# The N-Terminal Extracellular Domain 23−60 of the Calcitonin Receptor-Like Receptor in Chimeras with the Parathyroid Hormone Receptor Mediates Association with Receptor Activity-Modifying Protein 1†

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ABSTRACT: The calcitonin receptor-like receptor (CLR) requires the associated receptor activity-modifying protein (RAMP)1 to reveal a calcitonin gene-related peptide (CGRP) receptor. Here, the subdomain of the CLR that associates with RAMP1 has been identified in chimeras between the CLR and the parathyroid hormone (PTH) receptor 1 (PTHR). The PTHR alone does not interact with RAMP1. RAMP1 requires the CLR for its transport to the cell surface. Thus, receptor-dependent RAMP1 delivery to the plasma membrane and coimmunoprecipitation from the cell surface were used as measures for receptor/RAMP1 interaction. Several chimeric CLR-PTHR included the N-terminal amino acids 23−60 of the CLR transported RAMP1 to the surface of COS-7 cells much like the intact CLR. Moreover, RAMP1 coimmunoprecipitated with these receptors from the cell surface. A CLR deletion mutant, consisting of the N-terminal extracellular domain, the first transmembrane domain, and the C-terminal intracellular region, revealed the same results. Cyclic AMP was stimulated by CGRP in CLR/RAMP1 expressing cells (58 ± 19-fold, $EC_{50}$ = 0.12 ± 0.03 nM) and by PTH-related protein in cells expressing the PTHR (50 ± 10-fold, $EC_{50}$ = 0.25 ± 0.03 nM) or a PTHR with the N-terminal amino acids 23−60 of the CLR (23 ± 5-fold, $EC_{50}$ > 1000 nM). Other chimeric CLR-PTHR were inactive. In conclusion, structural elements in the extreme N-terminus of the CLR between amino acids 23−60 are required and sufficient for CLR/RAMP1 cotransport to the plasma membrane and heterodimerization.

The calcitonin receptor-like receptor (CLR)[1] belongs to the family B1 of G protein-coupled receptors with seven transmembrane domains that includes the receptors for secretin, vasoactive intestinal polypeptide, pituitary adenylate cyclase activating polypeptides, glucagon, glucagon-like peptide, growth hormone releasing hormone, gastric inhibitory peptide, corticotropin releasing factor, parathyroid hormone (PTH), and calcitonin (*1*). The CLR, unlike the other receptors of the B1 family, requires associated receptor activity-modifying protein (RAMP)1 for functional expression as a calcitonin gene-related peptide (CGRP) receptor (*2*). RAMP1 consists of an N-terminal extracellular domain of 90 amino acids, a single transmembrane domain, and an intracellular C-terminal tail of 10 amino acids. RAMP1 requires the CLR for its transport to the plasma membrane (*3*). Progressive truncation of the intracellular C-terminal tail of human RAMP1 identified the QSKRT sequence adjacent to the transmembrane domain as the signal for intracellular retention (*4*). This signal is overridden upon interaction with the CLR. The transport of RAMP1 to the cell surface is furthermore facilitated by the calcitonin receptor and the vasoactive intestinal polypeptide receptor 1 (*5*, *6*). The PTH receptor 1 (PTHR), on the other hand, does not interact with RAMP1, and as a consequence, fails to transport RAMP1 to the plasma membrane (*7*).

The sites of interaction between the CLR, RAMP1, and CGRP are largely unknown. Site-directed mutagenesis, targeting the N-terminal extracellular domain, identified $Asn^{117}$ in the human CLR and $Asp^{69}$ in the mouse CLR as hotspots for inactivating mutations (*8*, *9*). With $Asn^{117}$ to Ala, Gln, Thr, and Pro mutations in the human CLR, CGRP binding was abolished, but N-glycosylation and the cell surface expression were maintained, and CLR/RAMP1 heterodimerization, as revealed by coimmunoprecipitation, was only minimally affected. Similarly, substitution of $Asp^{69}$ in the mouse CLR to Asn, Glu, or Ala impaired or abolished the CGRP and adrenomedullin (AM) receptor functions in the presence of RAMP1 and 2, respectively. Much like with the human CLR, the mutations in the mouse CLR did not affect the expression of the receptor and the RAMP at the cell surface. Coimmunoprecipitation of RAMP1 was impaired with the mutant as compared to the intact mouse CLR, whereas the interaction with RAMP2 was not affected. The deletion of a TRNKIMT sequence (amino acid 14−20) in the extreme N-terminus of the mouse CLR abolished the AM receptor function in the face of normal functional expression of the

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[1] Abbreviations: CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PTH, parathyroid hormone; PTHR, PTH receptor 1; PTHrP, PTH related protein; RAMP, receptor-activity-modifying protein; SDS, sodium dodecyl sulfate.

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CGRP receptor (*10*). CLR/RAMP2 heterodimerization and cell surface expression remained unaltered. Taken together, mutations introduced into the N-terminal extracellular domain of the human and mouse CLR that impaired or abolished CLR functions only minimally affected CLR/RAMP1 cotransport and heterodimerization at the cell surface.

Here, a CLR deletion mutant and several chimeric CLR-PTHR were transiently coexpressed with RAMP1 in COS-7 cells. The expression of RAMP1 at the cell surface and receptor/RAMP1 coimmunoprecipitation were taken as measures for their interactions. The sequence of the amino acids 23−60 in the extreme N-terminus of the mouse CLR was found to contain the site(s) required and sufficient for cotransport of RAMP1 to the plasma membrane and for the noncovalent association of the CLR with RAMP1 at the cell surface.

## MATERIALS AND METHODS

*Materials.* Rat αCGRP(1−37) (CGRP) was purchased from Bachem (Bubendorf, Switzerland), and chicken PTH related protein (1−36) (PTHrP) was donated by E. Felder (Novartis, Basel, Switzerland). Restriction enzymes were obtained from Promega (Madison, WI). Other chemicals and reagents were purchased from Sigma and from VWR International GmbH (Darmstadt, Germany) at the highest grade available.

*Receptor and RAMP1 Expression Constructs.* The construct for expression of myc epitope-tagged mouse RAMP1 (myc-RAMP1) has been reported (*11*). There is little homology between the CLR and the PTHR (Figure 1A). V5 epitope tags were introduced into the intact mouse CLR and human PTHR after the respective signal sequences of 23 and 29 amino acids (www.cbs.dtu.dk/services/SignalP, ref *12*) with the QuickChange Multisite-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The deletion of the transmembrane domains 2−7 together with the intra- and extracellular loop structures, corresponding to amino acids 169−388 of the CLR, with the QuickChange Multisite-Directed Mutagenesis Kit revealed the CLR-TM1 (Figure 1B). DNA sequences encoding the chimeric CLR-PTHR were constructed with a two-step PCR-based strategy, using V5-CLR and V5-PTHR expression constructs in pcDNA3.1 (Invitrogen) as PCR product donor or respective acceptor templates. The first PCR was designed for amplification from the donor vector of the DNA fragment selected for substitution of the corresponding DNA sequence in the acceptor vector. The primers consisted from 5′ to 3′ of at least 25 nucleotides with the sequence of the DNA upstream (5′ primer) or downstream (3′ primer) of the integration site in the acceptor template, followed by at least 20 nucleotides with the sequence of the 5′-end (5′ primer) or complementary to the 3′-end (3′ primer) of the DNA fragment to be amplified from the donor template. Twenty-five cycles of PCR were carried out in a final volume of 50 μL, containing 1 ng of donor template, 10 pmol of the individual primers, and 2.5 units of the *Pfu*Ultra High-Fidelity DNA Polymerase (Stratagene). The annealing temperature was set to 58 °C. PCR products were separated from byproducts on a 2% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The complementary strands of 300 ng of purified PCR product were then used as megaprimers

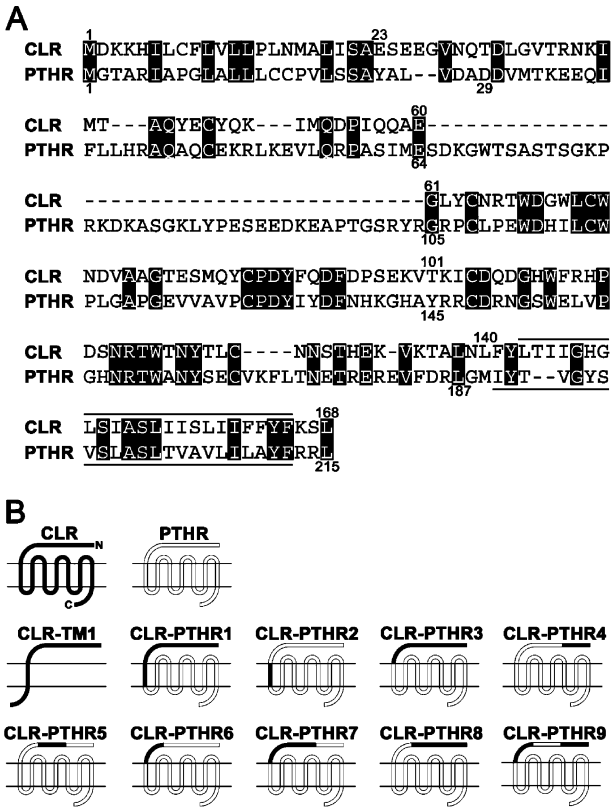


FIGURE 1: Amino acid sequence alignment of N-terminal regions of the CLR and PTHR and schematic illustration of receptor chimeras. (A) Alignment of the N-terminal extracellular domains and the first transmembrane domains (between lines) of the CLR receptor (GenBank accession Q9R1W5) and the PTHR (GenBank accession Q03431). Numbers above and below the sequences indicate the N- and C-termini of the respective CLR and PTHR subregions combined in chimeric receptors. The amino acids 65−104 of the PTHR were deleted in the receptor constructs CLR-PTH4, -5, -7, -8 and -9. (B) Schematic illustration of the intact CLR and PTHR and the CLR deletion mutant CLR-TM1 and the chimeric CLR-PTHR.

in the second PCR with 100 ng of acceptor vector as a template and 2.5 units of *Pfu*Ultra High-Fidelity DNA Polymerase (Stratagene) in a final volume of 50 μL. Twenty cycles of PCR were carried out at 95 °C for 30 s, at 50 °C for 45 s, and at 68 °C for 28 min. Subsequently, the templates were digested with 10 units of DpnI restriction enzyme at 37 °C for 3 h, and the PCR products were then transformed into calcium competent *Escherichia coli* XL-1 blue cells. Plasmid DNA was isolated from individual colonies, and the DNA encoding chimeric CLR-PTHR was sequenced from both directions with an ABI Prizm DNA Analyzer (Applied Biosystems, Foster City, CA). The CLR-PTHR encoding DNA fragment was then excised and separated from the coamplified pcDNA3.1 vector DNA and recloned into the pcDNA3.1 plasmid to avoid sequencing of the entire vector backbone.

The chimeric CLR-PTHR1 consists of the amino acids 23−167 of the CLR followed by the amino acids 215−593 of the PTHR. In the CLR-PTHR2, the amino acids 188−214 of the PTHR are replaced by amino acids 141−167 of the CLR. The CLR-PTHR3 has amino acids 23−139 of the CLR and amino acids 187−593 of the PTHR. Substitution of amino acids 29−104 of the PTHR by the CLR fragment 23−60 revealed the CLR-PTHR4. In the CLR-PTHR5,

amino acids 65−144 of the PTHR are replaced by amino acids 61−100 of the CLR. Substitution of amino acids 115−186 in the PTHR by amino acids 101−139 of the CLR revealed the CLR-PTHR6. In the CLR-PTHR7, amino acids 61−139 of the CLR replace amino acids 65−187 of the PTHR. The CLR-PTHR8 consists of amino acids 25−100 of the CLR and amino acids 145−593 of the PTHR. In the CLR-PTHR9, amino acids 23−60 and 101−139 of the CLR replace amino acids 29−100 and 145− 187 of the PTHR, respectively.

*Cell Culture, Transfection, [$^{125}$I]CGRP Binding, and Stimulation of cAMP Accumulation.* COS-7 cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ in HamF12/Dulbecco's modified Eagle medium (1:1) (4.5 g/L glucose) supplemented with 2 mM glutamine and 10% fetal calf serum (cell culture medium). The cells were grown to 40−60% confluence and transfected with the constructs encoding myc-RAMP1 and the indicated V5-tagged receptors and linear polyethylenimine (25kDa) (Polysciences, Warrington, PA) as described (*11*, *13*). Briefly, 0.3 $\mu$g/cm$^2$ plasmid DNA was diluted with 6.25 $\mu$L/cm$^2$ cell culture medium. This solution was then combined with 0.9 $\mu$g/cm$^2$ polyethylenimine in 6.25 $\mu$L/cm$^2$ cell culture medium and vortexed immediately. After incubation at room temperature for 15 min, the volume was adjusted to 125 $\mu$L/cm$^2$ with cell culture medium, and the solution was added to the cells. The cells were incubated at 37 °C for 16−24 h. The volume was then increased to 250 $\mu$L/cm$^2$ with cell culture medium, and the cells were cultured for 2 days.

[$^{125}$I]CGRP binding experiments in COS-7 cells were carried out at 15 °C as described (*14*). The stimulation of cAMP by 0.1 nM to 1 $\mu$M CGRP and PTHrP was estimated at 37 °C for 15 min in cell culture medium without fetal calf serum but supplemented with 0.1% bovine serum albumin and 1 mM isobutylmethylxanthine. Cyclic AMP was extracted from the cells and measured by radioimmunoassay as described (*14*).

*Protein Extraction, Cell Surface Immunoprecipitation, and Western Blot Analysis.* The cells were lysed with 50 mM Hepes, pH 7.5, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 3 $\mu$g/mL aprotinin, 3 $\mu$g/mL leupeptin, and 0.5% Triton X-100 (cell lysis buffer). The proteins in 50 $\mu$L of cell lysate were deglycosylated with 2 units of *N*-glycosidase F (F. Hoffmann-La Roche, Basel, Switzerland) at 37 °C for 18 h. The reactions were stopped with protein gel loading buffer.

Immunoprecipitation of V5-tagged receptors from the surface of COS-7 cells that coexpressed myc-RAMP1 was carried out as described (*15*). Briefly, cells cultured in 12.5 cm$^2$ flasks were incubated at 15 °C for 3 h with mouse monoclonal antibodies to V5 (Invitrogen) diluted 1:200 in cell culture medium with the fetal calf serum replaced by 0.1% bovine serum albumin (staining medium). The cells were then washed with staining medium and lysed in 500 $\mu$L of cell lysis buffer. The lysates were cleared by centrifugation, and the supernatants were incubated with 35 $\mu$L of ImmunoPure Immobilized Protein G (Pierce Biotechnology, Rockford, IL) at 4 °C for 2 h on an end-over-end rotator. The immobilized proteins were washed twice with cell lysis buffer and then deglycosylated with 2 units of *N*-glycosidase F at 37 °C for 18 h. The reactions were stopped with protein gel loading buffer.

Proteins in cell extracts or immunoprecipitated proteins were separated by SDS−PAGE and electrotransferred to nitrocellulose Hybond ECL membranes (Amersham Biosciences UK Ltd., Buckinghamshire, UK) in a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA) at 20 V for 60 min. The membranes were blocked with 5% low fat milk. The V5-CLR and myc-RAMP1 were detected with alkaline phosphatase-conjugated monoclonal V5 and myc antibodies (Invitrogen) (diluted 1:25 000 and 1:15 000, respectively, in 1% low fat milk). Actin as a reference for protein loading was visualized with monoclonal antibodies to actin (Chemicon International, Temecula, CA) (diluted 1:5000 in 5% low fat milk) and secondary alkaline phosphatase-conjugated goat antibodies to mouse IgG (The Jackson Laboratory, West Grove, PA) (1:15 000 in 1% low fat milk). The alkaline phosphatase-conjugated antibodies were visualized by chemiluminescence with the Immun-Star AP substrate Pack (Bio-Rad) using a VersaDoc Imaging System (Bio-Rad). The membranes were reprobed with different antibodies after being washed with distilled water for 5 min, 2 M NaOH for 10 min, and with distilled water for 5 min.

*Immunofluorescent Staining of Intact and Mutant V5-CLR, Chimeric V5-CLR-PTHR, and myc-RAMP1 at the Surface of COS-7 Cells.* V5 and myc immunofluorescent staining was carried out 2 days after transfection as described (*10*). Briefly, COS-7 cells fixed with 4% formalin were simultaneously incubated with rabbit anti-myc (Abcam, Cambridge, UK) and mouse anti-V5 (Invitrogen) sera (diluted 1:500 in staining medium) at room temperature for 2 h. The cells were then washed with phosphate-buffered saline and incubated in the dark with Alexa488-labeled goat anti-rabbit (Molecular Probes, Eugene, OR) and Cy3-labeled sheep anti-mouse sera (Sigma) (diluted 1:200 in staining medium) at room temperature for 30 min. The cells were again washed with phosphate-buffered saline and mounted with Immu-Mount (Shandon Scientific, Pittsburgh, PA). The fluorescence was detected with a Kappa DX20 CCD camera (Gleichen, Germany) connected with a 0.45× projection lens to an Eclipse E600 Nikon microscope that was equipped with a Plan Fluor 20x/0.5 DLL objective and G-2A (Cy3) and B-2A (Alexa488) filter blocks.

## RESULTS

*Expression of V5-Tagged Receptors and myc-RAMP1.* The proteins in cell extracts were deglycosylated, and the relative expression levels of V5-tagged receptors and myc-RAMP1 were estimated on Western blots (Figure 2). The expression levels of the V5-CLR-TM1, -CLR-PTHR2, -5, -6, and -7 were comparable to those of the intact V5-CLR and -PTHR, and those of the V5-CLR-PTHR1, -3, -4, -8, and -9 were higher. The expression of myc-RAMP1 was similar in the absence and the presence of the V5-CLR, -CLR-TM1, -CLR-PTHR1, -2, -3, -4, -5, -7, -8, and -9 and only slightly lower in the presence of the V5-PTHR and the CLR-PTHR6.

*Expression of the V5-Tagged Receptors and of myc-RAMP1 at the Cell Surface.* V5 and myc immunofluorescent staining of intact, transiently transfected COS-7 cells was a measure for the expression of the V5-tagged receptors and myc-RAMP1 at the cell surface (Figure 3). myc-RAMP1 was undetectable at the surface of intact cells in the absence of

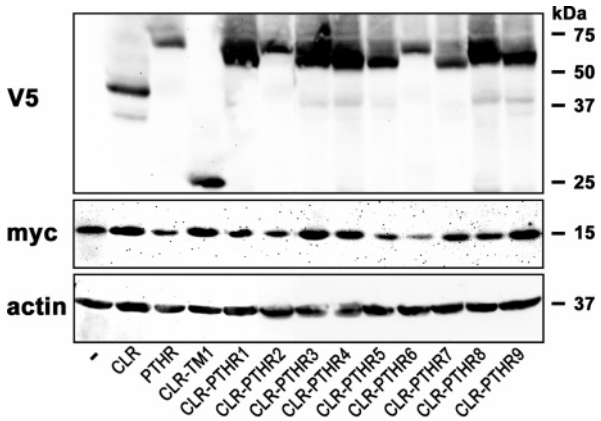


FIGURE 2: Western blot analysis of total cell extracts. COS-7 cells were transfected with myc-RAMP1 expression construct alone or together with the indicated V5-receptor constructs. Two days after transfection, the cells were harvested, and the proteins were extracted with cell lysis buffer. Extracts were treated with N-glycosidase F, and the proteins were separated on a 10−20% SDS−polyacrylamide gel and electro-transferred to a nitrocellulose membrane. V5-tagged receptors and myc-tagged RAMP1 were visualized with alkaline phosphatase-conjugated antibodies to V5 and myc. Actin as a reference for protein loading was detected with mouse monoclonal antibodies to actin and secondary alkaline phosphatase-conjugated goat antibodies to mouse IgG. Representative experiment was carried out three times.

the V5-CLR, but it was recognized in permeabilized COS-7 cells (not shown). myc-RAMP1 appeared at the cell surface in the presence of the V5-CLR, confirming the previously described cotransport of RAMP1 with the CLR to the plasma membrane (*3*). Importantly, myc-RAMP1 remained undetectable at the surface of cells coexpressing the V5-PTHR. This indicated that the PTHR, unlike the CLR, lacked domains for interaction with myc-RAMP1 required for its cotransport to the cell surface. Thus, the transport of myc-RAMP1 to the cell periphery served as an indicator for its interaction with coexpressed CLR mutants and CLR-PTHR chimeras. Much like in mock transfected COS-7 cells or in cells expressing myc-RAMP1 in the absence or presence of the V5-PTHR, myc immunofluorescent cell surface staining was undetectable in cells expressing myc-RAMP1 together with the V5-CLR-PTHR2, -5, -6, and -7. In cells coexpressing myc-RAMP1 with the V5-CLR-TM1 or the V5-CLR-PTHR1, -3, -4, -8, and -9, on the other hand, myc-RAMP-1 was transported to the cell surface much like in myc-RAMP1/V5-CLR expressing cells. These receptors had in common the extreme N-terminus consisting of the amino acids 23−60 of the CLR. Chimeric CLR-PTHR that lacked this CLR segment failed to transport the myc-RAMP1 to the cell surface. Taken together, RAMP1 interacts with the extreme N-terminus of the CLR during cotransport to the cell surface.

*Coimmunoprecipitation of V5-Tagged Receptors and myc-RAMP1 from the Cell Surface.* Cotransport of the V5-CLR with myc-RAMP1 to the plasma membrane results in the formation of corresponding immunoprecipitable receptor complexes at the cell surface (Figure 4). In contrast and as expected from the myc-RAMP1 cell surface delivery experiments, the V5-PTHR or the V5-CLR-PTHR2, -5, -6, and -7 that lacked the amino acid sequence 23−60 of the CLR failed to coprecipitate myc-RAMP1. Precipitation of myc-RAMP1, on the other hand, with the V5-CLR-TM1 and the V5-CLR-PTHR1, -3, -4, -8, and -9 that contained the CLR fragment

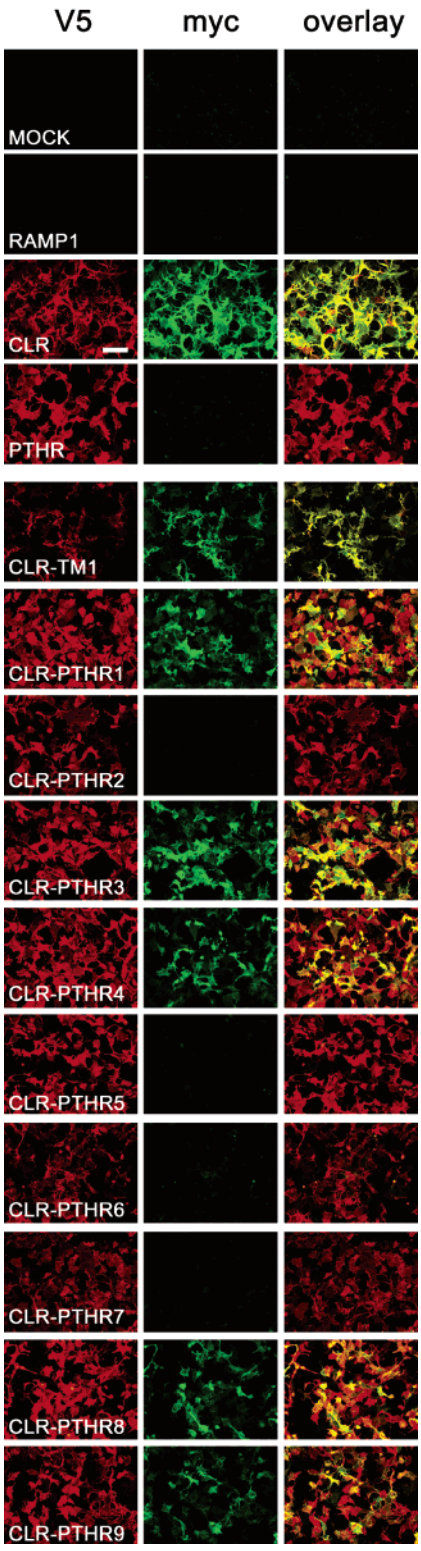


FIGURE 3: Cell surface immunofluorescence of intact and mutant V5-CLR and chimeric V5-CLR-PTHR and of myc-RAMP1. COS-7 cells were transfected with the myc-RAMP1 expression construct alone in the presence of pcDNA3 or together with the indicated V5-receptor constructs. After fixation with 4% formalin, the cells were simultaneously incubated with mouse monoclonal antibodies to V5 and rabbit antibodies to myc and subsequently stained with Cy3-conjugated sheep anti-mouse and Alexa488-conjugated goat anti-rabbit sera. Photomicrographs were taken for the detection of V5 (red) and myc (green) epitopes from the same field of inspection with respective filter blocks. Overlays in yellow indicate coexpression of the V5 receptors and myc-RAMP1. Scale bar: 100 $\mu$m.

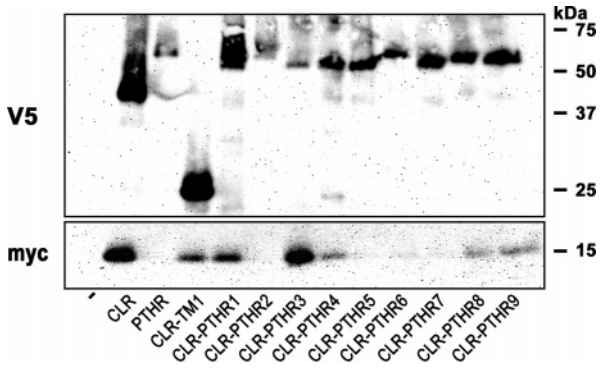


FIGURE 4: Western blot analysis of proteins immunoprecipitated with V5 antibodies from the surface of COS-7 cells. The cells were transfected with myc-RAMP1 expression construct alone in the presence of pcDNA3 or together with the indicated V5 receptor constructs. Two days after transfection, intact cells were incubated with mouse monoclonal antibodies to V5. V5 antibody bound proteins were extracted from cell lysates with immobilized protein G. They were then separated on a 12% SDS−polyacrylamide gel and visualized on Western blots with mouse monoclonal alkaline-phosphatase-conjugated V5 and myc antibodies. Representative experiments were carried out three times.

23−60 at the N-terminus, indicated RAMP1/receptor interactions comparable to those of myc-RAMP1 with the intact V5-CLR. Thus, immunoprecipitation of myc-RAMP1 from the cell surface only occurred with V5-tagged receptors that contained the amino acid sequence 23−60 of the CLR.

*[$^{125}$I]CGRP Binding and Stimulation of cAMP Production.* High affinity binding of [$^{125}$I]CGRP was only observed in COS-7 cells that coexpressed the intact V5-CLR and myc-RAMP1. Stimulation of cAMP by CGRP and PTHrP was furthermore assessed with the intact V5-CLR and -PTHR, the V5-CLR-TM1, and the chimeric V5-CLR-PTHR in COS-7 cells that transiently expressed the receptors together with myc-RAMP1. In cells expressing the intact V5-CLR or V5-PTHR, basal cAMP levels were 6 pmol/$10^6$ cells and 8 pmol/$10^6$ cells, respectively. In V5-CLR/myc-RAMP1 expressing cells, cAMP formation was stimulated 58 ± 19-fold by 1 $\mu$M CGRP with an $EC_{50}$ of 0.12 ± 0.03 nM ($n$ = 3) but not with up to 1 $\mu$M PTHrP. In V5-PTHR/myc-RAMP1 expressing cells, on the other hand, cAMP production was stimulated 50 ± 10-fold by 1 $\mu$M PTHrP with an $EC_{50}$ of 0.25 ± 0.03 nM ($n$ = 3), and here up to 1 $\mu$M CGRP was inactive. The cAMP response to 1 $\mu$M PTHrP in V5-CLR-PTHR4 expressing cells was 46 ± 7% of that in cells expressing the intact V5-PTHR, and CGRP was inactive. COS-7 cells expressing other chimeric V5-CLR-PTHR or the V5-CLR-TM1 did not respond to up to 1 $\mu$M CGRP and PTHrP.

## DISCUSSION

CLR/RAMP1 heterodimers are CGRP receptors, but the sites and mechanisms of interaction of the CLR and RAMP1 at the molecular level are largely unknown. Here, a subdomain of the CLR required for the association with RAMP1 has been identified.

The deletion of the transmembrane domains 2−7 of the CLR together with the respective loop structures did not affect the expression of corresponding mutant CLR-TM1/RAMP1 heterodimers at the cell surface, but high affinity [$^{125}$I]CGRP-binding and the stimulation of cAMP production by CGRP was no longer observed. This is in line with results obtained with the receptors for PTH, secretin, calcitonin, glucagon, and corticotropin releasing factor (*16*−*19*). There, interactions between juxtamembrane regions of the receptors and the N-terminal portions of the corresponding ligands were required for receptor ligand binding and signal transduction. The N-terminal extracellular domains of the receptors contributed to high affinity binding of the peptide ligand in a major way. Here, the results with the chimeric CLR-PTHR confirmed the findings of the previous studies with the PTHR. Micromolar concentrations of PTHrP were required for the stimulation of cAMP production in V5-CLR-PTHR4 expressing cells, and the other chimeric CLR-PTHR were inactive. The CLR-TM1 and all chimeric CLR-PTHR lacked the extracellular loop regions of the CLR, and CGRP failed to stimulate cAMP production in the presence of RAMP1. This strongly suggests that the CLR, the closest homologue of the calcitonin receptor in the B1 family of G protein-coupled receptors, in addition to RAMP1 also requires both the N-terminal extracellular domain and the juxtamembrane regions for high affinity interaction with CGRP. Earlier studies revealed chemical cross-linking of [$^{125}$I]CGRP to both CLR and RAMP1 at the cell surface, suggesting that the N-terminal extracellular domain of RAMP1 also contributes to the CGRP binding pocket in CLR/RAMP1 heterodimers (*15*).

The transport of RAMP1 to the plasma membrane by the CLR-TM1 deletion mutant and the formation of immunoprecipitable CLR-TM1/RAMP1 heterodimers at the cell surface indicated that the interactions between mutant CLR and RAMP1 were maintained. This implies that the N-terminal extracellular region, the transmembrane domain 1, and the intracellular C-terminal tail of the CLR contain all the structural elements required for the association with RAMP1.

Additional deletions introduced into the CLR-TM1 impaired or abolished the expression at the cell surface. As a consequence, chimeric CLR-PTHR were generated to narrow interaction domains down to short segments in the N-terminal extracellular region, the transmembrane domain 1, and/or the intracellular C-terminal tail of the CLR. Here, we took advantage of the fact that the CLR and the PTHR are both family B G protein-coupled receptors and that the PTHR, unlike the CLR, does not interact with RAMP1. The relative expression levels of the chimeric receptors in total cell extracts on Western blots (Figure 2) and at the cell surface, as revealed by V5-immunostaining of intact cells (Figure 3), were comparable. The exchange of subregions between the CLR and the PTHR maintained or increased the expression levels of the chimeric receptors when compared to the intact CLR and PTHR, but it did not affect the transport to the cell surface.

Interestingly, the PTHR was converted into a RAMP1-interacting receptor upon substitution of the amino acid sequence 29−104 in the extreme N-terminus of the PTHR by the CLR segment 23−60. RAMP1 transport and coimmunoprecipitation from the cell surface with this chimeric CLR-PTHR were similar to that of the intact CLR. This indicated that important sites for interaction with RAMP1 are localized within the amino acid sequence 23−60 of the CLR.

Alignment of this CLR segment with the corresponding subregions of the calcitonin receptor and the VPAC1 receptor

that also interact with RAMP1 revealed similarities between the sequences 43−60 of the CLR and 50−67 of the calcitonin receptor. But the corresponding VPAC1 receptor segment presents a different sequence. Thus, RAMP1-defined ligand selectivity of the CLR and the calcitonin receptor that is not observed with the VPAC1 receptor appears to require the interaction between the respective CLR and calcitonin receptor segments 43−60 and 50−67 and RAMP1. This is supported by the results of a previous study (*10*). There, the deletion of the amino acids 36−42 adjacent to the sequence 43−60 in the CLR abolished the AM receptor function in the presence of RAMP2 but maintained the CLR/RAMP1 CGRP receptor activity. Taking the results of the present and the previous study together, it is conceivable that RAMP1, through its association with sites within the CLR domain 23−60, masks the segment 36−42, required for high affinity interaction of AM with the CLR in the presence of RAMP2, and thereby determines in part the specificity of CLR/RAMP1 heterodimers for CGRP.

In conclusion, the CLR, unlike the PTHR, heterodimerizes with RAMP1 and reveals a CGRP receptor. The N-terminal extracellular region 23−60 of the CLR contains important structural elements for the association with RAMP1. Introduction of this CLR subdomain into the PTHR mediates interactions of chimeric CLR-PTHR with RAMP1 indistinguishable from those with the intact CLR. The CLR segment 23−60 is important for CLR/RAMP1 association and therefore for CGRP receptor function.

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