

The Amino Terminus of Receptor Activity Modifying Proteins Is a Critical Determinant of Glycosylation State and Ligand Binding of Calcitonin Receptor-Like Receptor

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ABSTRACT

The calcitonin receptor-like receptor (CRLR) can function as either a receptor for calcitonin gene-related peptide (CGRP) or for adrenomedullin (ADM), depending upon the coexpression of a novel family of single transmembrane proteins, which we have called receptor activity modifying proteins or RAMPs. RAMPs 1, 2, and 3 transport CRLR to the plasma membrane with similar efficiencies, however RAMP1 presents CRLR as a terminally glycosylated, mature glycoprotein and a CGRP receptor, whereas RAMPs 2 and 3 present CRLR as an immature, core glycosylated ADM receptor. Characterization of the RAMP2/CRLR and RAMP3/CRLR receptors in HEK293T cells by radioligand binding (^{125}I -ADM as radioligand), functional

assay (cAMP measurement), or biochemical analysis (SDS-polyacrylamide gel electrophoresis) revealed them to be indistinguishable, even though RAMPs 2 and 3 share only 30% identity. Chimeric proteins were created with the transmembrane and cytosolic portions of RAMP1 associated with the amino terminus of RAMP2 (RAMP2/1) and vice versa (RAMP1/2). Coexpression of RAMP2/1 with CRLR formed a core glycosylated ADM receptor, whereas the RAMP1/2 chimera generated both core glycosylated and mature forms of CRLR and enabled both ADM and CGRP receptor binding. Hence, the glycosylation state of CRLR appears to correlate with its pharmacology.

Calcitonin gene-related peptide (CGRP) and adrenomedullin (ADM) are related members of the calcitonin family of regulatory peptides, which also includes amylin and calcitonin (Muff et al., 1995). We recently showed that a seven-transmembrane receptor, the calcitonin receptor-like receptor (CRLR), can function as either a CGRP receptor or an ADM receptor, depending upon the coexpression of a family of three, single transmembrane receptor activity modifying proteins or RAMPs. RAMP1 presents CRLR at the plasma membrane as a terminally glycosylated, mature glycoprotein and a CGRP receptor, whereas RAMPs 2 and 3 present CRLR as an immature, core glycosylated ADM receptor (McLatchie et al., 1998).

We have considered a number of possible mechanisms of RAMP activity: First, RAMPs may be directly involved in the binding of CGRP and ADM to CRLR. This hypothesis is supported by the observation that ^{125}I -CGRP cross-links to a protein that could be RAMP1 and to CRLR following coexpression in human embryonic kidney (HEK) 293T cells (Stangl et al., 1991; McLatchie et al., 1998). Second, carbohydrate residues linked to CRLR during trafficking and glycosylation might determine the binding of CGRP and ADM.

Finally, coexpression of RAMPs might alter the conformation of CRLR and thereby alter its binding characteristics.

In this study we carried out a number of experiments to examine the structural basis of RAMP activity. Detailed pharmacological characterization showed that, in spite of their disparate amino acid sequences, RAMPs 2 and 3 form an identical ADM receptor following coexpression with CRLR in HEK293T cells. Furthermore, experiments using RAMP chimeras showed that both the glycosylation state of CRLR and its ligand specificity are determined by the amino terminus of the coexpressed RAMP protein.

Experimental Procedures

Oocytes. Adult female *Xenopus laevis* (Blades Biologicals) were anesthetized using 0.2% tricaine (3-aminobenzoic acid ethyl ester; Sigma Chemical Co., St. Louis, MO), sacrificed, and the ovaries rapidly removed. Oocytes were defolliculated by collagenase digestion (1.5 mg ml^{-1} ; Sigma type I) in divalent cation-free OR2 solution (82.5 mM NaCl , 2.5 mM KCl , $1.2\text{ mM NaH}_2\text{PO}_4$, and 5 mM HEPES , pH 7.5 at 25°C). Single stage V and VI oocytes were transferred to ND96 solution (96 mM NaCl , 2 mM KCl , 1 mM MgCl_2 , and 5 mM

ABBREVIATIONS: CRLR, calcitonin receptor-like receptor; CGRP, calcitonin gene-related peptide; ADM, adrenomedullin; RAMPs, receptor activity modifying proteins; EndoF, endoglycosidase F; EndoH, endoglycosidase H.

HEPES, pH 7.5 at 25°C), which contained 50 $\mu\text{g ml}^{-1}$ gentamycin and stored at 18°C.

Coexpression of the cystic fibrosis transmembrane regulator, which contains a chloride channel that is regulated by a cAMP-dependent kinase, was used to assay for CGRP receptor activation and subsequent cAMP accumulation.

Cystic fibrosis transmembrane regulator (in pBluescript), RAMPs 1 and 2, RAMP chimeras, and CRLR (all in pcDNA3) were linearized and transcribed to RNA using T7 or T3 polymerase (Promega Wizard kit, Madison, WI). m⁷G(5')pp(5')GTP capped mRNA was injected into oocytes (20–50 ng per oocyte) and whole-cell currents were recorded using two-microelectrode voltage-clamp (Geneclamp amplifier, pClamp6 software, Axon Instruments, Burlingame, CA) 3 to 10 days post-RNA injection. All recordings were made in Ca²⁺-free ND96 solution and drugs were applied by addition to the superfusate. Microelectrodes had a resistance of 0.5 to 2 M Ω when filled with 3 M KCl. Peptides were obtained from Bachem Bioscience (Philadelphia, PA).

Molecular Biology. CRLR was modified to provide a consensus Kozak sequence as described (Aiyar et al., 1996). The Haemagglutinin (HA) epitope tag was fused in frame to the 5' end of cDNA encoding CRLR. The native signal sequence was removed and replaced with that for T8. HA-CRLR was cloned into pCIN6 (a pcDNA3-based vector that contains an internal ribosomal entry site element for the construction of stable cell lines). HA-CRLR behaved in the same way as "native" CRLR in the assays described (data not shown).

Cell Culture and cDNA Transfection. HEK293T cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine and maintained at 37°C and 95% humidity. NG108-15 cells were cultured in DMEM/Ham's F12 (1:1) supplemented with hypoxanthine aminoptevine (1 \times) and 10% FCS. Five micrograms of linearized DNA per plasmid was transfected for each T75-cm² flask containing 80% confluent cells. Cells were left 24 h before plating into 96-well plates and were cultured for a further day. For cAMP determinations, the cells were washed with PBS and preincubated in DMEM containing 300 μM 3-isobutyl-1-methylxanthine (Sigma) for 30 min at 37°C. CGRP or ADM was added for a further 30 mins and the cells washed with ice-cold PBS. cAMP levels were determined using scintillation proximity-based assays (Amersham, Arlington Heights, IL).

Radioligand Binding, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Western Blotting. HEK293T cells were harvested into PBS 48 h following transfection. The cells were pelleted by centrifugation and homogenized in 50 mM HEPES-KOH, pH 7.6 (containing 15 $\mu\text{g/ml}$ aprotinin, 0.25 $\mu\text{g/ml}$ antipain, 0.25 $\mu\text{g/ml}$ leupeptin, 0.1 mg/ml benzamide, and 0.1 mg/ml bacitracin). After centrifugation at 500g for 20 min at 4°C, the supernatant was removed and centrifuged at 48,000g for 30 min at 4°C. The final pellet was resuspended in homogenization buffer and the protein content measured. SK-N-MC cells were harvested into PBS and subjected to the same membrane preparation in 50 mM HEPES-KOH, 1 mM EDTA, 100 μM leupeptin, and 25 $\mu\text{g/ml}$ bacitracin, pH 7.6. Immediately before the first homogenization, 1 mM phenylmethylsulfonyl fluoride and 2 μM pepstatin A were added. The final pellet was resuspended without phenylmethylsulfonyl fluoride or pepstatin A.

For the CGRP receptor assay, membranes (50 μg) were incubated for 90 min at 25°C in binding buffer (50 mM HEPES-KOH, 10 mM MgCl₂, and 1 mM EDTA, pH 7.4), containing 30 pM [¹²⁵I]-CGRP1 (Amersham) in a total volume of 200 μl . For the ADM receptor assay membranes (10–20 μg) were incubated for 30 min at 4°C in the same buffer with 100 pM [¹²⁵I]-rat ADM (Amersham) in 200 μl . Incubation for both assays was terminated by rapid filtration through GF/C filters soaked in 0.1% polyethylenimine using a Tomtek cell harvester (Tomtek, Orange, CT). Nonspecific binding was determined using a final concentration of either 1 μM CGRP or 1 μM ADM_{13–52}.

For other experiments, plasma membrane-containing P2 particulate fractions were prepared from transfected cell pastes that had been stored at –80°C following harvest. Membrane protein (75 μg)

was subjected to SDS-PAGE on either 10% gels or precast, 4 to 12%, gradient gels (NOVEX, Encinitas, CA). The epitope tags were visualized by immunoblotting with anti-myc or HA monoclonal antibodies and developed using enhanced chemiluminescence (Amersham). Deglycosylation was carried out exactly according to the supplier's protocols (Boehringer Mannheim, Indianapolis, IN). Samples were heated to 60°C, but not boiled, before analysis.

Fluorescence-Activated Cell-Sorting Analysis (FACS) Analysis. HEK293T cells were transiently transfected with cDNA as described above, were harvested 2 days later, and washed three times in PBS. The cells were resuspended in DMEM and incubated with the primary antibody, 9E10 (anti-C-myc) diluted 1:30 for 15 min. Following three further washes, the secondary antibody (sheep anti-mouse Fab₂ coupled with FITC) diluted 1:30 was incubated for 30 min in the dark. For permeabilized cells the Fix and Perm kit (Caltag Labs., South San Francisco, CA) was used. FACS sorting was performed on an EPICS Elite (Coulter Corp., Hialeah, FL); 10,000 cells were sorted in each experiment. Sham-transfected cells did not show any significant fluorescence (data not shown).

Results

We have previously reported that RAMPs 2 and 3 interact with CRLR to produce an ADM receptor when coexpressed in *Xenopus* oocytes (McLatchie et al., 1998). In the present study, detailed pharmacological characterization of the RAMP2/CRLR and RAMP3/CRLR receptors was carried out following transient expression in HEK293T cells. FACS analysis of cells coexpressing RAMPs 1, 2, or 3 with epitope-tagged CRLR demonstrated that the three RAMP proteins transport CRLR to the cell surface with a similar efficiency (Fig. 1). Thus, in cells expressing myc-CRLR but no RAMP, myc epitope appeared at the cell surface in only 2% of cells, whereas in cells coexpressing RAMP1, 2, or 3, approximately 25% of the cells expressed myc-CRLR at the cell surface (transfection efficiency for myc-CRLR was about 70%, as judged by FITC fluorescence of permeabilized cells).

Analyses of radioligand binding and cAMP accumulation were carried following transient transfection of RAMPs 2 or 3 with CRLR in HEK293T cells.

In nontransfected cells, or cells expressing CRLR alone, no [¹²⁵I]-rat ADM binding was detected. However, expression of RAMPs 2 or 3 with CRLR conferred specific, high-affinity [¹²⁵I]-rat ADM binding.

Displacement curves were constructed for this binding using a range of ADM and CGRP peptides. In each case, the IC₅₀ values obtained were similar for the receptors produced

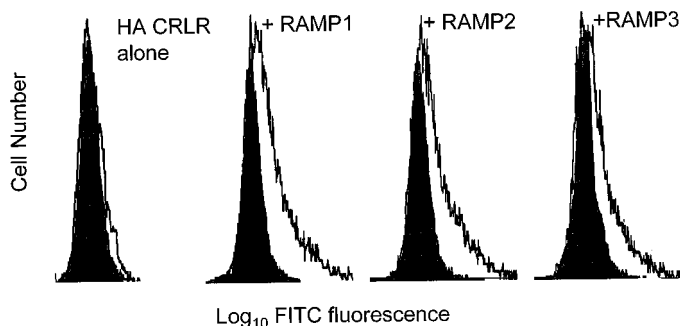


Fig. 1. RAMPs 1, 2, and 3 transport epitope-tagged CRLR to the cell surface with similar efficiencies. FACS analysis of HEK293T cells transiently expressing myc epitope-tagged CRLR with or without RAMPs 1, 2, or 3. Number of cells sorted as showing cell surface myc-CRLR increased from approximately 3 to 25% irrespective of which RAMP is coexpressed.

by RAMP2/CRLR or RAMP3/CRLR, with a rank order of binding affinity of $ADM_{1-52} > ADM_{13-52} > ADM_{22-52} > CGRP_{8-37} > CGRP2 > CGRP1$ (Table 1). Similar values were obtained using membranes from the rat/mouse hybrid NG108-15 cell line, which has been used extensively as a native source of ADM receptors (Zimmermann et al., 1996; Table 1), suggesting that the RAMPs 2- or 3-trafficked CRLR forms an ADM receptor.

Intracellular cAMP accumulation was measured in the same batch of cells using a range of CGRP and ADM receptor agonists. In cells expressing RAMP2/CRLR or RAMP3/CRLR (but not cells expressing CRLR alone), a dose-dependent increase in intracellular cAMP was recorded following incubation with ADM_{1-52} , ADM_{13-52} , CGRP1, or CGRP2. Once again, there was no significant difference in the responses of cells transfected with RAMP2/CRLR (Fig. 2, top) and those transfected with RAMP3/CRLR (Fig. 2, middle), with a rank order of agonist potency of $ADM_{1-52} = ADM_{13-52} > CGRP2 > CGRP1$ (ED_{50} values for these experiments shown in Table 2). These data contrast with the rank order of potency of $CGRP1 = CGRP2 > ADM_{1-52} > ADM_{13-52}$ (Fig. 2, lower panel, and Table 2) recorded in cells coexpressing RAMP1 and CRLR, which show a CGRP receptor pharmacology. Interestingly, in cells expressing RAMP2/CRLR and RAMP3/CRLR, CGRPs 1 and 2 produced a greater increase in cAMP than the ADM peptides.

Despite the relatively low level of homology between RAMPs 2 and 3, both proteins produce an ADM receptor when coexpressed with CRLR in *Xenopus* oocytes or in HEK293T cells. To further examine the RAMPs 2 and 3 processed CRLR, the glycosylation status of HA-epitope tagged CRLR was assessed by pretreatment with endoglycosidase F (EndoF) or H (EndoH), followed by SDS-PAGE and immunoblotting (McLatchie et al., 1998). As shown in Fig. 3, RAMP 2 and 3-trafficked CRLR showed an identical profile of endoglycosidase sensitivity, with both EndoF and EndoH reducing CRLR in size from a single immunoreactive band at 56 kDa to a band at 48 kDa. These data suggest that the glycosylation status of CRLR may be a key determinant of ligand specificity, with core glycosylation of CRLR (RAMPs 2 and 3) forming an ADM receptor, and terminal glycosylation (RAMP1) switching the ligand specificity to that of a CGRP receptor. To examine the regions of the RAMP protein involved in the interaction and glycosylation of CRLR, two chimeras were constructed between RAMPs 1 and 2. The amino terminal domain of RAMP1 was replaced with that of RAMP2 to produce a RAMP2/1 chimera, and conversely the RAMP1/2 chimera contained the amino terminus of RAMP1

and the transmembrane domain and carboxy-terminus of RAMP2. The junction of the chimera was a point of common sequence at the "extracellular" face of the transmembrane domain corresponding to an aspartic acid residue and two prolines (DPP in single-letter amino acid notation).

Initial characterization of the chimeric RAMP proteins was

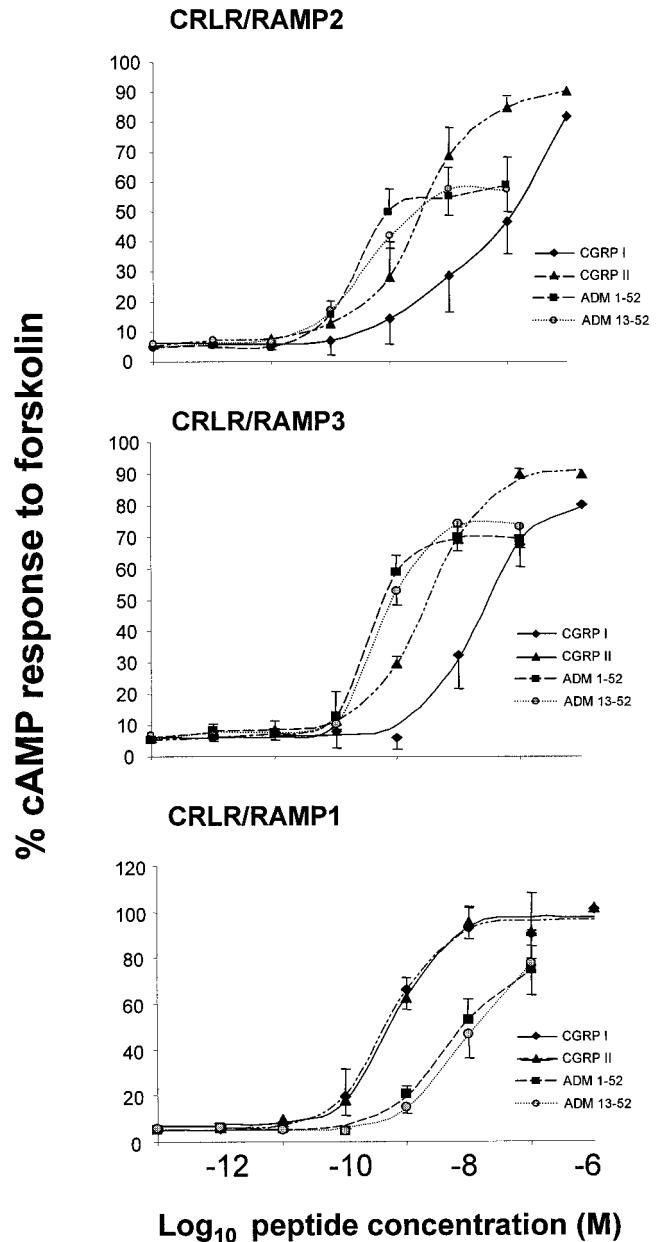


Fig. 2. Functional responses of cells transiently transfected with CRLR and RAMPs 1, 2, or 3 to CGRP and ADM peptides. HEK293T cells were cotransfected with CRLR and RAMPs 1, 2, or 3. Forty-eight hours later cells were incubated with CGRP1 (◆), CGRP2 (▲), ADM_{1-52} (■), and ADM_{13-52} (●) was added for a further 30 mins and intracellular cAMP measured and represented relative to cAMP response to forskolin. RAMP2/CRLR and RAMP3/CRLR transfected cells respond to ADM peptides more than CGRP. These cells discriminate between CGRP2 and CGRP1. RAMP1/CRLR receptor responds to CGRP peptides more than ADM and does not discriminate between CGRP1 and CGRP2. Maximum response to CGRP was greater than that to ADM ($p < .01$ for RAMP2/CRLR and RAMP3/CRLR). Figures are from a single experiment typical of three others. Values represent means \pm S.E.M. of quadruplicate determinations. ED_{50} values (means \pm S.E.M.) for intracellular cAMP responses to these peptides over three experiments are given in Table 2.

TABLE 1
Displacement curves for binding using a range of ADM and CGRP peptides

Peptide	^{125}I -Rat ADM binding/ IC_{50}		
	NG108-15	CRLR/RAMP2	CRLR/RAMP3
		nM	
CGRP1	606 \pm 196	546 \pm 136	311 \pm 105
CGRP2	68.3 \pm 13.4	168.3 \pm 36.9	91.7 \pm 9.3
CGRP ₈₋₃₇	73.4 \pm 13.3	67.1 \pm 7.6	56.3 \pm 12.1
ADM_{1-52}	0.2 \pm 0.06	0.8 \pm 0.04	4.6 \pm 1.05
ADM_{13-52}	12.0 \pm 1.4	5.5 \pm 2.4	40.7 \pm 14.7
ADM_{22-52}	46.8 \pm 8.4	20.5 \pm 1.2	72.2 \pm 10.6
Amylin	NS	NS	NS
Calcitonin	NS	NS	NS

carried out following expression in *Xenopus* oocytes. We have previously reported that RAMP1 will potentiate the endogenous oocyte "CRLR equivalent" to form a functional, type I CGRP receptor, which will respond to CGRP but not to ADM (McLatchie et al., 1998). In contrast, expression of RAMP2 in oocytes does not induce a response to either ADM or CGRP. It may be that RAMP2 is unable to interact with the *Xenopus* homolog of CRLR. Alternatively, the *Xenopus* receptor coexpressed with RAMP2 may not recognize human ADM. Whatever the mechanism, this lack of activity is shared by the RAMP2/1 chimera (amino terminus of RAMP2), which had no significant activity with the endogenous oocyte CGRP receptor (Fig. 4). In contrast, expression of the RAMP1/2 chimera induced a large CGRP response in oocytes, which was approximately 40% of the response seen with RAMP1 (Fig. 4).

These data suggest that the amino terminus of RAMP1 may be critical in determining the activity and ligand specificity of CRLR. To confirm this hypothesis, several experiments were carried out coexpressing RAMP chimeras with CRLR. Expression of RAMP1/2 with CRLR produced responses that were similar to those seen with RAMP1 alone or with RAMP1/2 alone: large responses to CGRP1 but no responses to ADM (Fig. 5). Conversely, coexpression of RAMP2/1 with CRLR produced responses that were similar to those seen with RAMP2 alone (McLatchie et al., 1998): responses to both ADM and, to a lesser extent, CGRP1 (although the magnitude of these responses was only about 10% of those observed with RAMP2 alone).

The two chimeric RAMP proteins were also examined in a mammalian background following coexpression with HA-CRLR in HEK293T cells. In agreement with the oocyte data, RAMP activity followed the amino terminus of the chimera, with RAMP1/2 and RAMP2/1 generating high-affinity ^{125}I -CGRP1 and ^{125}I -ADM specific binding, respectively. However, two inconsistencies were observed between the oocyte and mammalian data: 1) RAMP1 and RAMP1/2-trafficked CRLR showed ^{125}I -CGRP1 and ^{125}I -ADM binding sites (Fig. 6) even though the expression of the same constructs in oocytes produces no ADM responses. 2) RAMP2 and RAMP2/1-trafficked CRLR showed ^{125}I ADM but not ^{125}I -CGRP1 binding sites (Fig. 6), whereas oocytes expressing these constructs respond to CGRP and ADM.

HEK293T cell membranes from the above experiments were pretreated with EndoF or EndoH, subjected to SDS-PAGE, and immunoblotted to assess the glycosylation status of CRLR. HA-epitope tagged CRLR expressed alone, with RAMP2 or with RAMP2/1 ran as a 56-kDa protein that was digested to a 48-kDa form by EndoF (not shown) and EndoH (Fig. 6, bottom). HA-CRLR expressed with RAMP1 or with RAMP1/2 ran predominantly as a 66-kDa protein that was digested to a 48k-Da form by EndoF (not shown) but not by EndoH (Fig. 6, bottom).

An interesting observation from the HEK293T cell binding experiments was that RAMP1/2-trafficked CRLR showed an increased level of ^{125}I -ADM binding relative to RAMP1. To further examine the anomalous pharmacology of the RAMP1/2-trafficked CRLR, a dose-response experiment was carried out titrating increasing amounts of transfected RAMP1/2 cDNA against a fixed amount of cDNA encoding CRLR. At each concentration of RAMP1/2 cDNA, we measured ^{125}I -ADM and ^{125}I -CGRP1-specific binding as well as analyzing the molecular form of CRLR after EndoH treatment (Fig. 7). Even at low RAMP1/2 cDNA concentrations, the ratio of ADM-specific binding to CGRP-specific binding was greater than that observed with RAMP1, suggesting that differences in pharmacology did not result from the saturation of intracellular glycosylation pathways at high RAMP1/2 concentrations. Use of a gradient gel system provided better resolution of the molecular forms of CRLR following EndoH treatment. As observed previously (McLatchie et al. 1998 and Figs. 3 and 6 in this study), CRLR appeared to lose immunoreactivity following cotransfection with RAMP1, with only small amount of immunoreactive material visible at about 66 kDa. In contrast, the RAMP1/2 chimera generated a different profile, with a strong immunoreactive band at 48 kDa, and three higher molecular mass bands at around 66kDa. Although transfection of increasing amounts of RAMP1/2 led to an increase in the amount of immunoreactive material at 66 kDa, the gel profile never resembled that seen in RAMP1.

Discussion

We recently identified a family of three RAMP proteins (RAMPs 1, 2, and 3) that are required to traffic CRLR to the cell surface as a functional CGRP or ADM receptor. In this study, for the first time, we examined the expression and activity of RAMP3 in a mammalian cell line. In our experiments, RAMPs 2 and 3 appear to be functionally identical, with both proteins trafficking CRLR to the cell surface as an immature, core glycosylated protein. Pharmacological evaluation, using radioligand binding and cAMP assay, revealed that RAMP3-trafficked CRLR forms an ADM receptor that is indistinguishable from that seen following coexpression of RAMP2 and CRLR.

The pharmacology of the ADM receptor created by the coexpression of RAMPs 2 or 3 with CRLR is interesting in itself. CGRPs 1 and 2 differ by only 3 amino acids out of 37 and have previously been regarded as functionally identical (Muff et al., 1995). However, in our experiments, CGRP2 had a higher affinity (lower IC_{50}) in displacement binding assays and gave a lower ED_{50} in cAMP assays, suggesting that CGRP2 is more potent than CGRP1 at the RAMP2/CRLR and RAMP3/CRLR receptors. We have also observed that CGRPs 1 and 2 are more effective (in terms of maximal

TABLE 2
Intracellular cAMP accumulation using a range of CGRP and ADM receptor agonists

Transfection	Peptide ED_{50}			
	CGRP I	CGRP II	ADM ₁₋₅₂	ADM ₁₃₋₅₂
	<i>nM</i>			
CRLR/RAMP1	6.6 ± 2.7	7.8 ± 3.6	53.5 ± 7.9	87.4 ± 8.8
CRLR/RAMP2	194.5 ± 61.1	21.2 ± 2.3	4.7 ± 0.5	2.7 ± 0.1
CRLR/RAMP3	230.7 ± 50.2	35.3 ± 13.0	4.1 ± 0.8	6.2 ± 0.6

response) at increasing intracellular cAMP than the ADM peptides. This may relate to the kinetics of the responses to the two peptides, to their relative stability, or to their absolute efficacy at the RAMP/CRLR receptor.

In this study, we also examined the structural determinants of RAMP activity. RAMPs are type I membrane proteins with a relatively long extracellular amino terminus, a single membrane-spanning domain, and a short intracellular C-terminus. Several lines of evidence suggest that the extracellular N-terminus of RAMP1 plays an important role in determining ligand specificity. First, cross-linking data using ^{125}I -CGRP1 suggests that RAMP1 and CRLR are closely associated at the cell membrane (McLatchie et al., 1998). Second, the RAMP1/2 chimera (which contains the amino terminus of RAMP1) traffics CRLR as a CGRP receptor following expression in HEK293T cells. Finally, no ^{125}I -CGRP binding was detected in cells coexpressing the reverse chimera, RAMP2/1, with CRLR suggesting that the transmembrane domain and C-terminus of RAMP1 does not contribute to CGRP binding. Taken together these data suggest that RAMP1 could associate with the amino terminus of CRLR to form a binding pocket for CGRP. It is notable that other members of the family B class of G protein-coupled recep-

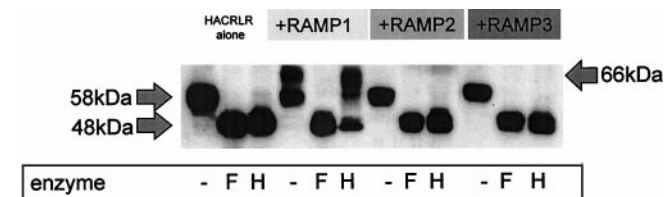


Fig. 3. The 56-kDa CRLR species (produced without RAMPs or with RAMPs 2 or 3) is a glycoprotein that reduced with either EndoF or EndoH to a 48-kDa band. The 66-kDa species produced after RAMP1 coexpression reduced to a 48-kDa band after EndoF treatment but it was resistant to EndoH, suggesting that it is a mature glycoprotein. Deglycosylation was carried out exactly according to the supplier's protocols (Boehringer Mannheim) before SDS-PAGE/immunoblotting. Similar results were obtained whether CRLR was tagged with the HA epitope (shown here) or that for myc.

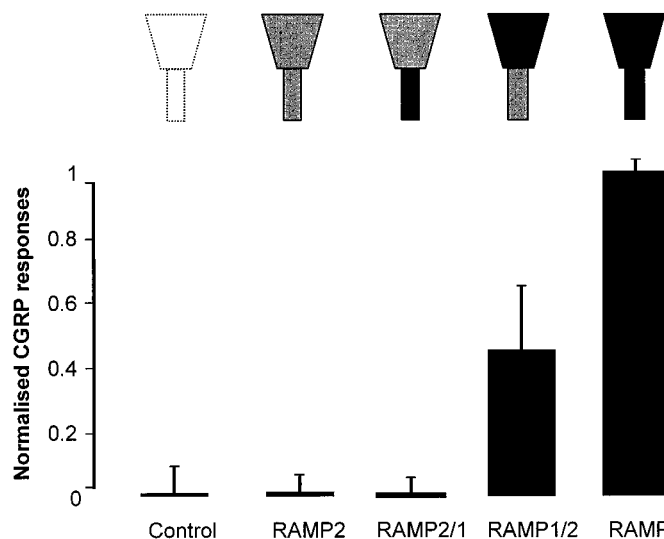


Fig. 4. CGRP responses by *Xenopus* oocytes injected with control cRNA and cRNA encoding RAMPs 2 and 1 and chimeric proteins RAMP2/1 and RAMP1/2. CGRP (100 nM) was used in each experiment and was maintained until a maximum response was reached. At least 15 individual oocytes from three different animals were used per determination. Results are presented normalized against the CGRP responses in RAMP1-expressing oocytes and as means \pm S.E.M.

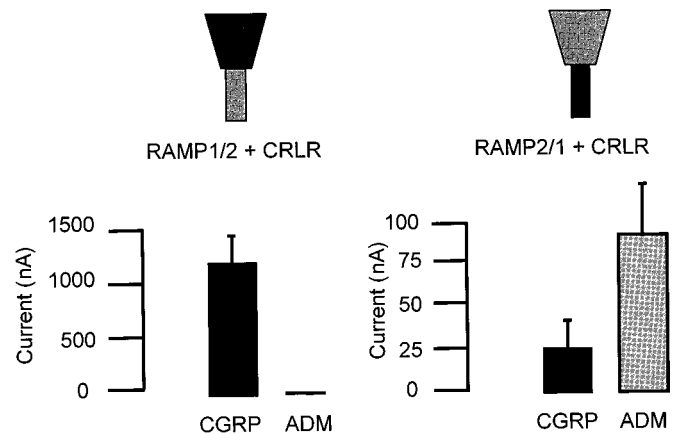


Fig. 5. CGRP and ADM responses by *Xenopus* oocytes injected with cRNA encoding chimeric proteins RAMP2/1 and RAMP1/2. CGRP1 (100 nM) and 10 nM ADM₁₋₅₂ were administered until a maximum response was recorded. At least 15 individual oocytes from three different animals were used per determination. Results are presented as size of elicited current to avoid obscuring different efficiencies of two chimeras in this system and as means \pm S.E.M.

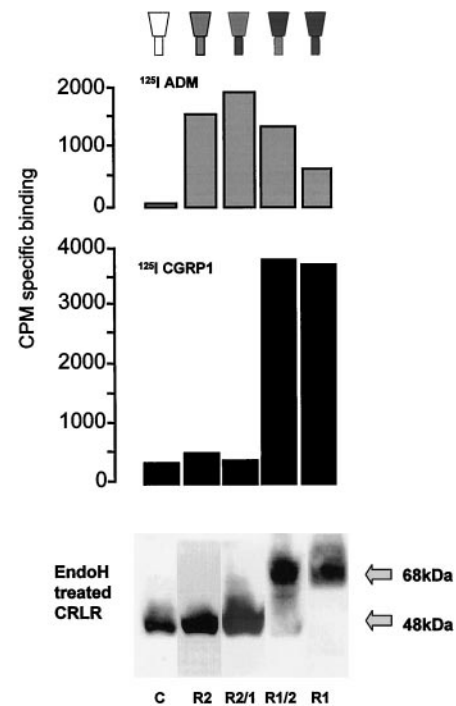


Fig. 6. ^{125}I -rat ADM- and ^{125}I -CGRP1-specific binding to HEK2993T cells cotransfected with cDNA encoding CRLR alone and in combination with RAMPs 1 or 2 or the chimeric proteins RAMP2/1 and RAMP1/2. ^{125}I -rat ADM (100 pM) or 30 pM ^{125}I -CGRP1 were used in radioligand binding studies. K_d values for ability of human ADM and CGRP receptors to bind their corresponding radiolabeled peptides have been reported as 200 pM and 0.05 pM, respectively (Sone et al. 1997; Stangl et al. 1991). Therefore results tend to underestimate CGRP binding relative to that for ADM. Plasma membrane from same cells were subject to SDS-PAGE and Western blot with anti-HA antiserum with or without treatment with EndoF (not shown) or EndoH. When expressed alone, with RAMP2 or RAMP2/1, HA-CRLR migrated as a 58-kDa protein (not shown) that was digested to 48-kDa by EndoF (not shown) or EndoH. When expressed with RAMP1, HA-CRLR migrated predominantly as a 66-kDa protein that was sensitive to EndoF (not shown) but not EndoH. When coexpressed with RAMP1/2, some HA-CRLR was sensitive to EndoH treatment. Results are from a single experiment performed in triplicate and are typical of two other experiments.

tors, such as the glucagon and parathyroid hormone receptors, are thought to bind ligand predominantly via their amino termini (Unson et al., 1995; Zhou et al., 1997). Alternatively, the N-terminus of RAMP1 may affect ligand binding indirectly through a change in the glycosylation or conformational state of CRLR.

RAMPs 2 and 3 traffic CRLR to the cell membrane with a similar efficiency to RAMP1, raising the possibility that they can contribute to the ligand binding of CRLR in a similar manner. However, such a hypothesis is difficult to reconcile with the fact that the N-termini of RAMPs 2 and 3 have only 27 out of 110 amino acids in common, 15 of which are shared by RAMP1. It is possible that the secondary and tertiary structure of RAMPs 2 and 3 are more similar than their primary amino acid sequence suggests, or alternatively, that a relatively small portion of RAMP2/RAMP3 may define their ability to generate an ADM receptor when coexpressed with CRLR.

The ligand specificity of CRLR could also be determined by differences in the level of glycosylation because RAMP1, but not RAMPs 2 or 3, traffics CRLR as a terminally glycosylated receptor. Thus, the ability of CRLR to bind and respond to CGRP could be conferred by terminal glycosylation. For several receptors, glycosylation state appears to have no effect upon function: the calcitonin (Quiza et al., 1997), leuteinizing hormone (Davis et al., 1997), parathyroid hormone (Bisello et al., 1996), and vasopressin V2 (Sadeghi et al., 1997) receptors are examples. However, some other receptors such as the calcium (Fan et al., 1997; Ray et al., 1997) and nicotinic acetylcholine receptors (Buller and White, 1990) are not expressed if glycosylation is inhibited. These data demonstrate that transit through the Golgi apparatus is essential for correct receptor processing;

whether glycosylation contributes directly to the pharmacology of the receptors is less certain (White et al., 1998). The importance of N-linked glycosylation to the functioning of CRLR as a CGRP or ADM receptor will be tested experimentally by the sequential mutation of its three putative N-linked glycosylation sites.

Despite our data demonstrating the biological significance of the amino terminus of RAMPs, other regions of the RAMP protein may also be involved in determining ligand specificity. This is suggested by experiments with the RAMP1/2 chimera (amino terminus of RAMP1) in which a significantly higher level of ^{125}I -ADM binding was seen than with the native RAMP1. Further structure-function studies will serve to elucidate the contribution of the RAMP protein to ligand binding.

These studies have demonstrated that the pharmacology of CRLR is the same whether it is expressed with RAMP2 or RAMP3. They have also suggested the importance of the amino terminal portions of RAMPs in determining their biological activity and provided the basis for a more complete molecular analysis of their mode of action.

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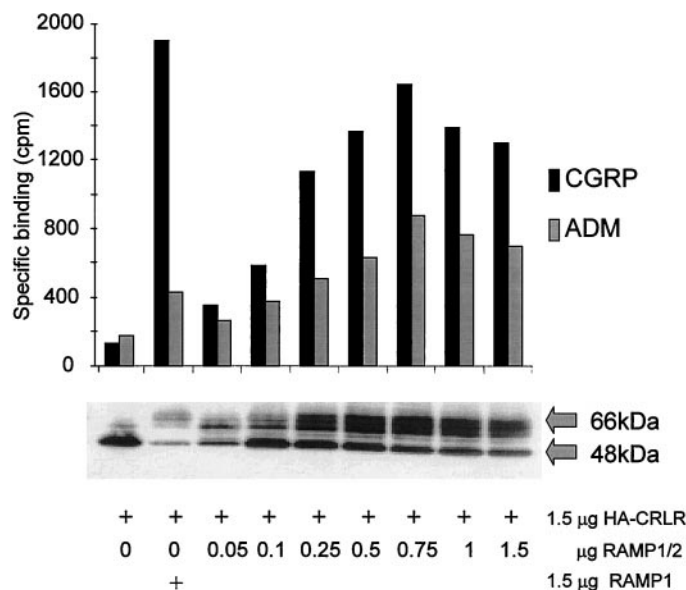


Fig. 7. HEK293T cells were transiently transfected with 1.5 μg of HA-CRLR and cotransfected with either vector control, RAMP1, or increasing amounts of RAMP1/2. The cells were analyzed for their expression of ^{125}I -rat ADM- and ^{125}I -CGRP1-specific binding and for their ability to generate EndoH-resistant CRLR as described in Fig. 6. Ratio of CGRP to ADM binding was approximately 4:1 when RAMP1 was coexpressed with CRLR, whereas ratio was no more than 2:1 with RAMP1/2 chimera irrespective of amount of cDNA transfected.

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