# The Transmembrane Domain of Receptor-Activity-Modifying Protein 1 Is Essential for the Functional Expression of a Calcitonin Gene-Related Peptide Receptor†

Sarah Steiner, Roman Muff, Remo Gujer, Jan A. Fischer, and Walter Born*

*Research Laboratory for Calcium Metabolism, Departments of Orthopedic Surgery and Medicine, University of Zurich, Klinik Balgrist, 8008 Zurich, Switzerland*

*Received April 15, 2002; Revised Manuscript Received July 11, 2002*

ABSTRACT: Three receptor-activity-modifying proteins (RAMP) define specific interactions between calcitonin (CT) gene-related peptide (CGRP), adrenomedullin (AM) and amylin, and a CT receptor or a CT receptor-like receptor (CRLR). Both form heterodimeric RAMP/receptor complexes at the cell surface. This association represents a novel principle of G protein-coupled receptor function. RAMP1 is transported to the cell surface together with the CRLR or the CT receptor. Here, we have investigated the functional relevance of the short C-terminal intracellular tail QSKRTEGIV and of the single transmembrane domain of human (h) RAMP1 for their interactions with the hCRLR to constitute a CGRP receptor. To this end, hRAMP1 has been sequentially truncated from the C-terminus, and [$^{125}$I]h$\alpha$CGRP/hRAMP1/hCRLR association at the cell surface and cAMP accumulation in response to h$\alpha$CGRP have been examined. With the C-terminal truncation of hRAMP1 by four amino acids wild-type hRAMP1 function was maintained, and the hCRLR was required for the transport of hRAMP1 to the cell surface. Further truncation of hRAMP1 through removal of the remaining five intracellular amino acids revealed CRLR-independent cell surface delivery but otherwise normal hRAMP1 activity. Sequential shortening of the hRAMP1 transmembrane domain resulted in progressively impaired association with the hCRLR and, as a consequence, abolished CGRP receptor function. In conclusion, the intracellular QSKRT sequence adjacent to the transmembrane domain of hRAMP1 provides a signal for intracellular retention. The sequence is unrelated to consensus endoplasmic reticulum retention/retrieval motives and overridden by the presence of the hCRLR. The entire single transmembrane domain of hRAMP1 together with one hydrophilic amino acid residue at its C-terminus is required for the formation of a fully functional CGRP/hRAMP1/hCRLR receptor complex.

Receptor-activity-modifying proteins (RAMP)[1] 1, -2, and -3 are 14−17 kDa single-transmembrane domain polypeptides. They are essential for the functional expression of initially orphan calcitonin (CT) receptor-like receptors (CRLR) of the family B of G protein-coupled receptors (*1*). Interdependence of the RAMP and the CRLR for their efficient delivery to the cell surface has been described. Importantly, heterodimeric RAMP/receptor complexes at the plasma membrane define the specificity of the CRLR and of the 60% homologous CT receptor (CTR) for CT gene-related peptide (CGRP), adrenomedullin (AM), and amylin (*2*−*5*). N-Glycosylation of the CRLR was ruled out as a mechanism explaining CGRP or AM specificity (*4*, *6*). Heterodimeric RAMP1/CRLR and RAMP1/CTR complexes represent CGRP receptor isotypes. Defined AM and amylin receptors consist of RAMP2/CRLR and RAMP3/CTR complexes, respectively. The peptide ligands of these receptors, $\alpha$- and $\beta$CGRP(1−37), AM(1−52), amylin(1−37), and CT-(1−32), are structurally related, and they exhibit overlapping biological actions (*7*). Along these lines, AM and the neuropeptide CGRP are potent vasodilators.

RAMP1, -2, and -3 have been identified in man, rat, and mouse. The individual RAMP of the three species are over 60% similar. All the RAMP have a common topology. They consist of an N-terminal signal sequence and an extracellular domain of approximately 100 amino acids and a short intracellular region beyond the single transmembrane domain. But the three RAMP within the same species exhibit less than 30% amino acid sequence similarity. RAMP2 and -3, unlike RAMP1, are N-glycosylated (*4*, *8*).

The mechanisms of the interaction between the CRLR and RAMP1, -2, or -3 remain largely to be investigated. CRLR-independent transport of the N-glycosylated RAMP2 and -3 to the cell surface has recently been described (*9*). The nonglycosylated RAMP1 requires coexpression of the CRLR to reach the plasma membrane. N-Terminal extracellular domains of chimeric human (h) RAMP are important for CGRP or AM specificity of the coexpressed CRLR (*10*). To this end, the seven extracellular amino acids 86−92 in hRAMP2 and the corresponding 59−65 amino acids in hRAMP3 are essential for AM binding to hRAMP2- or hRAMP3/hCRLR complexes (*11*). The transmembrane do-

† This study was supported by the Swiss National Science Foundation, the Kanton of Zurich, and the Schweizerischer Verein Balgrist.

* To whom correspondence should be addressed at the Research Laboratory for Calcium Metabolism, Klinik Balgrist, Forchstrasse 340, 8008 Zurich, Switzerland. Telephone: +41-1-386-1665. Fax: +41-1-386-1652. E-mail: wborn@balgrist.unizh.ch.

[1] Abbreviations: AM, adrenomedullin; CT, calcitonin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; CTR, CT receptor; RAMP, receptor-activity-modifying protein.

10.1021/bi020279r CCC: $22.00 
Published on Web 08/30/2002

mains and/or the C-terminus of the RAMP are thought to determine the level of expression of the human CTR isotype 2 as an amylin/CGRP receptor (*12*).

Here we have studied, through sequential C-terminal truncation of hRAMP1, the functional roles of the intracellular C-terminal tail and the single transmembrane domain. The individual mutated hRAMP1 were coexpressed with the hCRLR, and the effects of the mutations on [$^{125}$I]hαCGRP/hRAMP1/hCRLR cell surface complex formation and CGRP receptor activity were investigated. The results indicate that truncation of the intracellular C-terminal region of hRAMP1 by 9 amino acids maintains its association with the hCRLR to a functional CGRP receptor complex. The five amino acids next to the transmembrane domain function as a signal for intracellular retention in the absence of the hCRLR. Further truncation of the transmembrane domain progressively impaired complex formation and, as a result, abolished CGRP receptor function. hRAMP1 lacking the C-terminus and the predicted transmembrane domain was no longer associated with the hCRLR and retained intracellularly.

## MATERIALS AND METHODS

*Materials*. Human αCGRP(1−37) was purchased from Bachem. The membrane-impermeable cross-linker bis(sulfosuccinimidyl) suberate (BS3) and ImmunoPure immobilized protein G were obtained from Pierce. *N*-Glycosidase F was from Roche Diagnostics, and restriction enzymes were from Promega. ECL Western blot detection reagents and Hybond ECL nitrocellulose membranes were purchased from Amersham Pharmacia Biotech. Tissue culture media and fetal calf serum (FCS) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Geneticin 418, LipofectAMINE or LipofectAMINE 2000, and OptiMEM medium were supplied by Invitrogen. Other chemicals and reagents were purchased from Sigma and Merck at the highest grade available.

*Construction of Plasmids and of DNA Encoding hRAMP1 with Sequential C-Terminal Truncations and the hCRLR with an N-Terminal V5 Epitope Tag*. The cDNA encoding hRAMP1 with an N-terminal EQKLISEEDLL (myc) epitope (myc-hRAMP1) in the mammalian expression vector pcDNA3 (Invitrogen) was provided by S. Foord (GlaxoSmithKline). DNA fragments encoding myc-hRAMP1 with C-terminal truncations (Figure 1) were amplified by PCR from myc-hRAMP1 encoding cDNA, using cloned *Pfu* DNA polymerase (Promega). The 5′-oligonucleotide included a *Not*I restriction site and encoded part of the N-terminus of the CD33 signal sequence (CD33) (*13*) located upstream of the myc tag in myc-RAMP1. The C-terminal truncations were created by using 3′-primers that introduced a translational stop codon and a *Xho*I restriction site at the desired positions. An RSR ER retention/retrieval signal (*14*) in myc-hRAMP1Δ10RSR was also introduced with a corresponding 3′-primer. The PCR products obtained after 25 cycles were digested with *Not*I and *Xho*I restriction enzymes, gel purified, and cloned into *Not*I/*Xho*I-digested pcDNA3. The nucleotide sequence of all of the constructs was verified by sequencing in both directions.

The DNA encoding the hCRLR with an N-terminal GKPIPNPLLGLDST (V5) epitope tag was constructed as follows. A *Kpn*I/*Bam*HI DNA fragment encoding the CD33 signal sequence and the V5 epitope was amplified by PCR with cloned *Pfu* DNA polymerase from a construct encoding CD33-V5-rat CRLR (unpublished). The *Kpn*I and *Bam*HI restriction sites were provided by the 5′- and the 3′-oligonucleotide primers, respectively. The PCR product obtained after 25 cycles was gel purified and cloned into the pGEM-T Easy vector (Promega) using an A-tailing procedure. The CD33-V5 encoding DNA fragment flanked by the 5′ *Kpn*I and the 3′ *Bam*HI restriction site was removed with corresponding restriction enzymes. The fragment was used to replace a CD33-myc encoding *Kpn*I/*Bam*HI DNA fragment in a CD33-myc-hCRLR expression construct in pcDNA3 (provided by S. Foord, GlaxoSmithKline).

*Cell Culture and Lipotransfection*. COS-7 cells were cultured in HamF12/DMEM (4.5 g/L glucose) medium (1:1) supplemented with 10% FCS and 2 mM glutamine (tissue culture medium). SV40 T-antigen transformed human embryonic kidney HEK293 (TSA) cells were cultured in the same medium, supplemented with 400 μg/mL Geneticin 418. The cells were subcultured by treatment with 0.1% trypsin and 0.5 mM EDTA in PBS. Cells at 80% confluence were transfected for 4 h at 37 °C in OptiMEM medium containing the indicated concentrations of plasmid DNA and LipofectAMINE or LipofectAMINE 2000. The experiments were carried out 48 h after transfection. Cyclic AMP accumulation in transiently transfected COS-7 cells was measured as described (*15*).

*Cross-Linking, Immunoprecipitation, and Deglycosylation*. Cell surface protein cross-linking was carried out in the presence of [$^{125}$I]hαCGRP ($7.4 \times 10^{13}$ Bq/mmol). hαCGRP was labeled with Na$^{125}$I (Amersham Pharmacia Biotech) with a modified chloramine-T method (*16*). [$^{125}$I]hαCGRP was separated from noniodinated peptide by reversed-phase HPLC (*17*). Transiently transfected TSA cells in 100 mm tissue culture dishes were detached with 0.05% EDTA in PBS and washed with PBS; $2 \times 10^7$ cells were incubated in suspension for 2 h on ice with $10^5$ Bq of [$^{125}$I]hαCGRP in 400 μL of HamF12/DMEM (1:1) containing 0.1% BSA (binding medium). Subsequently, the cells were washed with PBS and incubated for 1 h on ice in 400 μL of 0.1 M PBS containing 1 mM cross-linker BS3. Cross-linking was quenched with 50 mM Tris-HCl (pH 8), and the cells were collected by centrifugation. Cell pellets were lysed in 300 μL of 50 mM Hepes (pH 7.5), 140 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 3 μg/mL aprotinin, and 3 μg/mL leupeptin (lysis buffer). The cell lysates were cleared by centrifugation for 5 min at 20000*g*. V5-tagged receptors were immunoprecipitated at 4 °C from cleared lysates of cross-linked and non-cross-linked samples by sequential incubation with 45 μL of ImmunoPure immobilized protein G for 1 h, 3 μg of V5 antibodies (Invitrogen) for 2 h, and with 45 μL of ImmunoPure immobilized protein G overnight on an end-over-end rotator. The precipitates were then collected by centrifugation for 3 min at 2700*g*. The pellets were washed once with lysis buffer. [$^{125}$I]hαCGRP in the immunoprecipitates of cross-linked samples, reflecting [$^{125}$I]hαCGRP binding, was measured in a γ-counter (Kontron). Deglycosylation of proteins in immunoprecipitates was carried out for 18 h at 37 °C with 2 units of *N*-glycosidase F in 50 μL of lysis buffer adjusted to 10 mM EDTA, 0.1% SDS, 0.5% octyl glucopyranoside, and 1% β-mercaptoethanol. Immunoextracted proteins were

eluted from immobilized protein G by incubation with 50 $\mu$L of SDS−PAGE loading buffer for 5 min at 60 °C. Similarly, N-glycosylated proteins in 50 $\mu$L of total cell lysate, equivalent to $3 \times 10^6$ cells, were deglycosylated with 4 units of *N*-glycosidase F.

*Western Blot Analysis*. Proteins in cell lysates and in immunoextracts were separated by SDS−PAGE and electrotransferred to nitrocellulose Hybond ECL membranes in a Trans-Blot cell (Bio-Rad Laboratories) overnight at 10 V and 4 °C. Immunoblots were blocked with 5% low-fat milk, and the epitope-tagged proteins were visualized on X-ray films (Amersham) by enhanced chemiluminescence with horseradish peroxidase (HRP) labeled monoclonal myc and V5 antibodies (1:2000 or 1:5000 final dilution) (Invitrogen). In cell surface protein cross-linking experiments myc-hRAMP1 was recognized by monoclonal myc antibodies (1:5000 final dilution) provided by T. Trueb (University Hospital of Zurich) and secondary HRP-conjugated sheep antibodies to mouse immunoglobulins (1:5000 final dilution) (Amersham). Actin as a reference protein for the amount of loaded cell lysates was visualized with monoclonal antibodies to actin (1:3500 final dilution) (Chemicon International) and secondary HRP-conjugated sheep antibodies to mouse immunoglobulins (1:5000 final dilution) (Amersham). Cross-linked [$^{125}$I]h$\alpha$CGRP was detected by autoradiography with Hyperfilm MP film (Amersham).

*Immunocytochemistry*. COS-7 cells were grown on cover slides in 24-well plates. At 50% confluence they were transfected for 4 h at 37 °C in 200 $\mu$L of OptiMEM medium/well containing 1 $\mu$L of LipofectAMINE 2000 and 200 ng of the indicated myc-hRAMP1 expression constructs together with 200 ng of pcDNA3 or of the hCRLR expression construct. Cell surface expression of intact and mutant myc-hRAMP1 was estimated 48 h later by myc immunofluorescence staining of intact cells. The cells were incubated for 1 h at room temperature with mouse monoclonal antibodies to myc (Invitrogen) diluted 1:300 in incubation medium [DMEM/HamF12 (1:1), 10% FCS]. The same medium was used to wash the cells twice and for the incubation with Cy3-labeled sheep anti-mouse antibodies (1:200 final dilution) (Sigma) for 30 min at room temperature. Subsequently, the cells were washed twice with incubation medium and once with PBS and then fixed with 4% formalin for 20 min at room temperature. After a final wash with PBS the cells were mounted with Immu-Mount (Shandon Scientific). The cells were then viewed with an Eclipse E600 Nikon microscope equipped with a Plan Fluor 40×/0.75 DLL objective and an excitation filter (510−560 nm), a dichroic mirror (575 nm), and a barrier filter (590 nm). Photomicrographs were taken through an additional 2.5× projection lens with a FDX-35 camera and a 1600 ASA film. Exposure times were measured with an U-III multipoint sensor system in eight randomly chosen fields of confluent cells with no fluorescence artifacts. Reciprocal exposure times were a measure for cell surface expression of intact and mutant myc-hRAMP1. The values obtained for intact myc-hRAMP1 coexpressed with the hCRLR were set to 100%.

*Statistical Analysis*. The values for half-maximal effective concentrations ($EC_{50}$) were calculated by nonlinear regression analysis using Fig. P 6.0 software (Biosoft, Cambridge, U.K.). Differences between mean values of percent [$^{125}$I]h$\alpha$CGRP binding in cells coexpressing the hCRLR with intact (set to 100%) or C-terminally truncated myc-hRAMP1 were analyzed by Student's *t*-test. Results are means ± standard error of the mean (SEM). *P* values of $<0.05$ were considered statistically significant.

```
Myc-hRAMP1   -DPPGSILYPFIVVPITVTLLVTALVVWQSKRTEGIV
-Δ4          -DPPGSILYPFIVVPITVTLLVTALVVWQSKRT
-Δ8          -DPPGSILYPFIVVPITVTLLVTALVVWQ
-Δ9          -DPPGSILYPFIVVPITVTLLVTALVVW
-Δ10         -DPPGSILYPFIVVPITVTLLVTALVV
-Δ10RSR      -DPPGSILYPFIVVPITVTLLVTALVVRSR
-Δ16         -DPPGSILYPFIVVPITVTLL
-Δ29         -DPPGSIL
```

FIGURE 1: Myc-hRAMP1 C-terminal truncation mutants. The amino acid sequence of the C-terminal regions of intact (top line) and mutant myc-hRAMP1 are aligned. Bold letters indicate amino acids in the predicted transmembrane domain of myc-hRAMP1. The gray box indicates the artificially introduced RXR ER retention/retrieval motif.

Table 1: Stimulation of cAMP Formation by CGRP in COS-7 Cells[a]

| | maximal (% myc-hRAMP1) | $EC_{50}$ (nM) |
|---|---|---|
| myc-hRAMP1 | 100[b] | 0.54 ± 0.09 |
| -Δ4 | 93 ± 6 | 0.28 ± 0.02 |
| -Δ8 | 88 ± 8 | 0.70 ± 0.11 |
| -Δ9 | 64 ± 7 | 6.30 ± 2.2 |
| -Δ10 | nd | >1000 |
| -Δ10RSR | 80 ± 7 | 0.35 ± 0.09 |
| -Δ16 | nd | >1000 |
| -Δ29 | ns | ns |

[a] Cells were cotransfected with cDNA encoding intact or C-terminally truncated myc-hRAMP1 and the hCRLR. Results are means ± SEM of three to five independent experiments. [b] Basal cAMP levels were 1.4 ± 0.1 pmol/well, and maximal cAMP accumulation amounted to 40 ± 2 pmol/well; nd, not determinable at up to 1 $\mu$M h$\alpha$CGRP; ns, no cAMP stimulation at up to 1 $\mu$M h$\alpha$CGRP.

## RESULTS

*Effects of C-Terminal Truncations of Myc-hRAMP1 on CGRP Receptor Activity of the hCRLR*. The hCRLR and intact or C-terminally truncated myc-hRAMP1 (Figure 1) were transiently coexpressed in COS-7 cells, and CGRP-evoked cAMP formation was measured. In cells coexpressing the hCRLR and intact myc-hRAMP1 a 30 ± 2-fold ($n$ = 14) stimulation of cAMP production by $10^{-6}$ M CGRP was considered as the maximal response and set to 100% (Table 1). C-Terminal truncation of myc-hRAMP1 by four or eight amino acids did not affect the cAMP response to CGRP to any great extent. Coexpression of the hCRLR with myc-hRAMP1Δ9 decreased the maximal cAMP formation by 36% and increased the $EC_{50}$ 12-fold. Further truncation to myc-hRAMP1Δ10 or to myc-hRAMP1Δ16, removing one or seven amino acids of the transmembrane domain, raised the $EC_{50}$ of CGRP over 1000-fold. Interestingly, the cAMP response was comparable in cells expressing myc-hRAMP1Δ8 or -Δ10RSR together with the hCRLR, indicating that the native sequence WQ could be replaced by an RSR endoplasmic reticulum (ER) retention/retrieval signal while maintaining normal CGRP receptor function of the associated V5-hCRLR. cAMP remained at basal levels at up to 1 $\mu$M CGRP in cells expressing the hCRLR together with myc-hRAMP1Δ29 that lacked the entire predicted transmembrane region except for I and L at its N-terminal end. Myc-

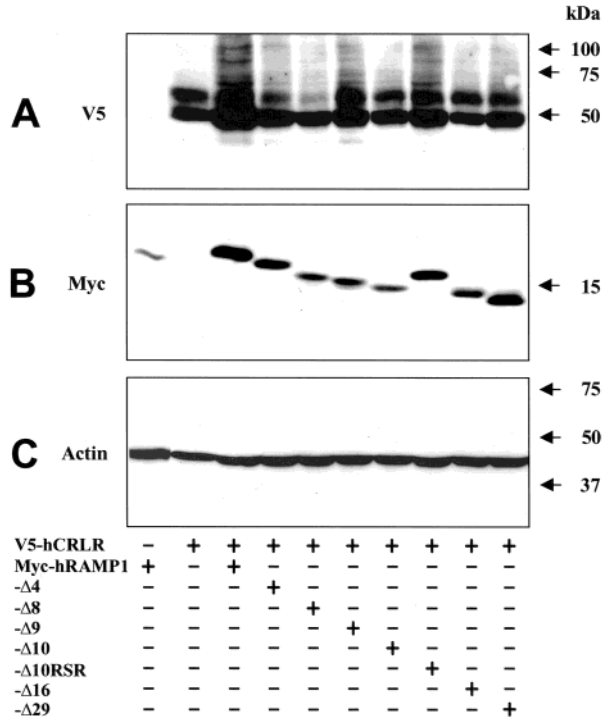


FIGURE 2: Expression levels of V5-hCRLR and of coexpressed intact and mutant myc-hRAMP1. TSA cells were grown in six-well plates to 80% confluency and transfected by incubation for 4 h at 37 °C in 2 mL of OptiMEM medium containing 4.8 $\mu$L of LipofectAMINE and the indicated combinations of 800 ng of intact or mutant myc-hRAMP1 and 800 ng of V5-hCRLR expression constructs per well. The total amounts of DNA in transfections with intact myc-hRAMP1 or V5-hCRLR alone were adjusted with the expression vector pcDNA3 to those of the double transfections. Two days later, aliquots of cell extracts were treated with 4 units of *N*-glycosidase F and subjected to 15% SDS−PAGE. V5-hCRLR (A) and intact and mutant myc-hRAMP1 (B) were visualized on Western blots with HRP-conjugated V5 and myc antibodies. Actin was detected with mouse monoclonal antibodies to actin and secondary HRP-conjugated sheep antibodies to mouse immunoglobulins (C). This representative experiment was carried out three times.

hRAMP1Δ31 lacking the entire transmembrane region but extended at the C-terminus by RSR remained inactive (not shown). Thus, C-terminal truncation of myc-hRAMP1 up to the presumed transmembrane domain, leaving at least one hydrophilic amino acid or an ER trafficking signal at the C-terminus, is tolerated by the hCRLR for normal CGRP receptor function.

*Total and Cell Surface Expression of C-Terminally Truncated Myc-hRAMP1*. Impaired expression and cell surface delivery of myc-hRAMP1Δ10, -Δ16, and -Δ29 were considered as mechanisms for defective CGRP receptor function of the coexpressed hCRLR. Intact and truncated myc-hRAMP1 were transiently expressed in TSA cells in the absence and presence of the hCRLR with an N-terminal V5 epitope tag (V5-hCRLR). Western blot analysis of total cell extracts revealed comparable levels of V5-hCRLR expression in the absence and presence of intact and the different mutant myc-hRAMP1 (Figure 2). The relative expression levels of intact and the different myc-hRAMP1 mutants in the presence of V5-hCRLR varied less than 2-fold.

Cell surface delivery of intact and C-terminally truncated myc-hRAMP1 was studied in the absence and presence of the hCRLR by myc immunofluorescent staining of intact COS-7 cells (Figure 3). Myc-hRAMP1 and -Δ4 required the coexpression of the hCRLR for their delivery to the cell surface. Interestingly, all of the other mutants, except for myc-hRAMP1Δ29, exhibited hCRLR-independent cell surface expression. Myc-hRAMP1Δ29 was not expressed at the cell surface even in the presence of the hCRLR but was readily recognized in saponin-permeabilized cells (not shown). It was also detected in trace amounts in tissue culture medium (not shown). A quantitative analysis of the cell surface delivery of intact and truncated myc-hRAMP1 is summarized in Table 2. Taken together, the results indicate that the cell surface expression of myc-hRAMP1 with 8, 9, 10, and 16 amino acids removed from the C-terminus is no longer hCRLR dependent. Moreover, impaired expression and cell surface delivery of myc-hRAMP1Δ10 and -Δ16 were ruled out as mechanisms for defective CGRP function of the hCRLR. Interestingly, substitution of the intracellular C-terminal tail of myc-hRAMP1 by the ER retention/retrieval signal RSR restored in part intracellular retention in the absence of the hCRLR.

*[$^{125}$I]hαCGRP Binding and Cell Surface Complex Formation of C-Terminally Truncated Myc-hRAMP1 with V5-hCRLR*. TSA cells transiently expressing intact myc-hRAMP1 or the V5-hCRLR alone, or the intact myc-hRAMP1 or the indicated myc-hRAMP1 mutants together with the V5-hCRLR, were incubated with [$^{125}$I]hαCGRP and subsequently treated with the membrane-impermeable cross-linker BS3. Protein components carrying V5 epitope tags were immunoprecipitated from cell homogenates with the corresponding antibodies and deglycosylated with *N*-glycosidase F. The amounts of coprecipitated [$^{125}$I]hαCGRP were measured in a $\gamma$-counter and reflected [$^{125}$I]hαCGRP binding. Immunoextracted proteins were size-separated on SDS−PAGE and further analyzed on Western blots. [$^{125}$I]hαCGRP binding protein components were recognized by autoradiography, and V5 and myc immunoreactive proteins were visualized with the corresponding antibodies. [$^{125}$I]hαCGRP immunopreciptated from cell extracts expressing the myc-hRAMP1 or the V5-hCRLR alone was less than 1% of added ligand and considered to reflect nonspecific binding (Figure 4A). Comparable amounts of [$^{125}$I]hαCGRP immunoextracted from cells expressing intact myc-hRAMP1 or myc-hRAMP1Δ4 or -Δ8 together with the V5-hCRLR reflected maximal binding. Further C-terminal truncation of myc-hRAMP1Δ8 by Q and W (myc-hRAMP1Δ9 and -Δ10) and coexpression with V5-hCRLR progressively decreased [$^{125}$I]hαCGRP binding. The amounts of immunoprecipitated [$^{125}$I]hαCGRP in myc-hRAMP1Δ10/V5-hCRLR coexpressing cells were 37 ± 4% ($P < 0.05$) of those observed in cells coexpressing intact myc-hRAMP1 together with the V5-hCRLR. [$^{125}$I]hαCGRP binding was fully restored when myc-hRAMP1Δ10 was extended at the C-terminus by the ER retention/retrieval sequence RSR. [$^{125}$I]hαCGRP binding of myc-hRAMP1Δ10 in the presence of V5-hCRLR was associated with stimulation of cAMP at over $10^{-8}$ M CGRP (Table 1). Thus, in the absence of the C-terminal intracellular region of hRAMP1 [$^{125}$I]hαCGRP binding was largely maintained, but $G_s$ coupling was reduced. [$^{125}$I]hαCGRP binding and cAMP stimulation by CGRP were both abolished when the myc-hRAMP1Δ16 or -Δ29, lacking one-third or most of the transmembrane domain, was coexpressed with the V5-hCRLR.

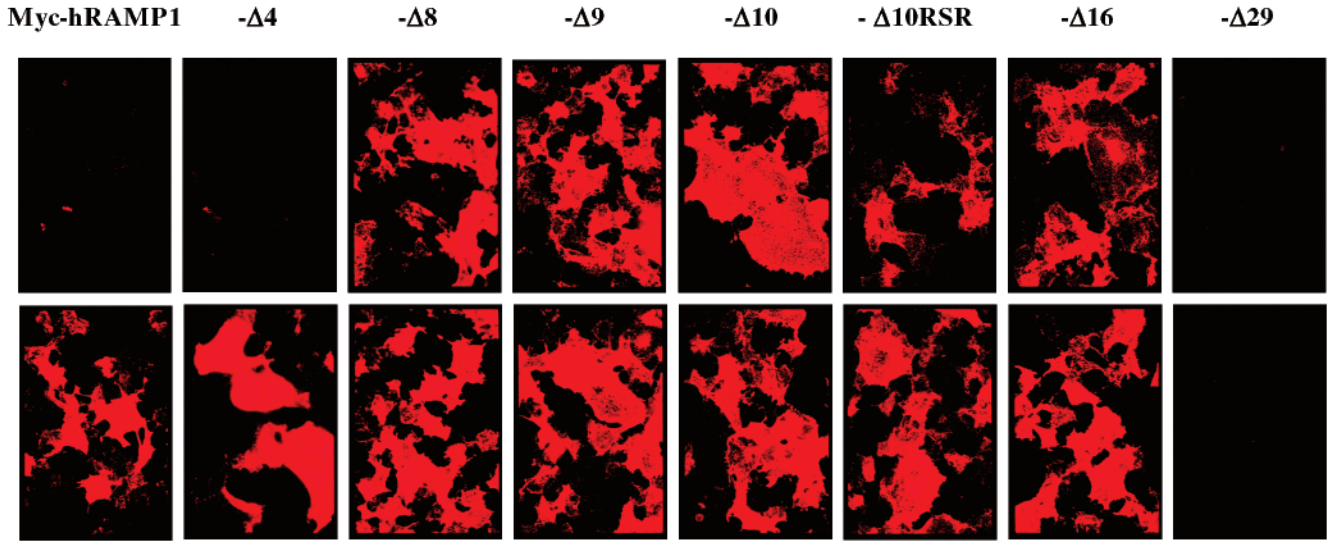


FIGURE 3: Myc immunofluorescent staining of intact COS-7 cells. The cells were transfected with expression constructs of intact or the indicated mutant myc-hRAMP1 alone (top) or together with the hCRLR (bottom). Mouse antibodies to myc and Cy3-labeled sheep antibodies to mouse immunoglobulins were used to visualize, by fluorescence microscopy, myc presented at the surface of intact cells. The cells were prepared as described in Materials and Methods. Cell surface expression from three to four independent experiments is calculated in Table 2.

Table 2: Cell Surface Expression of Myc-hRAMP1 in COS-7 Cells in the Absence and Presence of the hCRLR

| | −hCRLR | +hCRLR |
|---|---|---|
| myc-hRAMP1 | <5[a] | 100[b] |
| -Δ4 | <5[a] | 69 ± 8 |
| -Δ8 | 113 ± 13 | 138 ± 19 |
| -Δ9 | 135 ± 12 | 144 ± 11 |
| -Δ10 | 127 ± 17 | 138 ± 4 |
| -Δ10RSR | 40 ± 6 | 78 ± 5 |
| -Δ16 | 90 ± 11 | 108 ± 22 |
| -Δ29 | <5[a] | <5[a] |

[a] Fluorescence indistinguishable from background at up to 400 s exposure time. [b] Surface expression of myc-hRAMP1 with the hCRLR was set to 100%. Results are means ± SEM of three to four independent experiments; exposure times ranged from 10 to 30 s.

The autoradiography of the Western blot indicated that the immunoprecipitated [$^{125}$I]hαCGRP was cross-linked to 50 and 64 kDa protein components (Figure 4B). The 14 kDa size difference corresponds to the molecular mass calculated for myc-hRAMP1. The [$^{125}$I]hαCGRP cross-linking products had the size of major protein components detected with HRP-conjugated antibodies to the V5-hCRLR (Figure 4C). Moreover, the 64 kDa [$^{125}$I]hαCGRP binding components contained also intact or mutant myc-hRAMP1, detected with antibodies to myc and secondary HRP-conjugated antibodies to mouse immunoglobulins (Figure 4D). Myc-immunoreactive 64 kDa proteins were undetectable in immunoextracts of cells transfected with myc-hRAMP1 or V5-hCRLR expression constructs alone. They were barely detected in the samples derived from cells that expressed myc-hRAMP1Δ16 or -Δ29 together with the V5-hCRLR. A 50 kDa protein band, recognized in all samples of Figure 4D, represents the heavy chain of the V5 mouse monoclonal antibodies used for immunoprecipitation of the V5-hCRLR. It was visualized by the secondary HRP-conjugated sheep antibodies to mouse immunoglobulins.

Taken together, the results indicate that sequential C-terminal truncation of myc-hRAMP1 by up to 10 amino acids, including the presumed C-terminal tryptophan of the transmembrane domain, maintains [$^{125}$I]hαCGRP binding to the myc-hRAMP1/V5-hCRLR complexes at the cell surface. Further truncation by one-third or most of the transmembrane domain of myc-hRAMP1 greatly impaired cell surface association with the V5-hCRLR and as a consequence [$^{125}$I]hαCGRP binding.

*N-Glycosylation of the V5-hCRLR and Heterodimer Formation with Myc-hRAMP1 and Its Truncation Mutants*. V5 epitope-carrying protein components were immunoextracted from TSA cell homogenates and characterized on Western blots with V5 and myc antibodies (Figure 5). In extracts of mock-transfected cells (not shown) or of cells expressing myc-RAMP1 alone, myc and V5 immunoreactive proteins remained undetectable. The V5-hCRLR expressed alone was a 50 kDa protein doublet. A 100 kDa band was considered as a V5-hCRLR homodimer. In cells coexpressing the V5-hCRLR together with intact or myc-hRAMP1Δ4, the 50 and 100 kDa bands disappeared in favor of a predominant, previously described 73 kDa N-glycosylated form of the V5-hCRLR and a minor nonidentified V5 58 kDa immunoreactive component. Both V5-hCRLR components were also observed in cells coexpressing myc-hRAMP1Δ8, -Δ9, -Δ10, -Δ10RSR, -Δ16, or -Δ29, and variable amounts of the 50 kDa V5-hCRLR doublet and the presumed 100 kDa V5-hCRLR homodimer were still observed. This indicated varying efficiency of V5-hCRLR N-glycosylation in the presence of the different C-terminally shortened myc-hRAMP1. Heterodimer formation of intact and mutant myc-hRAMP1 with V5-hCRLR occurred at comparable efficiency when at least the hRAMP1 transmembrane domain remained intact. The variable expression levels of intact and mutant myc-hRAMP1 in the face of comparable amounts of coexpressed V5-hCRLR (Figure 2) did not affect hRAMP1/receptor heterodimer formation. N-Glycosylation of the V5-hCRLR and its coimmunoprecipitation with myc-hRAMP1Δ16 lacking the C-terminal intracellular region and one-third of the transmembrane domain were impaired. Interestingly, further truncation of myc-hRAMP1, removing most of the transmembrane domain, restored N-glycosylation

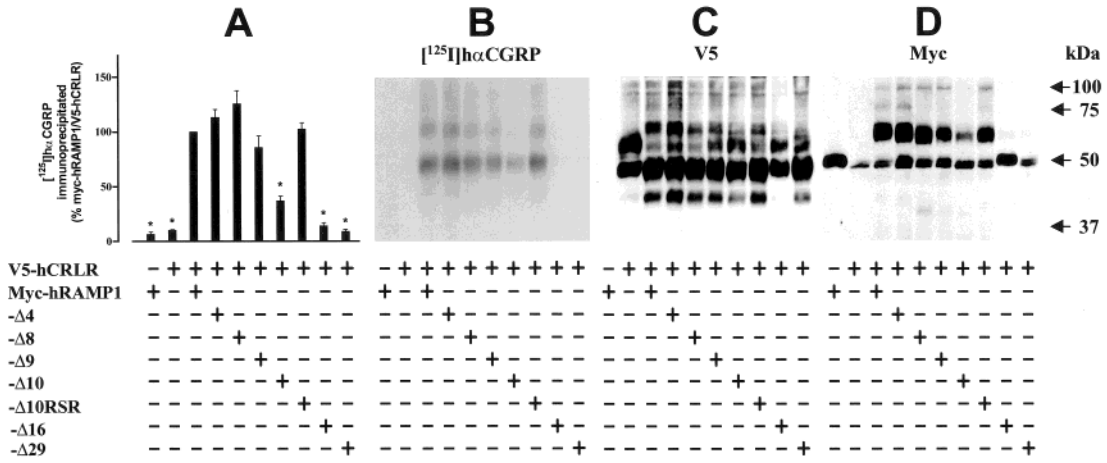


FIGURE 4: Immunoextraction with V5 antibodies of [$^{125}$I]hαCGRP cross-linked to intact and mutant myc-hRAMP1/V5-hCRLR complexes (A) and analysis by autoradiography (B) and on Western blots with HRP-labeled V5 antibodies (C) and with mouse antibodies to myc and HRP-labeled antibodies to mouse immunoglobulins (D). TSA cells were grown in 100 mm dishes to 80% confluency. They were then transfected in 12 mL of OptiMEM medium containing 29 $\mu$L of LipofectAMINE and 4.8 $\mu$g of myc-hRAMP1 or V5-hCRLR expression construct together with 4.8 $\mu$g of pcDNA3 or with 4.8 $\mu$g of the indicated intact or mutant myc-hRAMP1 together with 4.8 $\mu$g of V5-hCRLR expression construct per dish. Two days after transfection [$^{125}$I]hαCGRP binding, protein cell surface cross-linking, immunoextraction, deglycosylation, autoradiography, and Western blot analysis of immunoextracted proteins, subjected to 12% SDS−PAGE, were carried out as described in Materials and Methods. This representative experiment was carried out three times. Asterisks denote $P < 0.05$ ($n = 5$) versus myc-hRAMP1/V5-hCRLR set to 100%.

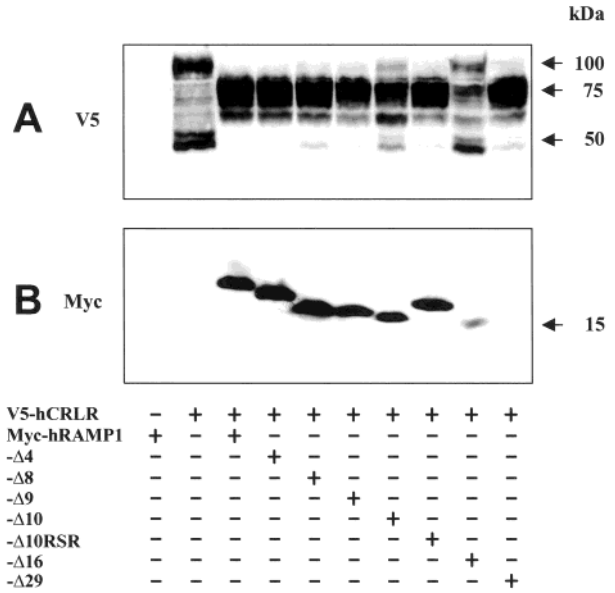


FIGURE 5: Coimmunoprecipitation of intact and the indicated mutant myc-hRAMP1 with V5-hCRLR. TSA cells were grown and transfected as described in the legend to Figure 4. Proteins immunoprecipitated with V5 antibodies from cell lysates were subjected to 15% SDS−PAGE, and Western blots were analyzed with HRP-conjugated V5 (A) and myc antibodies (B). This representative experiment was carried out three times.

of the V5-hCRLR, but coimmunoprecipitation, indicating heterodimer formation, was no longer observed. Thus an intact transmembrane domain in myc-hRAMP1 lacking the intracellular region is essential for its noncovalent association with the V5-hCRLR. Coexpression of the hRAMP1 extracellular domain alone is sufficient for the normal N-glycosylation of the hCRLR.

## DISCUSSION

Heterodimer formation between the individual RAMP1, -2, and -3 and the CRLR or a 60% homologous CTR defines G protein-coupled receptors for the related CGRP, AM, and amylin. But, structural elements in RAMP and receptors, essential for their functional interactions, remain largely to be identified.

Here, myc-hRAMP1 was sequentially truncated from the C-terminus, and the effects of the mutations on myc cell surface expression and on CGRP receptor function of the coexpressed V5-hCRLR have been analyzed. Myc-hRAMP1 lacking at the C-terminus eight of nine predicted intracellular amino acids maintained the association with the V5-hCRLR and close to normal CGRP receptor function. With the removal of the next two amino acids Q and W at the presumed boundary between the transmembrane domain and the intracellular region, cell surface association with the V5-hCRLR was still maintained, but [$^{125}$I]hαCGRP binding was reduced as compared to intact myc-hRAMP1/V5-hCRLR, and adenylyl cyclase activation by CGRP was impaired. Interestingly, readdition of RSR, an ER trafficking signal, in place of WQ restored CGRP receptor function of the V5-hCRLR. Presumably, the transmembrane domain of hRAMP1 and the presence of at least one hydrophilic residue reaching into the cytoplasm are essential for the association with the hCRLR to a functional CGRP receptor. This was confirmed by more extensive C-terminal truncation of myc-hRAMP1 through removal of the intracellular C-terminal region together with 7 (myc-hRAMP1Δ16) or 20 (myc-hRAMP1Δ29) of the predicted 22 transmembrane domain amino acids. In cells coexpressing myc-hRAMP1Δ16 or -Δ29 with the V5-hCRLR [$^{125}$I]hαCGRP binding and adenylyl cyclase activation by CGRP were abolished. Reduced or undetectable coimmunoprecipitation of myc-hRAMP1Δ16 and -Δ29, respectively, with the V5-hCRLR from total cell extracts indicated defective complex formation. As a consequence, negligible amounts of myc-hRAMP1Δ16 or -Δ29 as compared to myc-hRAMP1 were cross-linked to the V5-hCRLR at the cell surface. Importantly, reduced and undetectable association of myc-hRAMP1Δ16 and -Δ29 with the V5-hCRLR and low amounts of corresponding cell

surface complexes were observed at comparable expression levels of myc-hRAMP1, -Δ16, and -Δ29 detected in total cell extracts. Taken together, defective myc-hRAMP1Δ16- and -Δ29/V5-hCRLR complex formation abolished CGRP receptor function. Our results are in agreement with those obtained with influenza hemagglutinin where the length of the transmembrane domain and a single hydrophilic amino acid at the C-terminus were crucial for proper insertion into the plasma membrane and as a result normal influenza virus fusion (*18*).

Interestingly, myc-hRAMP1-dependent N-glycosylation of the V5-hCRLR was suppressed by myc-hRAMP1Δ16 lacking the C-terminal intracellular domain and 7 of the 22 amino acids of the transmembrane domain. But N-glycosylation was nearly restored in the presence of myc-hRAMP1Δ29 consisting of the intact N-terminal extracellular domain and I and L of the predicted transmembrane region. This implies that the N-terminal extracellular domain of hRAMP1 alone is a predominant determinant and sufficient for normal N-glycosylation of the hCRLR in mammalian cells. The association with the hCRLR into an immunoprecipitable stable complex is not required.

N-glycosylated hRAMP2 and -3 are transported to the plasma membrane in the absence of the coexpressed hCRLR (*9*). The nonglycosylated hRAMP1, on the other hand, requires the hCRLR for cell surface expression. The introduction of N-glycosylation sites into the extracellular region of hRAMP1 revealed hCRLR-independent cell surface delivery comparable to that of nonglycosylated hRAMP1 in the presence of the hCRLR (*9*). Here, the transport of nonglycosylated hRAMP1 to the plasma membrane occurred in the absence of the hCRLR when 8, 9, 10, or 16 amino acids were removed from the C-terminus. Consensus KDEL, dilysine, or RXR ER retention/retrieval motives within the sequence of the nine intracellular C-terminal amino acids of hRAMP1 have not been recognized (*14*, *19*). Along these lines, myc-hRAMP1 was shown to traffic from the ER to the Golgi but not to the cell surface in the absence of the hCRLR (*5*). A hemagglutinin-tagged hCRLR, on the other hand, was retained in the ER in the absence of hRAMP1. Thus, the QSKRT sequence in the intracellular C-terminal tail next to the transmembrane domain of intact myc-hRAMP1 apparently functions as a signal for retention in the Golgi that is overridden upon association with the hCRLR. Similarly, heterodimerization of $GABA_BR1$ and $GABA_BR2$ through their C-terminal coiled-coil α helices masks an RXR ER retention signal in the $GABA_BR1$ C-terminal intracellular region to reveal fully functional $GABA_BR1$/-2 complexes at the cell surface (*20*). Importantly, the present findings with myc-hRAMP1Δ8 or those with intact N-glycosylated hRAMP1 (*9*) indicate that hRAMP1/hCRLR complex formation, a prerequisite for CGRP recognition, did not depend on hRAMP1 retention in an intracellular compartment. On the other hand, QSKRT sequence dependent intracellular retention in the absence of the hCRLR may be required for the regulation of the transport of hRAMP1 to the cell surface.

In conclusion, anchoring of hRAMP1 in the plasma membrane, brought about by the transmembrane region and a single hydrophilic amino acid in place of the C-terminal intracellular tail, is required for CGRP recognition by the hRAMP1/hCRLR complex.

## REFERENCES


1. McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thomson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998) *Nature 393*, 333−339.
2. Christopoulos, G., Perry, K. J., Morfis, M., Tilakaratne, N., Gao, Y., Fraser, N. J., Main, M. J., Foord, S. M., and Sexton, P. M. (1999) *Mol. Pharmacol. 56*, 235−242.
3. Leuthäuser, K., Gujer, R., Aldecoa, A., McKinney, R. A., Muff, R., Fischer, J. A., and Born, W. (2000) *Biochem. J. 351*, 347−351.
4. Aldecoa, A., Gujer, R., Fischer, J. A., and Born, W. (2000) *FEBS Lett. 471*, 156−160.
5. Hilairet, S., Bélanger, C., Bertrand, J., Laperrière, A., Foord, S. M., and Bouvier, M. (2001) *J. Biol. Chem. 276*, 42182−42190.
6. Hilairet, S., Foord, S. M., Marshall, F. H., and Bouvier, M. (2001) *J. Biol. Chem. 276*, 29575−29581.
7. Wimalawansa, S. J. (1996) *Endocr. Rev. 17*, 533−585.
8. Sexton, P. M., Albiston, A., Morfis, M., and Tilakaratne, N. (2001) *Cell. Signalling 13*, 73−83.
9. Flahaut, M., Rossier, B. C., and Firsov, D. (2002) *J. Biol. Chem. 277*, 14731−14737.
10. Fraser, N. J., Wise, A., Brown, J., McLatchie, L. M., Main, M. J., and Foord, S. M. (1999) *Mol. Pharmacol. 55*, 1054−1059.
11. Kuwasako, K., Kitamura, K., Ito, K., Uemura, T., Yanagita, Y., Kato, J., Sakata, T., and Eto, T. (2001) *J. Biol. Chem. 276*, 49459−49465.
12. Zumpe, E. T., Tilakaratne, N., Fraser, N. J., Christopoulos, G., Foord, S. M., and Sexton, P. M. (2000) *Biochem. Biophys. Res. Commun. 267*, 368−372.
13. Simmons, D., and Seed, B. (1988) *J. Immunol. 141*, 2797−2800.
14. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron 22*, 537−548.
15. Bühlmann, N., Leuthäuser, K., Muff, R., Fischer, J. A., and Born, W. (1999) *Endocrinology 140*, 2883−2890.
16. Hussain, A. A., Jona, J. A., Yamada, A., and Dittert, L. W. (1995) *Anal. Biochem. 224*, 221−226.
17. Stangl, D., Born, W., and Fischer, J. A. (1991) *Biochemistry 30*, 8605−8611.
18. Armstrong, R. T., Kushnir, A. S., and White, J. M. (2000) *J. Cell Biol. 151*, 425−437.
19. Teasdale, R. D., and Jackson, M. R. (1996) *Annu. Rev. Cell Dev. Biol. 12*, 27−54.
20. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) *Neuron 27*, 97−106.


BI020279R