The receptors for the neuropeptide calcitonin (CT) gene-related peptide (CGRP) and the multifunctional peptide hormone adrenomedullin (AM) are calcitonin-like receptor (CLR)/receptor-activity-modifying protein (RAMP) 1 and CLR/RAMP2 heterodimers, respectively. Here, the amino acid sequence TRNKIMT, corresponding to the residues 14–20 of the N terminus of the mouse (m) CLR, was found to be required for a functional mCLR/RAMP2 AM receptor. The deletion of amino acids 14–20 (∆14–20) or their substitution by alanine (14–20A) did not affect the heterodimerization of the mCLR with mRAMP1 or mRAMP2, and the levels of expression at the surface of transiently transfected COS-7 cells were not altered. In mRAMP1/mCLR- or mRAMP1/mCLR-(∆14–20)-expressing cells CGRP stimulated cAMP formation with $EC_{50}$ values of 0.12±0.01 and 1.5±0.4 nm, respectively. In mRAMP2/mCLR-expressing cells the $EC_{50}$ of AM was 0.8±0.2 nm. However, in cells expressing mRAMP2/mCLR-(∆14–20) up to $10^{-6}$ M AM failed to stimulate cAMP production. In mRAMP2/mCLR-(14–20A) expressing cells the cAMP response to AM was minimally restored, and the $EC_{50}$ was >100 nm. In conclusion, the deletion of the amino acid sequence TRNKIMT of the extreme N terminus of the mCLR maintained CGRP receptor function in the presence of mRAMP1/receptor heterodimers, but AM no longer activated the mutant mCLR-(∆14–20) in the presence of mRAMP2. The TRNKIMT sequence is required for normal mCLR/mRAMP2 association, and as a consequence, high affinity AM binding signaling the activation of adenylyl cyclase.

The neuropeptides α- and β-calcitonin (CT)1 gene-related peptide (CGRP) (1–37) and the multifunctional peptide hormone adrenomedullin (AM) of 52 amino acids in man and 50 amino acids in the rat are potent vasodilators (1). CGRP and AM are only 20% identical but have in common ring structures of six amino acids linked by disulfide bonds between cysteine residues and amidated C termini, both required for biological activity.

Molecularly defined receptors for CGRP and AM are CT-like receptor (CLR)/receptor-activity-modifying protein (RAMP) 1 and CLR/RAMP2 heterodimers, predominantly linked to cAMP production (2). The CLR belongs to the family B of G protein-coupled receptors with seven transmembrane domains that includes the 60% homologous CT receptor and receptors for secretin and parathyroid hormone (PTH). The CLR, unlike the other known members of this receptor family, requires as associated proteins the RAMP for the functional expression. RAMP1 and RAMP2 and a third, RAMP3, consist of between 147 and 189 amino acids. They are single transmembrane domain proteins with 30% amino acid sequence similarity. Their N-terminal extracellular domains of between 90 and 110 amino acids define in part CGRP and AM selectivity of the CLR (3). Substitution of the 18 N-terminal amino acids of the human CLR by the corresponding domain of the porcine CT receptor revealed a fully functional CGRP receptor in the presence of human RAMP1, but the RAMP2-dependent AM receptor function was impaired (4). This implies that subdomains of both the CLR and the RAMP determine distinct binding interaction sites for CGRP and AM in the corresponding heterodimers.

In other receptors of the B family of G protein-coupled receptors critical determinants for high affinity ligand binding are localized near the N terminus. Photochemical cross-linking of PTH analogs and site directed mutagenesis identified Thr33 and Glu37 in the N-terminal extracellular domain of the rat PTH receptor as determinants for PTH binding (5). Similarly, 125I-CT analogs with photoactive p-benzoyl-l-phenylalnine in positions 16 and 26 labeled Thr30 close to the N terminus and Phe137 adjacent to the first transmembrane domain of the human CT receptor (6). Similarly, in the secretin receptor amino acids 1–10 were indispensable for high affinity ligand binding (7).

Here, amino acids 14–20 of the mouse CLR with the sequence TRNKIMT were identified as a critical determinant for the functional interaction of the mCLR/mRAMP2 with AM. Importantly, the deletion of the TRNKIMT sequence in the mCLR maintained the GGRP receptor function in the presence of mRAMP1. Substitution of amino acids 14–20 by alanine revealed a low affinity mCLR/mRAMP2 AM receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture products were supplied by Invitrogen. Rat oCGRP (CGRP) and rat AM (AM) were obtained from Bachem AG (Bubendorf, Switzerland). chemicals and other reagents were purchased from Sigma and VWR International GmbH (Darmstadt, Germany).

**Construction of mCLR Mutants**—A DNA fragment encoding amino acids 1–292 of the mCLR was subcloned into the pBluescript SK– vector (Stratagene, La Jolla, CA). The amino acid deletions and substi-
Inactivation of Adrenomedullin over CGRP Receptor Function

In the experiment described, COS-7 cells were grown in 6-well plates and transfected with indicated combinations of myc-mRAMP1 or myc-mRAMP2 and V5-mCLR or V5-mCLR mutant expression constructs. In transfections with the V5-mCLR alone, empty pcDNA3 expression vector was added to keep the DNA concentrations constant. 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 separated on a 10–20% gradient SDS-polyacylamide gel. Proteins were blotted on a nitrocellulose membrane, and V5-tagged receptors and myc-tagged RAMP were visualized with alkaline phosphatase-conjugated V5 and myc antibodies. 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 experiments were carried out three times.

**Cell Culture and Transfection**—COS-7 cells were cultured in Ham’s F-12/Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) medium (1:1) supplemented with 10% fetal calf serum and 2 mM glutamine (cell culture medium). For transfection the cells were seeded in 24- or 6-well plates or in 100-mm culture dishes at a density of 15,000 cells/cm². 48 h later the cells were transfected with the indicated receptor and RAMP expression constructs and linear polyethyleneimine (25 kDa) (PolyPlus Transfection, Illkirch, France). DNA fragments carrying the desired mutations were excised from plasmid DNA and cloned into a pcDNA 3.1 construct that provided an in-frame DNA fragment encoding the non-modified C-terminal region of the mCLR.

**Protein Cross-linking, Immunoprecipitation, and Deglycosylation**—Cell surface protein cross-linking was carried out with 10 mM bis(sulfosuccinimidyl) suberate (BS3; Pierce). The cells were lysed with 50 mM Hepes, pH 7.5, 7 mM MgCl₂, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 1 mg/ml dodecyl-β-maltoside, and 0.2 mg/ml cholesterolhemisuccinate (cell lysis buffer). V5-tagged receptors were immunoprecipitated from the cleared lysates at 4 °C by sequential incubation with 50 μl of Immunopure® Immobilized Protein G (Pierce) for 1 h, 3 μl of V5 antibodies for 2 h, and 50 μl of Immunopure® Immobilized Protein G overnight on an end-over-end rotator. The immobilized proteins were washed twice with cell lysis buffer and then deglycosylated in 45 μl of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 0.5% acetic acid, and 1% β-mercaptoethanol supplemented with 2 units of N-glycosidase F (F. Hoffmann-La Roche, Ltd., Basel, Switzerland) at 37 °C for 18 h. Proteins bound to Immunopure® Immobilized Protein G were eluted twice with 15 μl SDS-PAGE sample buffer, and the eluates were pooled. Proteins in 50 μl of total cell extracts were deglycosylated with 2 units of N-glycosidase F at 37 °C for 18 h. The reactions were stopped with protein gel loading buffer.

**Western Blot Analysis**—Proteins in cell extracts obtained with lysis buffer or immunoprecipitated proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose Hybond™ membranes (Amersham Biosciences UK Ltd., Buckinghamshire, UK) in a Trans-Blot® SD semidry transfer cell (Bio-Rad) at 20 V for 80 min. Immunoblots were washed with water for 5 min, 2 mM NaOH for 10 min, and with water for 5 min. They were then blocked with 5% low fat milk. The epitope-tagged proteins were detected with alkaline phosphatase-labeled monoclonal V5 and myc antibodies (Innogenetix) (diluted 1:3,000 and 1:15,000 in 1% low fat milk, respectively) or with mouse monoclonal myc antibodies 9E10 (diluted 1:50 in 5% low fat milk) (9) and secondary alkaline phosphatase-conjugated goat antibodies to mouse IgG (1:15,000 final dilution in 1% low fat milk) (The Jackson Laboratory, Bar Harbor, ME). Actin as a reference for total protein loading was visualized with monoclonal antibodies to actin (diluted 1:5,000 in 5% low fat milk) (Chemicon International, Temecula, CA) and secondary alkaline phosphatase-conjugated goat antibodies to mouse IgG (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).

**Data Analysis**—The values for half-maximal effective concentrations (EC₅₀) were calculated by non-linear regression analysis using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). The results are means ± S.E. Differences between mean values were analyzed by analysis of variance. p < 0.05 was considered statistically significant.

**RESULTS**

**Total and Cell Surface Expression of Intact and Mutant V5-mCLR and of myc-mRAMP1 and myc-mRAMP2**—The relative expression levels of the V5-mCLR and of the N-terminal deletion mutants and of myc-mRAMP1 and myc-mRAMP2 in transiently transfected COS-7 cells were estimated on Western blots of cell extracts (Fig. 2). The expression of the intact V5-mCLR was the same in the absence and presence of myc-
mRAMP1. The expression levels of the V5-mCLR(Δ14–20) and -(Δ18–20) in the presence of myc-mRAMP1 were comparable and those of V5-mCLR(Δ14–20A) and -(Δ14–17) slightly higher than that of the intact V5-mCLR. Myc-mRAMP1 was expressed at equal levels with intact and mutant V5-mCLR.

The expression levels of the V5-mCLR in the absence and the presence of myc-mRAMP2 were similar and comparable with those of the V5-mCLR(Δ18–20) in the presence of myc-mRAMP2. The V5-mCLR(Δ14–20) and -(Δ14–17) were expressed at lower and the V5-mCLR(Δ14–20A) at higher levels than the intact V5-mCLR in the presence of myc-mRAMP2. Myc-mRAMP2 was lower in the presence of the V5-mCLR(Δ14–17) than together with the V5-mCLR or the other mutants.

The expression of the intact and mutant V5-mCLR and of the myc-mRAMP1 and myc-mRAMP2 at the cell surface was visualized by V5 and myc immunofluorescence staining of intact COS-7 cells (Fig. 3). Cells transfected with the empty pcDNA3 expression vector or with myc-mRAMP1 alone did not stain (Fig. 3A), but myc-mRAMP1 was recognized in permeabilized COS-7 cells (not shown). Thus, myc-mRAMP1 in the absence of the V5-mCLR was not transported to the cell surface. But the V5-mCLR and myc-mRAMP2 expressed alone were both recognized at the cell surface. The cell surface expression of the intact V5-mCLR and of the mutants in the presence of myc-mRAMP1 or myc-mRAMP2 was comparable (Fig. 3B). Moreover, the expression of myc-mRAMP1 and myc-mRAMP2 at the cell surface was the same in cells co-expressing the intact V5-mCLR or the different mutants. Taken together, the mutations introduced into the N-terminal extracellular domain of the V5-mCLR did not affect the expression of the receptors and the RAMP at the cell surface.

Stimulation of cAMP Production—In COS-7 cells transiently co-transfected with myc-mRAMP1 and V5-mCLR expression constructs basal cAMP concentrations were 1.1 ± 0.2 pmol/100,000 cells. The stimulation of cAMP formation by 10−6 M CGRP was 48 ± 9-fold (100%) and indistinguishable in cells expressing myc-mRAMP1/V5-mCLR(Δ14–17), -(Δ14–20A), -(Δ14–20), or -(Δ18–20) (Fig. 4, Table I). The EC50 of CGRP in myc-mRAMP1/V5-mCLR(Δ14–17), -(Δ14–20A), -(Δ14–20)-expressing cells were 0.12 ± 0.01 nM, 0.24 ± 0.12 nM, 1.0 ± 0.2 nM, and 1.5 ± 0.4 nM, respectively. In cells co-expressing myc-mRAMP1 and the V5-mCLR(Δ18–20), on the other hand, the EC50 of CGRP was >100 nM.
values were 0.8 ± 0.2 nM, 9.3 ± 3.4 nM, 59 ± 7.2 nM, and >100 nM, respectively (Fig. 4, Table I). In contrast, in myc-mRAMP2/V5-mCLR-(Δ14–20)-expressing cells the cAMP levels were not affected at up to 10⁻⁷ M AM. Taken together, the deletion of amino acids 14–20 of the extreme N terminus of the mCLR maintains CGRP receptor function in the presence of mRAMP1 but abolishes the mRAMP2-dependent AM receptor activity.

**Cell Surface Association of Intact and Mutant V5-mCLR with myc-mRAMP1 and myc-mRAMP2**—The association at the cell surface of the V5-mCLR or its mutants with myc-mRAMP1 or myc-mRAMP2 was assessed by chemical cross-linking of corresponding myc-mRAMP/V5-mCLR heterodimers in transiently transfected, intact COS-7 cells. Protein components with a V5 epitope tag were immunoprecipitated from cell homogenates and deglycosylated. The extracted proteins were then separated on SDS-PAGE and analyzed on Western blots with V5 and myc antibodies. Predominant V5-immunoreactive protein components with an apparent size of ~50 kDa represented the V5-mCLR and the indicated mutant (Fig. 5). The amounts of the V5-mCLR and of the mutants in the presence of myc-mRAMP1 were comparable and not different from V5-mCLR in the absence of myc-mRAMP1. In the presence of myc-mRAMP2 the amounts of the V5-mCLR and of the V5-mCLR-(Δ14–20A) and -(Δ18–20) were similar, but those of the V5-mCLR-(Δ14–20) and of the V5-mCLR-(Δ14–17) were lower. The amount of the V5-mCLR in the absence of myc-mRAMP2 was also lower than in its presence.

The extracts obtained with V5 antibodies from cells that expressed the V5-mCLR or its mutants together with myc-mRAMP1 contained myc-immunoreactive protein components of 16 kDa and ~66 kDa apparent size. They represented intact myc-mRAMP1 and myc-mRAMP1 cross-linked at the cell surface to the V5-mCLR and the mutants, respectively. This indicated that the deletions and substitutions introduced into the N terminus of the mCLR did not affect the formation and cell surface expression of mCLR/mRAMP1 heterodimers.

Immunoprecipitation with V5 antibodies of cells co-expressing myc-mRAMP2 together with the intact and mutant V5-mCLR revealed predominant myc-immunoreactive protein components of ~18 kDa apparent size. They represented deglycosylated myc-mRAMP2 co-precipitating with the intact and mutant V5-mCLR. Interestingly, receptor/myc-mRAMP2 cell surface cross-linking products of ~70 kDa were only recognized in extracts of cells that co-expressed myc-mRAMP2 together with the intact V5-mCLR or with the mutant V5-mCLR-(Δ14–20A) and -(Δ18–20). All together, this indicated that the deletion of amino acids 14–17 or 14–20 of the extreme N terminus of the mCLR did not affect co-immunoprecipitation of myc-mRAMP2 but prevented chemical cross-linking at the cell surface. Since the V5-mCLR-(Δ14–20A) and myc-mRAMP2 revealed a cross-linking product, the Lys¹⁷ residue is excluded as the cross-linking site between the V5-mCLR and myc-mRAMP2.

**DISCUSSION**

The structures of CLR/RAMP1 or CLR/RAMP2 defining CGRP or AM selectivity are largely unknown. The N-terminal extracellular domains of RAMP1 and RAMP2 determine in part CGRP and AM specificity of the CLR (3). Along these lines, chemical cross-linking of [¹²⁵I]-CGRP or [¹²⁵I]-AM to intact cells expressing the CLR with RAMP1 or RAMP2 revealed [¹²⁵I]-CGRP/RAMP1 and [¹²⁵I]-AM/RAMP2 cross-linking products (10). This indicated that extracellular subdomains of the RAMP are presumably in close proximity and contribute to the CGRP and AM binding sites of CLR/RAMP1 and CLR/RAMP2 heterodimers. Accordingly, RAMP1, through association with the CTR, the closest homologue of the CLR in the family B of G protein-coupled receptors, brings about a CGRP receptor isoform (11). The CTR, on the other hand, unlike the CLR, does not recognize AM in the presence of RAMP2. This indicates that structural elements in the CLR, that are not present in the CTR, are required for the AM receptor function of RAMP2/CLR heterodimers. Interestingly, the overall homology of the amino acid sequences of the CLR and the CTR is 60%, but the extreme N-terminal region of the receptors differs more widely. Here, targeted deletion and alanine substitution mutagenesis in the N-terminal regions of the mCLR identified amino acids 14–20 with the sequence TRNKIMT as a receptor domain that is
required for the high affinity interaction with AM in the presence of mRAMP2.

Deletion of the TRNKIMT sequence abolished the AM receptor function of the V5-mCLR without affecting the expression at the cell surface and heterodimerization with myc-mRAMP2. Chemical cross-linking of myc-mRAMP2 with the V5-mCLR at the surface of intact cells indicates the close proximity of the extracellular domains of the corresponding proteins. Cross-linking was no longer possible when the TRNKIMT sequence of the V5-mCLR was deleted. Cell-surface cross-linking of myc-mRAMP2 to the V5-mCLR brought together, the results demonstrate that structural alterations in the N-terminal extracellular domain of the V5-mCLR revealed the AM receptor function in the presence of mRAMP2 was maintained. The results may imply that the chemical cross-linker BS3 coupled myc-mRAMP2 to the Lys17 residue in the V5-mCLR, but this was ruled out by the substitution of the TRNKIMT sequence by 7 alanine residues revealing myc-mRAMP2/V5-mCLR-(14–20) cross-linking products indistinguishable from myc-mRAMP2/V5-mCLR. Taken together, the results demonstrate that structural alterations in the N-terminal extracellular domain of the V5-mCLR brought about by the deletion of the TRNKIMT sequence inhibit the formation of a high affinity AM recognition site in myc-mRAMP2/V5-mCLR-(14–20) heterodimers. The structural alterations in the N-terminal extracellular domain of the V5-mCLR are less severe when the TRNKIMT sequence is replaced by 7 alanines. Here, low affinity interaction with AM and chemical cross-linking of the myc-mRAMP2/V5-mCLR-(14–20A) complex at the cell surface was maintained.

In an attempt to assign the defect in AM receptor function of the V5-mCLR-(14–20) to parts of the TRNKIMT motif, the TRNK and the IMT sequences were individually deleted. Interestingly, the CGRP and AM receptor functions of the V5-mCLR were minimally affected by the TRNK deletion. The deletion of the IMT sequence, on the other hand, increased the half-maximal concentrations of both CGRP and AM for the stimulation of cAMP formation by 2 orders of magnitude. Thus, minor changes in the amino acid sequence of the mCLR N-terminal extracellular domain impair high affinity interactions of mCLR/mRAMP1 and mCLR/mRAMP2 heterodimers with CGRP and AM. Along these lines, the substitution of Asn17 in a consensus N-glycosylation site of the human CLR by amino acids other than Asp abolished the CGRP and AM receptor functions in the presence of RAM1 and RAM2 (12).

In conclusion, minimal amino acid deletions or substitutions in the N-terminal extracellular domain of the CLR reveal distinct CGRP and/or AM receptor defects of the respective CLR/RAMP1 and CLR/RAMP2 heterodimers. Here, the deletion of the sequence TRNKIMT, corresponding to amino acids 14–20 in the mCLR, selectively abolished its AM receptor function. This observation is important for the development of AM receptor deficient animals to reveal distinct biological functions of AM and CGRP in vivo.

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Selective Inactivation of Adrenomedullin over Calcitonin Gene-related Peptide Receptor Function by the Deletion of Amino Acids 14-20 of the Mouse Calcitonin-like Receptor
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