeabalized with 0.3% Tween 20 in PBS for 5 to 10 min, washed with PBS, and then incubated for 30 to 60 min with 10% normal goat serum 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% normal goat serum, for 1 h at room temperature. Cells were washed three times with PBS and 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 temperature for 1 h. Cells were washed with PBS three times, and coverslips were mounted with Dako-fluorescent mounting media (Dako North America, Inc., Carpinteria, CA). Fluorescence was visualized on a Zeiss Acioplan-2 microscope (Carl Zeiss, Jena, Germany) with an MRC-1024 confocal microscopy system (Bio-Rad Laboratories, Hercules, CA) and LaserSharp 2000 software (Bio-Rad).

Data Analysis. At least four independent repeats were performed for each of the above-mentioned experiments, and the results are presented as mean ± standard error of means. Curve fitting was done using Prism 4 (GraphPad Software Inc., San Diego, CA). pIC_{50} and pEC_{50} values were compared by two-way t tests or one-way analysis of variance as appropriate, where $P < 0.05$ was considered significant. Post hoc testing was performed with Dunnett's test (for comparison with vector) and Bonferroni's test for comparison of WT and mutant RAMPs.

Results

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 (data 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; Muff et al., 1999; Hay et al., 2005).

To determine the role of the C terminus of RAMPs in the induction of functional complexes from CLR, wild-type or deletion mutants of RAMP1, -2, and -3 were coexpressed with CLR in either COS-7 or HEK293 cells, and cAMP production in response to hCGRP and hAM was measured. At RAMP1- and c-Myc-RAMP1-induced phenotypes, hCGRP had high potency, and hAM had a lower potency (Fig. 2, A and B; Table 1), typical of classic $CGRP_1$ 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 and F; Table 1), although a reduction in AM potency was observed in COS-7 cells (Fig. 2E; Table 1). These data indicate that deletion of the RAMP1 C terminus had little effect on the functional $CGRP_1$ receptor.

Coexpression of CLR with RAMP2 gave a receptor phenotype with higher potency for adrenomedullin than hCGRP, typical of classic AM_1 pharmacology (Poyner et al., 2002; Fig. 2C; 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. 2G; Table 1) and only a small increase in AM potency observed in HEK293 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 HEK293 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. 2D; 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. 2H; Table 1) and only a small decrease in CGRP potency observed in the HEK293 cells (Table 1).

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

TABLE 1

pEC_{50} values for peptide-induced cAMP production in COS-7 or HEK293 cells cotransfected with hCLR and RAMPs

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

	hAM	hCGRPα
COS-7 cells		
CLR + RAMP1	8.51 ± 0.32	9.60 ± 0.14
CLR + RAMP1Δ-C	7.18 ± 0.30*	9.43 ± 0.14
CLR + c-Myc-RAMP1	7.78 ± 0.27	9.63 ± 0.13
CLR + c-Myc-RAMP1Δ-C	7.20 ± 0.17	9.32 ± 0.16
CLR + RAMP2	9.39 ± 0.16	6.97 ± 0.16
CLR + RAMP2Δ-C	9.32 ± 0.14	7.09 ± 0.14
CLR + RAMP3	9.48 ± 0.17	6.93 ± 0.16
CLR + RAMP3Δ-C	9.07 ± 0.17	6.94 ± 0.16
HEK293 cells		
CLR + RAMP1	7.07 ± 0.56	8.49 ± 0.17
CLR + RAMP1Δ-C	6.57 ± 0.11	8.76 ± 0.25
CLR + c-Myc-RAMP1	6.50 ± 0.06	8.99 ± 0.07
CLR + c-Myc-RAMP1Δ-C	6.39 ± 0.15	8.78 ± 0.12
CLR + RAMP2	7.63 ± 0.10	6.80 ± 0.08
CLR + RAMP2Δ-C	8.47 ± 0.14*	6.98 ± 0.08
CLR + RAMP3	8.29 ± 0.14	7.32 ± 0.04
CLR + RAMP3Δ-C	8.14 ± 0.14	6.76 ± 0.12*

* Significantly different from full-length RAMP ($P < 0.05$; unpaired t test).

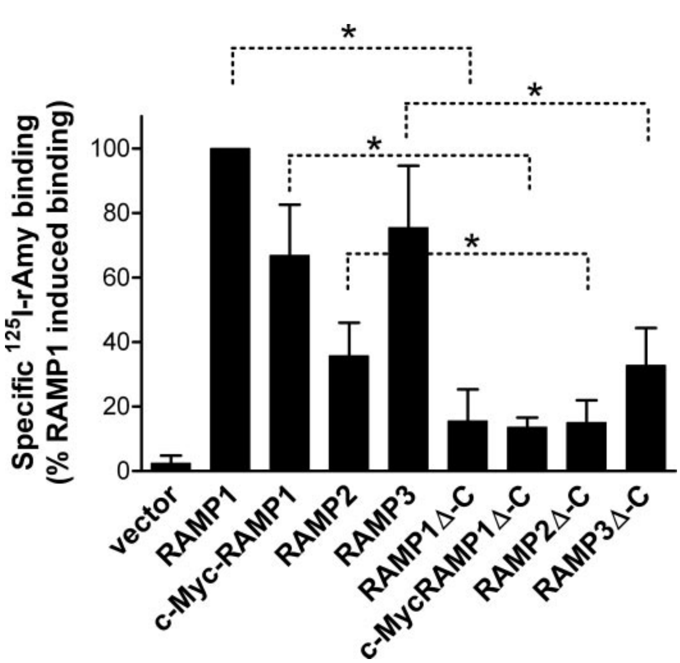

Fig. 3. Effect of RAMP C-terminal deletion on specific ^{125}I-rAmy binding to CTRa/RAMP receptors. COS-7 cells were transfected with 100 ng of CTRa and 150 ng of RAMP WT or C-terminal deletion mutants. Data are mean ± S.E.M. of six separate experiments, expressed as a percentage of RAMP1-induced binding (*, $P < 0.05$; paired t test).

only in COS-7 cells where no background phenotype was found.

Induction of ^{125}I-rAmy Binding by Wild-Type and Mutant RAMPs Cotransfected with CTRs. Consistent with previous findings (Christopoulos et al., 1999; Muff et al., 1999; Zumpe et al., 2000), when expressed with CTRa in COS-7 cells RAMP1 and RAMP3 induced high levels of rat amylin binding, whereas 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}I-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}I-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 Gα_s increases the low level of ^{125}I-rAmy binding to COS-7 cells cotransfected with CTRa and RAMP2 (Tilakaratne et al., 2003), suggesting that Gα_s may be interacting with the RAMP, presumably via the C terminus, to alter receptor behavior. 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 Gα_s protein (Fig. 4). Binding levels were normalized to HA-CTRa cell surface expression to minimize effects of variations in transfection efficiency. Cotransfection of Gα_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 Gα_s. Incubation of CTR/RAMP1 receptors with the GTP analog Gpp(NH)p led to a marked reduction in the level of ^{125}I-Amy binding (Fig. 5 A) with no change in the affinity of either rAmy (Fig. 5B) or hCGRP (Fig. 5C), consistent with a role for G protein coupling on the level of functional AMY$_1$ receptors. Coexpression of RAMP2Δ-C with Gα_s led to a pronounced increase in induced ^{125}I-rAmy binding to levels similar to those seen with the wild-type RAMP2, either with or without Gα_s. A similar effect was observed after, coexpression of RAMP3Δ-C with Gα_s (Fig. 4).

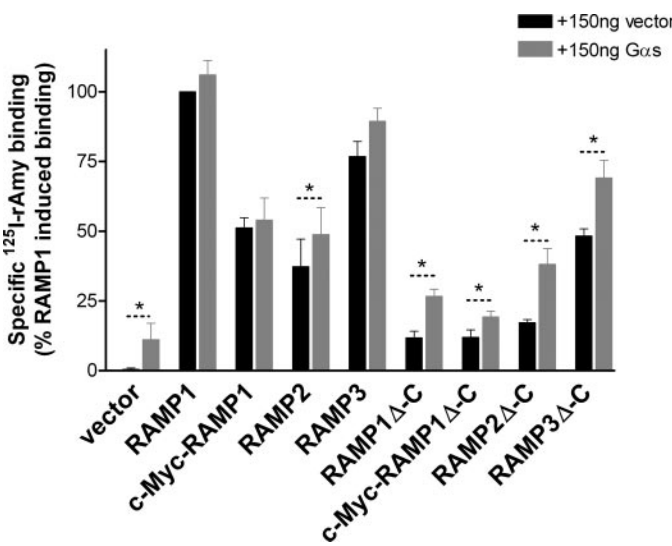

Fig. 4. Effect of Gα_s cotransfection on specific ^{125}I-rAmy binding to CTRa/truncated RAMPs. COS-7 cells were transfected with 100 ng of HA-CTRa and 150 ng of RAMP WT or C-terminal deletion mutants with (■) or without (▨) 150 ng of Gα_s protein. Data are mean ± S.E.M. of seven separate experiments expressed as percentage of the level or binding induced by RAMP1, normalized to HA-CTRa cell surface expression measured in parallel (*, $P < 0.05$; paired t test).

Binding Phenotype of AMY$_3$ Receptors after Deletion of RAMP3 C Terminus. Only very low ^{125}I-Amy binding was observed for cells cotransfected 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)

Effect of C-Terminal RAMP Deletion on Downstream Signaling 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.

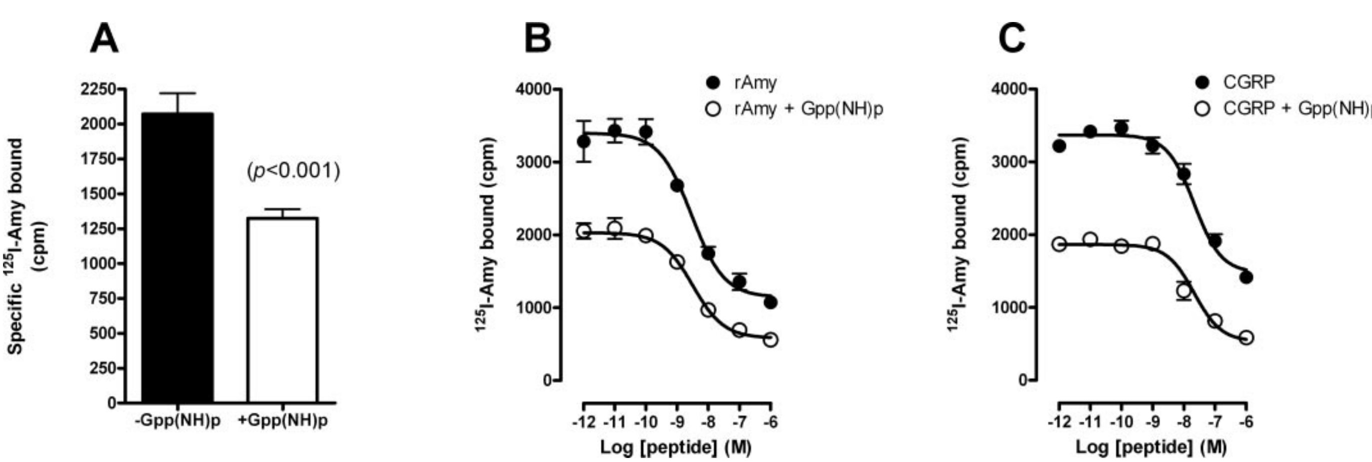

Fig. 5. Effect of Gpp(NH)p on binding of ^{125}I-Amy to lysed COS-7 cells coexpressing CTRa and RAMP1. Cells in 48-well plates were cotransfected with 50 ng of HA-CTRa and 75 ng of RAMP1. Before assay cells were lysed and incubated with buffer or 10^{-4} M Gpp(NH)p. A, specific ^{125}I-Amy binding. B, competition of ^{125}I-Amy binding by rAmy. C, competition of ^{125}I-Amy binding by hCGRP.

This CT receptor phenotype is characterized 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. 7A; Table 3). When the CTRa was coexpressed with RAMP1, both hCGRP and rAmy displayed increased potency (Fig. 7B; 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. 7F; Table 3). Coexpression of CTRa with c-Myc-RAMP1 led to increased potency of rAmy and hCGRP and a decrease in hCT potency (Fig. 7C; 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 after c-Myc-RAMP1 cotransfection was also observed (Fig. 7G; Table 3).

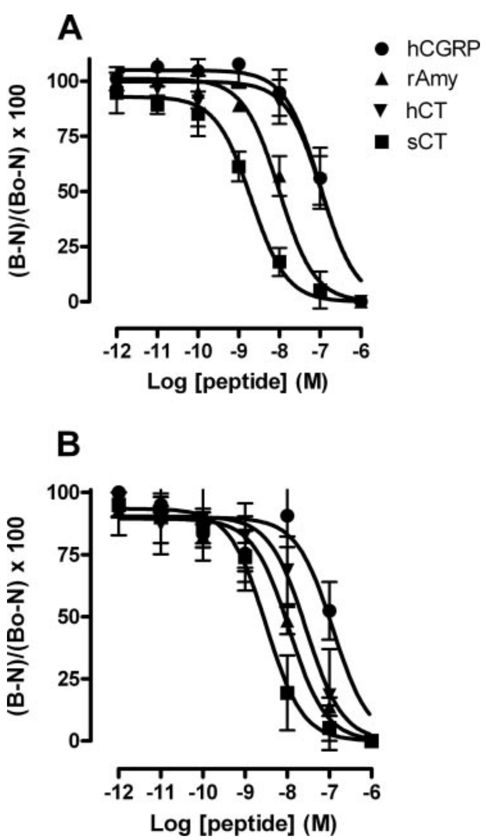

Fig. 6. Effect of C-terminal deletion on peptide competition for ^{125}I-rAmy binding to CTRa/RAMP3. COS-7 cells were cotransfected with 4 μg of CTRa and 6 μg of RAMP3 (A) or RAMP3Δ-C (B). hCGRPα (●), rAmy (▲), hCT (▼), and sCT (■) were used to compete for ^{125}I-rAmy binding. Data are mean ± S.E.M. of four or more separate experiments. B, ^{125}I-rAmy bound; B_0, total binding in the absence of competing peptide; N, nonspecific binding (measured in the presence of 10^{-6} M peptide). pIC_{50} values are given in Table 2.

In COS-7 cells, cotransfection 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, coexpression of CTRa and RAMP2 did not lead to an overt change in the response to peptides (Fig. 7D; Table 3). Deletion of the RAMP2 C terminus led to a significant decrease in hCT potency (Fig. 7H; Table 3).

Cotransfection of RAMP3 with the CTRa led to an increased potency of rAmy and hCGRP and a decreased potency of hCT (Fig. 7E; 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 with the wild-type RAMP3 (Fig. 7I; 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 coexpressed with RAMP3 (Table 3).

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 with the full-length tagged RAMP1 (Fig. 8A). When cotransfected with HA-CLR both c-Myc-RAMP1 and the deletion mutant translocated to the cell surface (Fig. 9B).

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 labeled secondary antibodies. HA-CLR showed relatively low cell surface expression in the absence of RAMPs (Fig. 9C, bottom left). This was increased upon cotransfection of either c-Myc-RAMP1 or c-Myc-RAMP1Δ-C (Fig. 9C, bottom right). When visualized by double staining, both c-Myc-RAMP1 and c-Myc-RAMP1Δ-C demonstrated colocalization 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 coexpressed 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

TABLE 2
pIC_{50} values for peptides in competition for ^{125}I-rAmy binding to COS-7 cells cotransfected with hCTRa and RAMPs
Data are represented as mean ± S.E.M. ($n \geq 4$).

	hCGRPα	rAmy	hCT	sCT
CTRa + RAMP3	7.00 ± 0.13	8.01 ± 0.14	6.96 ± 0.13	8.69 ± 0.12
CTRa + RAMP3Δ-C	6.91 ± 0.16	7.95 ± 0.11	7.55 ± 0.20*	8.50 ± 0.16

* Significantly different from RAMP3 ($P < 0.05$; unpaired t test).

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. 8B). 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

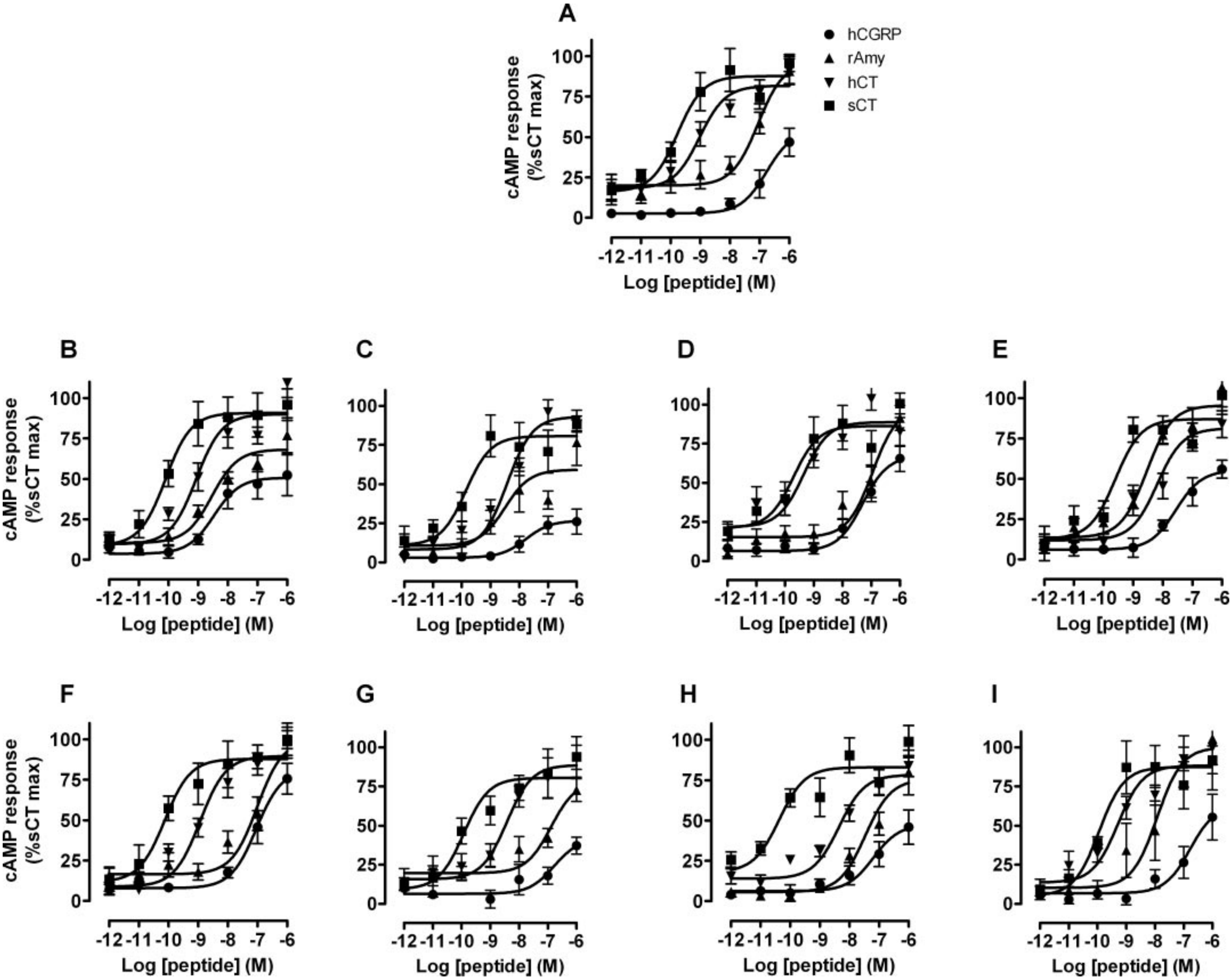

Fig. 7. Effect of RAMP C-terminal deletion on induction of cAMP accumulation at CTR/RAMP receptors. COS-7 cells were cotransfected with hCTRa and empty vector (A), RAMP1 (B), RAMP1Δ-C (F), c-Myc-RAMP1 (C), c-Myc-RAMP1Δ-C (G), RAMP2 (D), RAMP2Δ-C (H), RAMP3 (E), or RAMP3Δ-C (I) and stimulated with hCGRPα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

TABLE 3

pEC_{50} values for peptide-induced cAMP production in COS-7 cells cotransfected with hCTRa and RAMPs

Data are represented as mean ± S.E.M. ($n \geq 7$).

	hCGRPα	rAmy	hCT	sCT
CTRa + vector	6.88 ± 0.10	7.13 ± 0.19	9.43 ± 0.17	10.10 ± 0.22
CTRa + RAMP1	8.45 ± 0.15†	8.47 ± 0.16†	9.00 ± 0.14	10.12 ± 0.23
CTRa + RAMP1Δ-C	7.18 ± 0.13*	6.95 ± 0.18*	8.92 ± 0.13	10.16 ± 0.21
CTRa + c-Myc-RAMP1	8.50 ± 0.36†	8.44 ± 0.29†	8.72 ± 0.15	9.77 ± 0.21
CTRa + c-Myc-RAMP1Δ-C	6.78 ± 0.38*	6.89 ± 0.32*	8.42 ± 0.23†	9.35 ± 0.29
CTRa + RAMP2	7.11 ± 0.17	7.16 ± 0.18	9.39 ± 0.19	9.70 ± 0.25
CTRa + RAMP2Δ-C	6.90 ± 0.24	7.40 ± 0.20	8.32 ± 0.17*†	10.37 ± 0.22
CTRa + RAMP3	7.62 ± 0.19	8.61 ± 0.15†	8.17 ± 0.20†	9.58 ± 0.27
CTRa + RAMP3Δ-C	6.57 ± 0.26*	7.88 ± 0.17	9.21 ± 0.18*	9.94 ± 0.23

* Significantly different from full-length RAMP ($P < 0.05$; one-way analysis of variance).
† Significantly different from vector control.

(Fig. 9A). In contrast, in the presence of CLR both full-length and truncated c-Myc-RAMP1 translocated to the cell surface in these cells (Fig. 9B). 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. 9C, top). In these cells c-Myc-RAMP1Δ-C colocalized with HA-CLR at the cell surface (data not shown). HA-CTRa also exhibited colocalization with c-Myc-RAMP1Δ-C; however, this occurred with lower efficiency than seen with the full-length construct (data not shown), and it was not correlated with a functional phenotype.

To determine the effect of RAMP C-terminal deletion on cell surface localization of CTR, ^{125}I-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

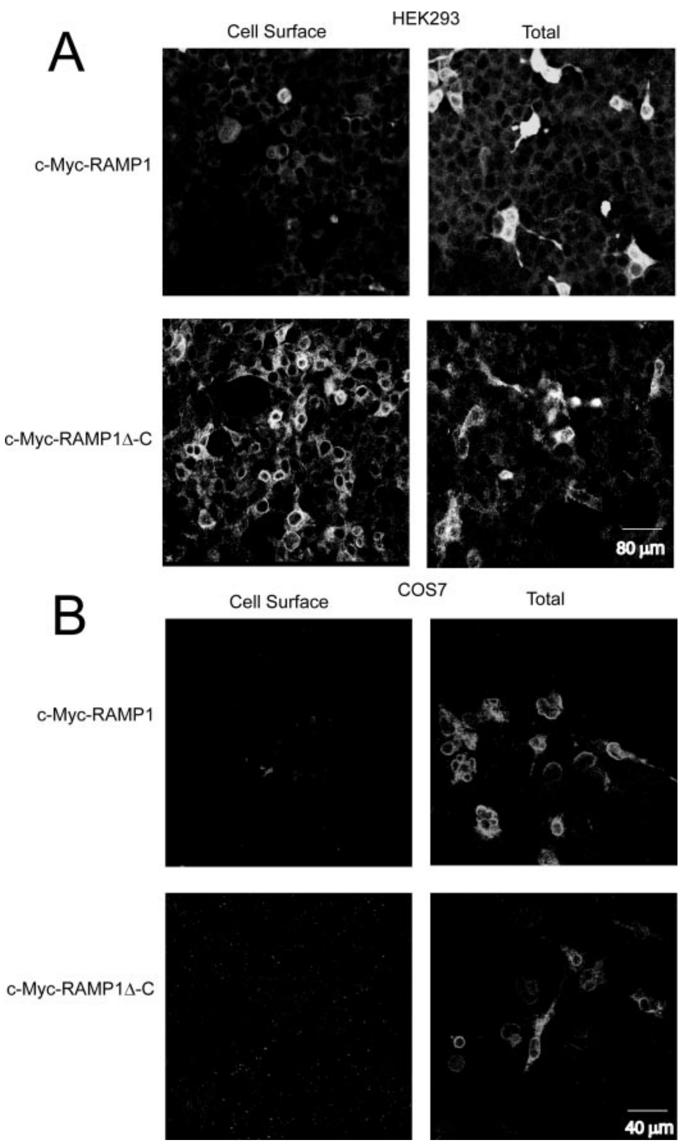

Fig. 8. Expression of c-Myc epitope in HEK293 cells (A) or COS-7 cells (B) transfected with 150 ng of c-Myc-RAMP1 (top) or c-Myc-RAMP1Δ-C (bottom). The left-hand column represents cell surface binding (nonpermeabilized) and the right-hand column represents total binding (permeabilized with 0.3% Tween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

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 compared with the CTRa with vector control (data not shown). This suggests that deletion of the C terminus of RAMPs does not have great impact on the intrinsic translocation of HA-CTRa 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 eight 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 colocalized. A similar observation was previously seen for RAMP1 truncated by nine 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 CGRP$_1$ receptor, as monitored by cAMP accumulation assay. These data were consistent with the previously published work of 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_{max} and potency of the receptor with deletion of nine 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 Gα_s signaling compared with those in COS-7 cells (Kuwasako et al., 2004). The two cell lines also differ in their profile of regulatory protein expression (Purdue, 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. It is noteworthy that 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 et al. (2006), where Tyr^0-CGRP but not CGRP exhibited reduced potency after deletion of nine amino acids of the RAMP1 C terminus.

Similar to RAMP1-based CGRP receptors, we observed only minor effects on receptor phenotype of AM receptors after truncation of RAMP2 or RAMP3 in each of the cell lines. The RAMP3 data are consistent with recent work with RAMP3 truncated at the C terminus by nine amino acids (Kuwasako et al., 2006). However, our data are in marked contrast to that seen for truncated RAMP2, where Kuwasako and colleagues observed a significant loss of AM binding and decreased E_{max} after cotransfection with CLR into their HEK293 cells. In those experiments, both CLR and RAMP2Δ-C mutants (of eight or nine amino acids) were primarily retained in the endoplasmic reticulum. In our COS-7 cells, expression of the CLR/ RAMP2Δ-C complex was at least as efficient as that seen with the wild-type RAMP2. However, we did see a trend toward a reduction in E_{max} in our HEK293 cells, which may be related to the observations reported in Kuwasako et al. (2006). The variation in data between the two studies is likely to be related to differences in cellular background of the HEK293 cells of the Japanese laboratory and those of our HEK293 and COS-7 cells, but it

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may also be related, in part, to effects of either the green fluorescent protein-fused to CLR or the epitope tagging of the RAMP2 because 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 et al. (2006) 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 nonspecific 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, with strong receptor-independent cell surface expression seen in the HEK293 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 after C-terminal truncation indicates that this can be altered and that the conserved Ser-Lys sequence may be important for the observed differences (Kuwasako et al., 2006). Together, 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 et al. (2005a,b).

In stark contrast to the minimal impact of RAMP C-terminal truncation on CLR-based receptor function, deletion of the C-terminal eight 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 signaling phenotypes. The lack of functional high-affinity AMY receptor phenotype, however, was due neither to destabilization of the CTR nor to its capacity to be expressed at the cell surface, because direct assay of the

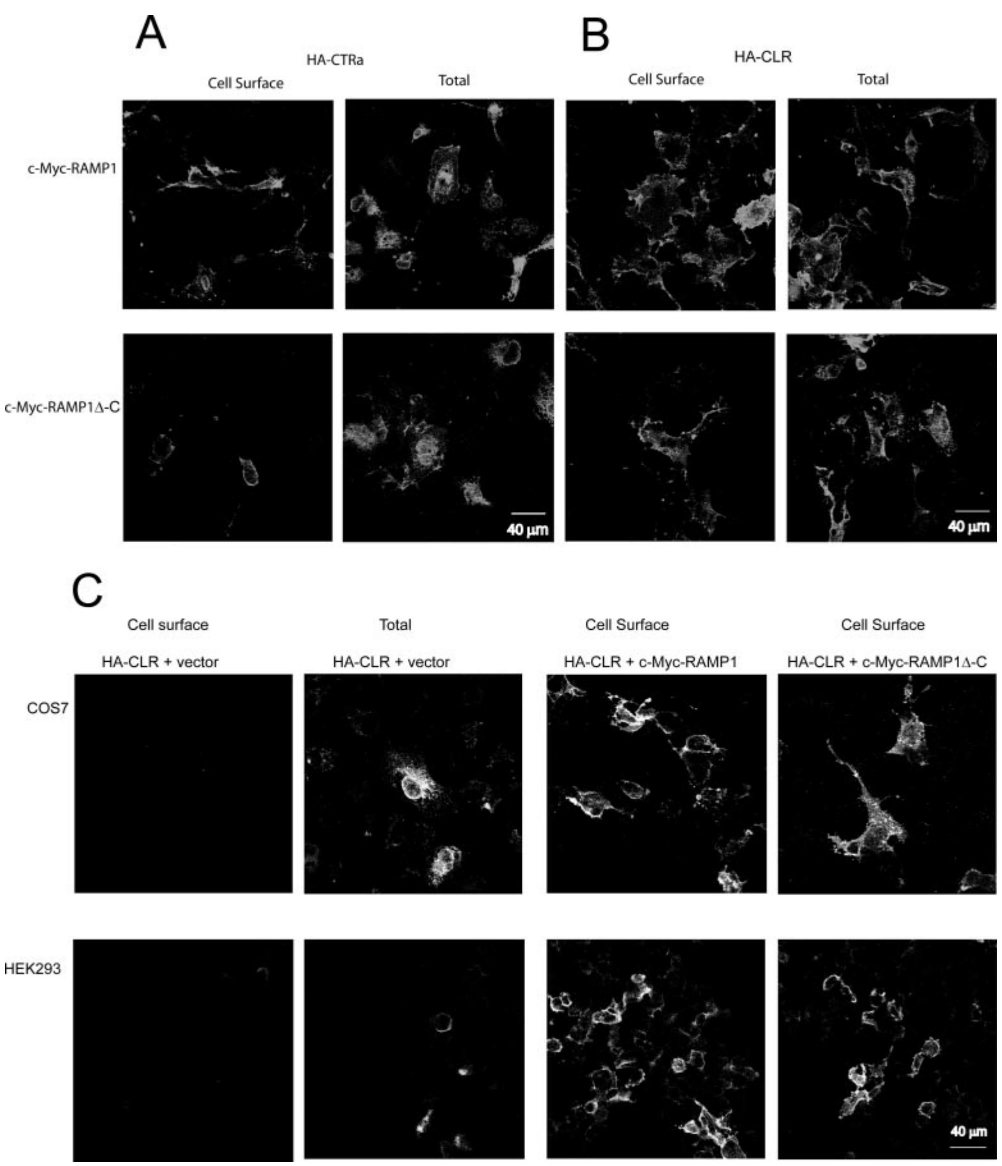

Fig. 9. A, expression of c-Myc epitope in COS-7 cells cotransfected with 100 ng of HA-CTRa and 150 ng of c-Myc-RAMP1 (top) or c-Myc-RAMP1Δ-C (bottom). The left column represents cell surface binding (nonpermeabilized), and the right column represents total binding (permeabilized with 0.3% Tween 20) to anti c-Myc antibody. B, expression of c-Myc epitope in COS-7 cells cotransfected with 100 ng of HA-CLR and 150 ng of c-Myc-RAMP1 (top) or c-Myc-RAMP1Δ-C (bottom). The left column represents cell surface binding (nonpermeabilized), and the right column represents total binding (permeabilized with 0.3% Tween 20) to anti c-Myc antibody. C, expression of HA epitope in COS-7 (top) or HEK293 (bottom) cells transfected with 100 ng of HA-CLR in absence of RAMPs (left) or in presence of 150 ng of c-Myc-RAMP1 or c-Myc-RAMP1Δ-C (right). The figure is representative of at least three independent experiments.

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 cotransfected 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 probably 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 three 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 signaling, presumably via contributing to receptor-G protein interaction, because knockdown of RCP expression leads to marked attenuation of cAMP signaling (Evans et al., 2000; Prado et al., 2001, 2002). More recent data indicate that RCP stabilizes the interaction between RAMP and CLR, with knockdown of RCP preventing coimmunoprecipitation of RAMP and CLR (Dickerson and Loiseau, 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 two receptors. For CLR-based receptors, the RAMP C terminus does not play a strong role in the efficiency of G protein coupling because of the additional complexing of the RAMP/receptor dimer with RCP; therefore, there is a relatively low impact of deletion on receptor phenotype. In contrast, for CTR/RAMP dimers, the RAMP C terminus seems 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 behavior 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 two RAMPs (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; 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, but 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 ^{125}I-Amy binding in RAMP1Δ-C/CTRa cotransfectants was increased with overexpression of $G\alpha_s$, the data suggest 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 cotranslocation together through endoplasmic reticulum-Golgi-plasma membrane, albeit that the overall stability of the complex is impaired, because 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 stabilizing 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.

Acknowledgments

We thank Ece Arel for excellent technical assistance and Souheir Houssami for assistance in preparation of the manuscript.

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