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**FUNCTIONAL INTERACTION OF
G PROTEIN-COUPLED RECEPTORS OF THE
CALCITONIN PEPTIDE FAMILY WITH
ACCESSORY RECEPTOR-ACTIVITY-
MODIFYING PROTEINS (RAMP)**

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Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ADM	adrenomedullin
Ala	L-alanine
Asn	L-asparagine
Asp	L-aspartic acid
BS3	bis(sulfosuccinimidyl)suberate
BSA	bovine serum albumin
cAMP	cyclic adenosine-5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CGRP	calcitonin gene-related peptide
COS-7	monkey kidney cell line
CRLR	calcitonin receptor-like receptor
CT	calcitonin
CTR	calcitonin receptor
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
EC ₅₀	half-maximal effective concentration
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
Gln	L-glutamine
h, r	human, rat
HEK	human embryonic kidney cell line
Hepes	4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid
HRP	horseradish peroxidase
IBMX	isobutylmethylxanthine
IC ₅₀	half-maximal inhibitory concentration
mRNA	messenger ribonucleic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pro	L-proline
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
RAMP	receptor-activity-modifying protein
S2	Drosophila Schneider 2
SDS	sodium dodecyl sulfate
SEM	standard error mean

Ser	L-serine
Thr	L-threonine
Tris	Tris(hydroxymethyl)aminoethane
TRITC	tetramethyl rhodamine isothiocyanate
TSA	SV40 T-antigen transformed HEK cells
VIP	vasoactive intestinal polypeptide

Summary

The calcitonin family of peptides includes calcitonin (CT), α - and β -calcitonin gene-related peptide (CGRP), adrenomedullin (ADM) and amylin. They exhibit sequence homology, including ring structures linked by a disulfide bridge between cysteine residues and amidated carboxyl-termini. The structural similarities result in overlapping biological activities such as vasodilatation and inhibition of bone resorption. In 1991, the structure of the CT receptor was identified by the group of Goldring (Harvard) through expression cloning. Two years later when the CT receptor-like receptor (CRLR) was discovered by Flühmann et al. (Zürich), its 60% sequence identity with the CT receptor suggested members of the CT peptide family as candidate ligands, but the CRLR failed to respond to any of them and therefore was deemed an orphan receptor. With the discovery of novel accessory proteins called receptor-activity-modifying proteins (RAMP) by Foord et al. (GlaxoWellcome) in 1997, a new mechanism for the regulation of G protein-coupled receptors was revealed. RAMPs constitute a group of three single transmembrane spanning proteins, designated RAMP1, -2 and -3 consisting of 148 to 175 amino acids. The expression of RAMP1 and -3 with previously cloned orphan CRLR reveals CGRP receptors whereas co-transfection with RAMP2 or -3 generates ADM receptors. Without co-expressed RAMP the CRLR remains inactive. In contrast, the CT receptor does not require RAMP for functional expression, but in the presence of RAMP1, the CT receptor recognizes CGRP and amylin, and together with RAMP3 amylin alone.

In my dissertation, I have investigated possible mechanisms of the human RAMP activity which is required for modulated receptor specificity. Differential glycosylation of human (h) and rat (r) CRLR, dependent on co-expressed RAMP, was considered to define CGRP or ADM receptors in mammalian cells. In the presence of RAMP1, CRLR was transported to the cell surface as a mature glycoprotein whereas core glycosylation was observed when CRLR was expressed alone or together with RAMP2. Studies in *Drosophila* Schneider 2 (S2) cells, stably expressing rCRLR together with RAMP1 or -2, revealed CGRP and ADM receptors that exhibited the pharmacological profile and coupling to cyclic AMP production indistinguishable from that of the CRLR and RAMP co-transfected mammalian cells. However, rCRLRs expressed in S2 cells were uniformly glycosylated proteins, independent of the presence of RAMP1 or -2. Thus, the pattern of CRLR glycosylation does not define ligand specificity in *Drosophila* Schneider 2 cells.

Crosslinking of [125 I]h α CGRP and [125 I]rADM to S2 cells co-expressing V5-tagged rCRLR and V5-tagged RAMP1 or -2 revealed the formation of rCRLR/RAMP1 and rCRLR/RAMP2 heterodimers on the cell surface that function as specific CGRP and ADM receptors. In cell extracts not treated with crosslinker the individual protein components with the size of rCRLR, RAMP1 and -2 were observed. Thus, direct interaction of RAMP with CRLR defines ligand specificity of the receptor for CGRP or ADM.

The functional relevance of the glycosylation of myc-tagged hCRLR was investigated through suppression of N-glycosylation with tunicamycin and site directed mutagenesis. Three putative N-glycosylation sites Asn⁶⁰, Asn¹¹² and Asn¹¹⁷ are present in the amino-terminal extracellular domain of the hCRLR. In human embryonic kidney (TSA) cells co-expressing myc-hCRLR and RAMP1 or -2, tunicamycin dose-dependently inhibited the glycosylation of the myc-hCRLR and in parallel [125 I]h α CGRP and [125 I]hADM binding. Site directed mutagenesis of Asn to Thr identified Asn⁶⁰ and Asn¹¹² as the predominant N-glycosylation sites in myc-hCRLR. Mutation of one of these two sites minimally decreased the expression of myc-hCRLR as CGRP or ADM receptor. But substitution of both Asn⁶⁰ and Asn¹¹² by Thr reduced cell surface expression and, as a result [125 I]h α CGRP and [125 I]hADM binding. The mutation of Asn¹¹⁷ to Thr revealed a receptor mutant which is delivered to the cell surface, but specific [125 I]h α CGRP and [125 I]hADM binding was not detectable. Thus, N-glycosylation of hCRLR at Asn⁶⁰ and Asn¹¹² is important for cell surface expression whereas Asn¹¹⁷ is essential for the interaction between ligand, receptor and RAMP.

With regard to CGRP receptor function, cell surface expression and association with RAMP1, further mutants of myc-hCRLR containing substitutions of Asn¹¹⁷ by Ala, Asp, Gln and Pro were investigated in cells expressing the individual Asn¹¹⁷ mutants together with RAMP1. CGRP-evoked stimulation of cyclic AMP formation was impaired by Asn¹¹⁷ to Ala or Gln substitutions and abolished when Asn¹¹⁷ was replaced by Thr or Pro. In contrast, CGRP receptor activity of the myc-hCRLR was not affected when Asn¹¹⁷ was substituted by Asp. Myc-immunofluorescence-staining of intact cells revealed cell surface expression of all Asn¹¹⁷ mutants similar to the non-modified myc-hCRLR. In TSA cells expressing V5-tagged RAMP1 together with myc-hCRLR and the various Asn¹¹⁷ mutations, cell surface crosslinking and co-immunoprecipitation revealed the formation of hCRLR/RAMP1 complexes on the cell surface. However, crosslinked [125 I]h α CGRP was only detected when non-modified myc-hCRLR or the Asp mutant were co-expressed with RAMP1. Moreover, V5-

tagged RAMP1 was shown to be co-immunoprecipitated with myc-hCRLR even in the absence of crosslinker, indicating direct molecular interaction of RAMP1 with the receptor protein. Thus, substitution of Asn¹¹⁷ by Ala, Asp, Gln, Pro and Thr does not affect N-glycosylation, cell surface expression and association with RAMP1. But with the exception of the Asn¹¹⁷ to Asp mutant the interaction with the receptor ligand CGRP is abolished.

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Zusammenfassung

Zur Calcitonin-Peptidfamilie gehören Calcitonin (CT), α - und β -*calcitonin gene-related peptide* (CGRP), Adrenomedullin (ADM) und Amylin. Allen gemeinsam sind amino-terminale Ringstrukturen, die durch Disulfidbrücken zustande kommen und amidierete Carboxyl-Enden. Die strukturellen Ähnlichkeiten führen zu überlappenden biologischen Wirkungen wie Gefässerweiterung und Hemmung des Knochenabbaus. 1991 wurde die Struktur des CT Rezeptors durch die Gruppe von Goldring (Harvard) mittels Expressions-Klonierung identifiziert. Als zwei Jahre später der *CT receptor-like receptor* (CRLR) durch Flühmann et al. (Zürich) entdeckt wurde, deutete dessen 60% -ige Sequenzhomologie zum CT Rezeptor darauf hin, dass Mitglieder der Calcitonin-Peptidfamilie als mögliche Liganden in Frage kämen. Da jedoch kein Ligand für den CRLR gefunden werden konnte, wurde er als „Waisenrezeptor“ publiziert. Durch die Entdeckung neuartiger, akzessorischer Proteine, genannt *receptor-activity-modifying proteins* (RAMP) durch Foord et al. (GlaxoWellcome) 1997, wurde ein neues Konzept für die Regulierung G Protein-gekoppelter Rezeptoren aufgezeigt. Bis anhin sind drei verschiedene RAMPs, genannt RAMP1, -2 und -3 bekannt, die eine einzige Transmembrandomäne aufweisen und aus 148 bis 175 Aminosäuren bestehen. Die Expression von RAMP1 und -3 mit dem ursprünglich als „Waisenrezeptor“ beschriebenen CRLR führt zur Bildung von CGRP Rezeptoren. Zusammen mit RAMP2 oder -3 bildet der CRLR ADM Rezeptoren. Ohne koexprimiertes RAMP hingegen bleibt der CRLR inaktiv. Im Gegensatz dazu wird für die funktionelle Expression des CT Rezeptors kein RAMP benötigt. Wird der CT Rezeptor jedoch zusammen mit RAMP1 exprimiert, so kann er CGRP und Amylin erkennen und mit RAMP3 lediglich Amylin.

In meiner Dissertation habe ich mögliche Mechanismen der RAMP Aktivität, welche für die modulierte Rezeptorspezifität benötigt wird, untersucht. In Säugetierzellen wurde die unterschiedliche Glykosylierung des humanen (h) und Ratten (r) CRLR in Abhängigkeit von ko-exprimiertem RAMP als möglicher Mechanismus für die modulierte Ligandspezifität des Rezeptors erachtet. In Anwesenheit von RAMP1 wurde der CRLR als terminal glykosylierter Rezeptor an die Zelloberfläche transportiert. Wurde er hingegen alleine oder zusammen mit RAMP2 exprimiert, so wies er lediglich eine Grund-Glykosylierung auf. Untersuchungen in *Drosophila Schneider 2* (S2) Zellen, welche den rCRLR zusammen mit RAMP1 oder -2 stabil exprimierten, zeigten die Expression von CGRP und ADM Rezeptoren, die bezüglich Ligandspezifität, -affinität

und Stimulation der zyklischen AMP Produktion von denjenigen in Säugetierzellen nicht zu unterscheiden waren. In S2 Zellen führte die Expression des rCRLR jedoch zu einem Glykoprotein, dessen Glykosylierung, unabhängig von RAMP1 oder -2, unverändert blieb. Folglich wird die Ligandspezifität des CRLR, zumindest in *Drosophila Schneider 2* Zellen, nicht durch das Glykosylierungsmuster des Rezeptors bestimmt. Crosslinking von [¹²⁵I]hαCGRP und [¹²⁵I]rADM an S2 Zellen, die V5-markierten rCRLR und V5-markiertes RAMP1 oder -2 exprimierten, führte zur Bildung von rCRLR/RAMP1 und rCRLR/RAMP2 Heterodimeren an der Zelloberfläche, die als spezifische Rezeptoren CGRP und ADM erkennen. Die einzelnen Proteinkomponenten rCRLR, RAMP1 und -2 wurden in Zellextrakten beobachtet, die nicht mit Crosslinker behandelt wurden. Somit bestimmt die direkte Interaktion von RAMP mit dem CRLR die Ligandspezifität des Rezeptors für CGRP und ADM.

Die funktionelle Bedeutung der Glykosylierung von myc-markiertem hCRLR wurde durch Hemmung der N-Glykosylierung mit Tunicamycin und durch Mutationen der N-Glykosylierungsstellen untersucht. Der hCRLR weist drei potentielle N-Glykosylierungsstellen bei Asn⁶⁰, Asn¹¹² und Asn¹¹⁷ am amino-terminalen Ende auf. In human embryonalen Nierenzellen (TSA), die myc-hCRLR und RAMP1 oder -2 koexprimierten, hemmte Tunicamycin dosisabhängig die Glykosylierung des myc-hCRLR und parallel dazu die [¹²⁵I]hαCGRP und [¹²⁵I]rADM Bindung. Durch die Mutation von Asn zu Thr wurden Asn⁶⁰ und Asn¹¹² als vorherrschende N-Glykosylierungsstellen des myc-hCRLR identifiziert. Die Mutation von nur einer dieser beiden Stellen verminderte die Expression des myc-hCRLR als CGRP oder ADM Rezeptor nur gering. Der gleichzeitige Austausch von Asn⁶⁰ und Asn¹¹² durch Thr bewirkte hingegen eine Reduktion der Zelloberflächenexpression und als Folge davon eine verminderte [¹²⁵I]hαCGRP und [¹²⁵I]rADM Bindung. Die Mutation von Asn¹¹⁷ zu Thr führte zur Bildung einer Rezeptormutante, die zwar an die Zelloberfläche transportiert wurde, deren spezifische [¹²⁵I]hαCGRP und [¹²⁵I]rADM Bindung jedoch nicht nachgewiesen werden konnte. Die N-Glykosylierung bei Asn⁶⁰ und Asn¹¹² des myc-hCRLR ist somit wichtig für dessen Zelloberflächenexpression. Asn¹¹⁷ hingegen ist wesentlich für die Interaktion zwischen Ligand, Rezeptor und RAMP.

Im Hinblick auf die CGRP Rezeptorfunktion, der Zelloberflächenexpression und der Assoziation mit RAMP1, wurden weitere myc-hCRLR Mutanten untersucht bei denen Asn¹¹⁷ durch Ala, Asp, Gln oder Pro ersetzt wurde. Die neuen Mutanten wurden jeweils zusammen mit RAMP1 exprimiert. Die durch CGRP hervorgerufene Stimulation der zyklischen

AMP Bildung war vermindert durch den Austausch von Asn¹¹⁷ durch Ala oder Gln und wurde vollständig aufgehoben bei Substitution von Asn¹¹⁷ durch Thr oder Pro. Im Gegensatz dazu wurde die CGRP Rezeptor Aktivität des myc-hCRLR nicht beeinflusst, wenn Asn¹¹⁷ durch Asp ersetzt wurde. Die myc-Immunfluoreszenzfärbung intakter Zellen liess eine Zelloberflächenexpression aller Asn¹¹⁷ Mutanten erkennen, die vergleichbar war mit derjenigen von nicht-modifiziertem myc-hCRLR. In TSA Zellen, die V5-markiertes RAMP1 zusammen mit myc-hCRLR und den verschiedenen Asn¹¹⁷ Mutanten exprimierten, führte crosslinking und Ko-Immunopräzipitation zur Bildung von hCRLR/RAMP1 Komplexen an der Zelloberfläche. Gecrosslinktes [¹²⁵I]hαCGRP wurde jedoch nur beim nicht-modifizierten myc-hCRLR und der Asp Mutante, koexprimiert mit RAMP1, nachgewiesen. Zudem wurde gezeigt, dass V5-markiertes RAMP1 auch in Abwesenheit von crosslinker mit myc-hCRLR koimmunopräzipitiert werden konnte und somit auf eine direkte molekulare Interaktion zwischen RAMP1 und dem Rezeptor hinweist. Die N-Glykosylierung, die Zelloberflächenexpression und die Assoziation mit RAMP1 wird durch die Substitution von Asn¹¹⁷ durch Ala, Asp, Gln, Pro und Thr nicht beeinflusst. Die Interaktion mit dem Rezeptorliganden CGRP hingegen ist, mit Ausnahme der Asp Mutante, aufgehoben.

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Introduction

G protein-coupled receptors

G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors. Over 100 structures have so far been defined in mammals. Many have been identified by homology cloning, in which hybridisation with existing cDNA probes is used to detect related DNA sequences. Other family members have been found by expression cloning, using their ligand-binding or cell-activation properties to identify them [1]. The visual pigments were the first G protein-coupled receptors for which sequence data were obtained. In the early 1980s, the complete amino acid sequences for bovine and ovine rhodopsin were reported and found to display marked similarities. When the gene encoding a mammalian β -adrenergic receptor was subsequently cloned, it was apparent from the deduced sequence that this receptor exhibited a structure similar to that of the rhodopsins, and suggested the existence of a family of signal receptors [2].

GPCRs are involved in many physiological processes and are attractive targets for pharmacological intervention to modify these processes in normal and pathological states [3]. GPCRs share a predicted structure consisting of an extracellular amino-terminus, seven transmembrane-spanning α -helices connected by alternating extracellular and intracellular loops, and a cytoplasmic carboxyl-terminal region [4]. The amino-terminal sequence of most GPCRs contains putative sites for N-linked glycosylation [2].

Intracellular signaling

GPCRs respond to a wide variety of stimuli including peptide hormones, neurotransmitters, large glycoproteins, photons and ions [5]. The core formed by the arrangement of the seven transmembrane-spanning helices and the extracellular domains provide specific recognition/binding sites for ligands, while the intracellular portions interact with heterotrimeric guanine nucleotide-binding proteins (G proteins) to initiate signaling [4]. G proteins are membrane-associated heterotrimeric proteins composed of α -, β -, and γ -subunits (Fig. 1) [6].

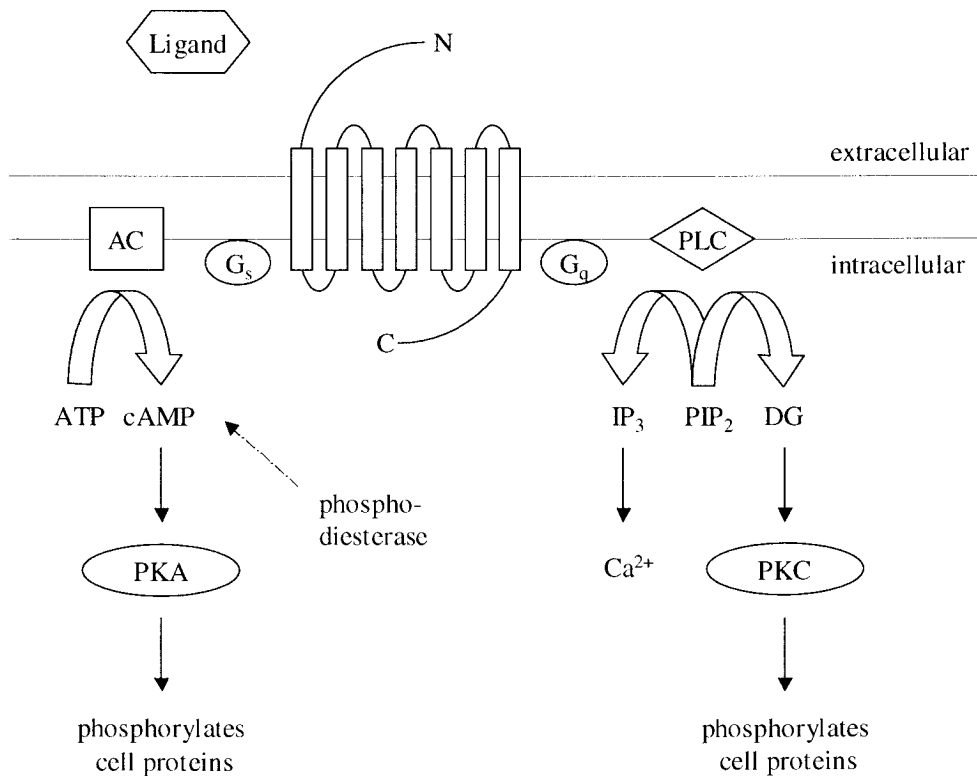


Fig. 1. The cAMP and the inositol phospholipid signaling pathway of GPCRs. Following binding of an extracellular ligand, a conformational change of the GPCR is transmitted to a stimulatory G protein (G_s) resulting in activation of adenylyl cyclase (AC), a plasma membrane-bound enzyme. AC synthesizes cAMP, which activates protein kinase A (PKA) and leads to phosphorylation of target proteins. Some GPCRs bind to a G_q protein (G_q) and activate phospholipase C (PLC), which cleaves phosphatidylinositol bisphosphate (PIP₂) to generate inositol trisphosphate (IP₃) and diacylglycerol (DG). IP₃ releases Ca²⁺ from the endoplasmic reticulum into the cytosol and DG activates protein kinase C (PKC).

Activation of GPCRs results in the modulation of intracellular second messenger levels via the coupling of receptors to a wide variety of effector systems via G proteins. Following agonist binding, receptors activate G proteins by catalysing the exchange of bound GDP for GTP on the G protein α -subunit. Binding of GTP leads to the dissociation of the $G\alpha$ -subunit from the $G\beta\gamma$ -subunits, which when dissociated regulate the activity of effector systems such as adenylyl cyclase and phospholipase C depending on the G proteins involved [5]. Signaling is terminated when the $G\alpha$ -subunits, which have slow intrinsic GTPase activities, hydrolyse GTP, return to the GDP-bound state, and reassociate with $G\beta\gamma$ -dimers to form inactive heterotrimers [7].

Two of the most widely used intracellular molecules are cyclic AMP (cAMP) and cytosolic free Ca^{2+} . The former is synthesized from ATP by the plasma membrane-bound enzyme adenylyl cyclase, and is inactivated by cAMP phosphodiesterases. cAMP exerts its effects by activating the enzyme protein kinase A, which catalyses the transfer of the terminal phosphate group from ATP to specific serines or threonines of selected proteins to regulate the activity of target proteins [1].

The activation of membrane-bound phospholipase C, which cleaves phosphatidylinositol bisphosphate (PIP_2) upon stimulation of GPCRs results in the formation of second messengers, diacylglycerol (DG), a direct activator of protein kinase C, and inositol trisphosphate (IP_3). The latter binds to receptors on the endoplasmic reticulum, causing transient release of calcium from the endoplasmic reticulum into the cytosol [8].

B family of G protein-coupled receptors

Based on primary amino acid sequence homology, the superfamily of G protein-coupled receptors can be divided into several subfamilies. Family B of GPCRs, which typically recognizes regulatory peptides, includes receptors for calcitonin (CT), parathyroid hormone (PTH), secretin, glucagon, vasoactive intestinal peptide (VIP), GH-releasing hormone, and pituitary adenylyl cyclase-activating polypeptide (PACAP) [9]. The amino acid sequence identity within this subfamily is over 30% (Fig. 2).

In 1991, the CT receptor was cloned by expression of a cDNA library from a porcine kidney epithelial cell line. By interacting with distinct G proteins, CT receptors have been shown to stimulate adenylyl cyclase and phospholipase C [10]. Two isoforms of the human CT receptor (hCTR) have been identified, which differ by the presence (hCTR1) or absence (hCTR2) of a 16-amino acid insert within the first intracellular loop. The

presence of the insert attenuates coupling to the phospholipase C signaling pathway [11]. Additional receptor isoforms occur through alternative splicing of initial transcription products of single genes.

In 1993, novel seven transmembrane receptors, named calcitonin receptor-like receptors (CRLR), were identified through molecular cloning from rat and human cerebellum cDNA libraries [12, 13]. Despite 60% sequence identity with cloned CT receptors, CRLR failed to respond to any of the CT family of peptides and therefore was originally considered as an orphan receptor [13]. In situ hybridisation analysis of CRLR mRNA during rat development revealed predominant expression in the lung and in telencephalic areas of the brain [14].

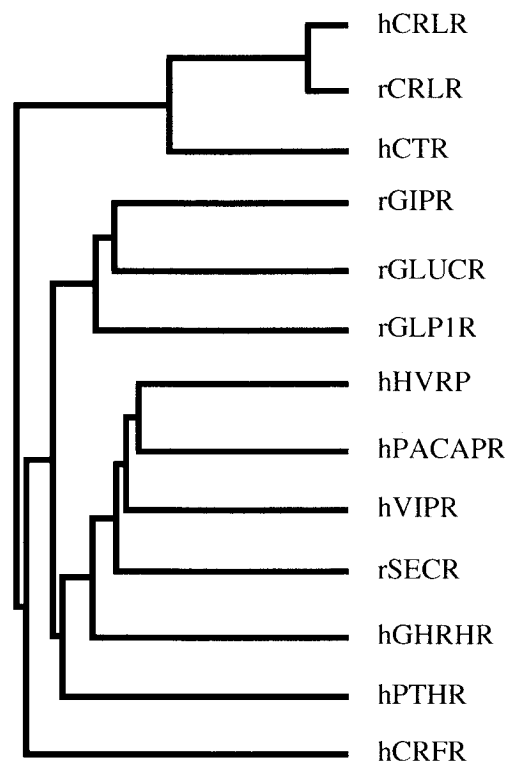


Fig. 2. **Structural relationships within the B family of GPCRs.** Besides human (h) and rat (r) calcitonin receptor-like receptor (CRLR), the B family of GPCRs includes the human calcitonin receptor (hCTR), the rat gastric inhibitory peptide receptor (rGIPR), the rat glucagon receptor (rGLUCR), the rat glucagon-like peptide 1 receptor (rGLP1R), the human helodermin-preferring vasoactive intestinal peptide receptor (hHVRP), the human pituitary adenylate cyclase-activating polypeptide receptor (hPACAPR), the human vasoactive intestinal peptide receptor (hVIPR), the rat secretin receptor (rSECR), the human growth hormone-releasing hormone receptor (hGHRHR), the human parathyroid hormone receptor (hPTHr) and the human corticotropin-releasing factor receptor (hCRFR) [13].

Calcitonin family of peptides

Calcitonin (CT), calcitonin gene-related peptide (CGRP), adrenomedullin (ADM) and amylin are structurally related peptides that belong to the calcitonin peptide family (Fig. 3). They have in common an amino-terminal ring structure of 6- or 7-amino acids linked by a disulfide bridge between cysteine residues, and amidated carboxyl-termini required for biological activity [15-17]. Carboxyl-terminal fragments of CT, CGRP and ADM lacking the ring structure are antagonists [18]. Structural similarities between the peptides of the calcitonin family result in overlapping biological actions mediated through cross-reaction with their specific receptors.

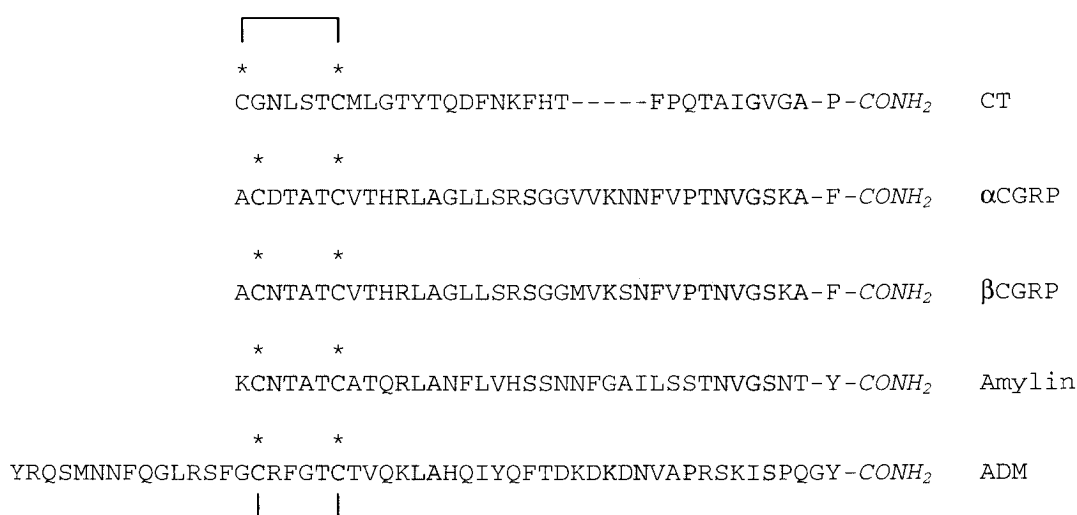


Fig. 3. Comparison of the amino acid sequences of the CT family of peptides. Homologous regions of human CT, α- and βCGRP, amylin and ADM are shaded. Cysteine residues involved in disulfide linkage are indicated by asterisks.

Calcitonin

Calcitonin, a polypeptide of 32 amino acids, was discovered in 1961 as a hypocalcaemic hormone [19]. CT is synthesized and secreted from thyroid C-cells in response to increased extracellular calcium concentrations. CT lowers serum calcium levels through inhibition of osteoclastic bone resorption and stimulation of the urinary calcium excretion. Inhibition of bone resorption is obtained through suppression of osteoclastic activity

mediated by stimulation of cyclic AMP and raised cytosolic calcium. In this way CT regulates calcium homeostasis and maintains skeletal mass during periods of calcium stress, as during growth, pregnancy, and lactation [10]. In the kidney, CT stimulates the excretion of calcium, phosphate, sodium and chloride through inhibition of the tubular reabsorption. Central actions of CT include antinociceptive properties as well as inhibition of gastric secretion and inhibition of food intake [20].

CT receptors have been recognized in osteoclasts, in the kidney cortex and in the central nervous system [21].

Calcitonin gene-related peptide

Calcitonin gene-related peptide is a neuropeptide generated by alternative tissue-specific splicing of the initial mRNA transcript of the CT gene [22-24]. Two forms of CGRP, α - and β CGRP, differing in 3 and 1 of the 37 amino acid residues, respectively, have been identified in man and rat.

CGRP is synthesized in neurons of the central and peripheral nervous system and released through stimulation of voltage-dependent calcium channels from afferent nerve fibres. CGRP acts mainly locally in paracrine fashion. CGRP is the most potent vasodilator acting directly on vascular smooth muscle through activation of adenylyl cyclase. In the heart, CGRP has positive chronotropic and inotropic effects [15]. In addition to its cardiovascular actions, CGRP enhances the glomerular filtration rate, renal blood flow, and the secretion of renin [25-27]. Moreover, CGRP inhibits gastric acid secretion [28, 29].

Specific CGRP binding sites have been identified throughout the nervous system, including the cerebellum, brainstem, and spinal cord, and among peripheral organs in the heart, spleen, liver, kidney, and skeletal muscle [30].

Amylin

Amylin is a 37 amino acid peptide synthesized and co-secreted with insulin from pancreatic β -cells in response to glucose. Amylin is also known as diabetes associated peptide or islet amyloid polypeptide (IAPP), reflecting its original identification as the major peptide product of pancreatic amyloid plaques in type II diabetic patients [31]. Amylin may be involved in the pathogenesis of non-insulin-dependent diabetes mellitus. But, the precise role of amylin remains to be elucidated [32].

Biological actions of amylin include inhibition of glucose-stimulated insulin release and inhibition of insulin-stimulated glucose uptake and glycogen synthesis in the skeletal musculature [33]. Moreover, amylin acts to potently inhibit gastric emptying, postprandial glucagon secretion, and food intake [34]. In the kidney, amylin regulates ion transport and enhances the activity of renin [35].

Consistent with its functional targets, specific amylin binding sites have been recognized in brain, kidney and skeletal muscle [36-39]. Interaction of amylin with a CT/CGRP receptor may mediate the vasodilatory effects and inhibition of osteoclast-mediated bone resorption [10].

Adrenomedullin

Adrenomedullin was isolated in 1993 from human pheochromocytoma, a tumor arising from the adrenal medulla [40]. Unique among the CT family of peptides, human and rat ADM of 52 and 50 amino acids have linear amino-terminal extensions of the ring structure of 15 and 13 amino acids, respectively [41, 42]. But, removal of these amino acids does not result in reduced biological activity.

ADM is synthesized and secreted from the adrenal medulla, endothelium and vascular smooth muscle cells. Since the discovery of ADM, it has attracted intensive interest for hypertension research because of its powerful vasodepressor effect [43]. The hypotensive activity of ADM is comparable to that of CGRP, which has been established as one of the most potent vasorelaxants [44]. It is supposed that cross-reaction of ADM with CGRP receptors mediates, at least in part, the vasodilatory response caused by ADM. Moreover, ADM plays an important role in the regulation of renal function showing diuretic and natriuretic effects [45].

Specific ADM binding sites have been recognized in heart, lung, spleen, liver, brain cortex, cerebellum, and skeletal muscle [46, 47].

Taken together, CT, CGRP, ADM and amylin have overlapping biological effects owing to their related structures and cross-reaction between receptors (Fig. 3, Table 1). The highest sequence homology is about 45% between human CGRP and amylin, followed by about 25% between human CGRP and ADM. Human CT shares approximately 15% of the amino acids with human CGRP and amylin [15, 21, 32].

Amino acid sequences from various mammalian species of CT, CGRP and amylin suggest that all three peptides have been highly conserved. The invariant residues are clustered at the amino- and carboxyl-termini of these molecules, therefore implying their importance for biological activity.

CT, CGRP, ADM and amylin have different distributions in various organs. CGRP is a widely distributed neuropeptide, CT is a hormone mainly located in the C-cells of the thyroid, and amylin is located in the β -cells of the pancreas, while adrenomedullin is predominantly localized in the adrenal medulla [10].

Table 1. **Biological effects of CT, CGRP, ADM and amylin.^a**

	CT	CGRP	ADM	Amylin
Cardiovascular effects				
Vasodilatation	↑	↑↑	↑↑	↑
Chronotropic action	- ^b	↑	↑/-	↑
Inotropic action	-	↑	↑/-	↑
Gastrointestinal effects				
Food intake	↓	↓	ND ^c	↓
Gastric acid secretion	↓	↓	ND	ND
Renal effects				
Blood flow	-	↑	ND	ND
Fractional excretion (Na, Cl, Ca, PO ₄)	↑	-	ND	ND
Osteoclastic bone resorption				
	↓↓	↓	ND	↓
Serum calcium				
	↓	-	-	↓
Pain perception				
	↓	↑/↓	ND	ND
Glycogen synthesis in skeletal muscle				
	↓	↓	ND	↓

^a modified after [10, 15]

^b no effect

^c ND, not done

Receptor-activity-modifying proteins

Rat and human CT receptor-like receptor (CRLR) have been cloned as orphan receptors since initially a specific ligand could not be found [12, 13]. In 1997, a 14 kDa protein, designated human receptor-activity-modifying protein 1 (hRAMP1) was identified and cloned from CGRP receptor expressing human SK-N-MC neuroblastoma cells in *Xenopus* oocytes where it enhanced the stimulation of cyclic AMP (cAMP) production by endogenous CGRP receptors [48].

Subsequently, CRLR became a functional CGRP receptor when co-transported with RAMP1 to the cell surface. Neither RAMP1 nor CRLR induced significant responses to CGRP when transfected alone, but expression of both produced cells that responded to CGRP with specific binding of [¹²⁵I]-labelled CGRP and activation of adenylyl cyclase.

Human RAMP1 is a 148 amino acid protein with an amino-terminal signal sequence and a putative single transmembrane region close to the carboxyl-terminus (Fig. 4). Limited nucleotide sequence homology of hRAMP1 encoding cDNA to expressed sequence tags in public databases has been used for identification of structurally related hRAMP2 and -3 of 175 and 148 amino acids, respectively. They share 30% sequence identity with hRAMP1. Hydrophobicity plots of the RAMP amino acid sequences are similar despite low sequence homology. In the amino-termini, four cysteine residues are conserved, suggesting some common secondary structure. RAMPs are type I membrane proteins with a relatively long extracellular amino-terminus, a single transmembrane domain, and a short intracellular carboxyl-terminus.

The hRAMP1 gene is expressed in many tissues, including the uterus, bladder, brain, pancreas and gastrointestinal tract. Human RAMP2 and -3 have similar, but not identical, tissue distributions and are expressed strongly in the lung, breast, immune system and fetal tissues.

Co-transfection of RAMP2 or -3 with CRLR leads to its expression at the cell surface as an ADM receptor. This change in specificity of CRLR as CGRP or ADM receptor, dependent on the co-expressed RAMP, correlates in mammalian cells with differential glycosylation of the receptor. RAMP1 presents CRLR at the plasma membrane as a terminally glycosylated, mature glycoprotein whereas RAMP2 and -3 present CRLR as an immature, core glycosylated receptor. Without the co-expression of RAMPs the CRLR is not expressed at the cell surface.

```

hRAMP1 -----MARALCRLPRRGLWL----LLAHHLFMTTACQE-----ANYG
hRAMP2 MASLRVERA-GGPRLPRTVRVGRPAAVRLLLLLLGAVLNPHEALAQPLPTTG
hRAMP3 -----METGALRRPQ-----LLPLLLLLCGGCPRAGG--CNETG
      ←----- Signal peptide -----→

                                     *
hRAMP1 -----ALLRELCLTQFQVDMEAVGETLWCDWGRTIRSYRELAD
hRAMP2 TPGSEGGTVKKNYETAVQFCWNHYKDQMDPIEK-DWCDWAMISRPYSTLRD
hRAMP3 -----MLERLPLCGKAFADMMGKVDVWVKWCNRAEFIVYYESFTN

                                     *
hRAMP1 CTWHMAEKLGCFWPNAEVD RFFLA VHGRYFRSCPI SGRAVRDPPGSILYP
hRAMP2 CLEHFAELFDLGFNPPLAERII FETHQIHFANCSLVQPTFSDPPEDVLLA
hRAMP3 CTEMEANVVGCYWPNPLAQGFITGIHRQFFSNCTVDRVHLEDPPDEVLI P
                                                                 ←-----

hRAMP1 FIVVPITVTLVLTALVVWQSKRTEGIV
hRAMP2 MIIAPICLIPFLITLVVWRSKDSEAQA
hRAMP3 LIVIPVVLTVAMAGLVVWRTRKRTDTLL
      ----- TM domain ----->

```

Fig. 4. Comparison of the amino acid sequences of human RAMPs 1, 2 and 3. Putative signal sequences and transmembrane (TM) domains are indicated. Conserved amino acids are shaded and conserved cysteine residues are indicated by asterisks [48].

Chimeric proteins have been created in which the transmembrane and cytosolic portions of RAMP2 were associated with the amino terminus of RAMP1 (RAMP1/2) and vice versa (RAMP2/1). In HEK293T cells, RAMP1/2 and RAMP2/1 formed CGRP or ADM receptors much like native RAMP1 and RAMP2 co-expressed with CRLR. The extracellular amino-termini of RAMPs are considered to be important for ligand specificity [49].

The discovery of RAMPs provided a novel mechanism to define receptor specificity. Possible mechanisms of RAMP activity include the glycosylation of CRLR which, in turn, correlates with receptor phenotypes (Fig. 5a) or RAMPs may be directly involved in the binding of ligand to the receptor (Fig. 5b).

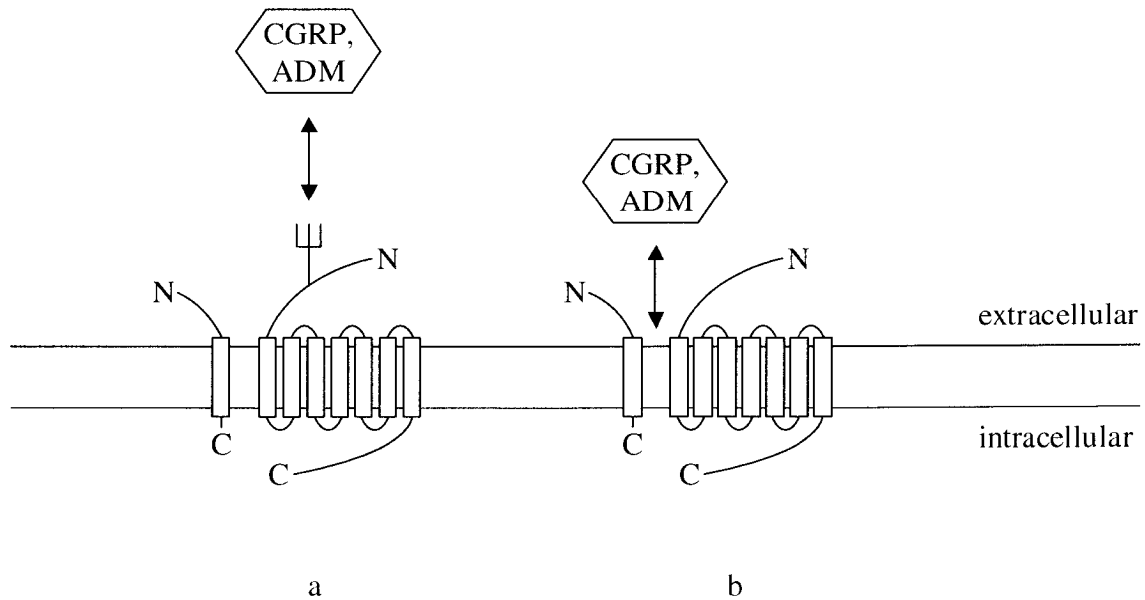


Fig. 5. Possible mechanisms of RAMP activity in regulating the specificity of CRLR for CGRP or ADM. The glycosylation state (ψ) of CRLR may determine which ligand, CGRP or ADM, binds to the receptor (a). RAMP may define CRLR specificity by participating directly in ligand binding (b).

Glycosylation

Many proteins presented at the external surface of mammalian cells are glycosylated. They are predominantly N-glycosylated, i.e. they contain oligosaccharides covalently linked to an asparagine residue of a polypeptide chain within the consensus peptide sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. Some membrane proteins are O-glycosylated, with the carbohydrate chains attached to serine or threonine residues. The importance of O-glycosylation has yet to be elucidated [50]. A variety of roles have been suggested for N-linked oligosaccharides of glycoproteins, including involvement in the binding of ligands and signal transduction, trafficking of the receptor to the cell membrane, and contribution to the overall conformation of the receptor [51].

Synthesis and trimming of N-linked oligosaccharides

The covalent addition of sugars to proteins takes place in the endoplasmic reticulum (ER) [52]. A core unit of 14 saccharides ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) (Fig. 6) is synthesized as a dolichol pyrophosphate precursor by enzymes located in the ER membrane. It is then transferred to the asparagine side chain of Asn-X-Ser/Thr consensus sequences by a membrane-bound oligosaccharyl transferase. The addition of core oligosaccharides occurs as the nascent polypeptide emerges into the lumen of the ER through a translocation complex that forms a channel through the rough ER membrane. Immediately after addition of the core oligosaccharide, trimming begins with the removal of the three terminal glucose residues and one mannose residue. As a result, most glycoproteins leave the ER with $\text{Man}_8\text{GlcNAc}_2$ side chains. Upon entry into the Golgi complex, the majority of the oligosaccharide chains undergo further trimming followed by terminal glycosylation.

For most glycoproteins N-linked oligosaccharides play a central role in the conformational maturation, but individual glycoproteins depend on their oligosaccharide side chains to varying degrees and in different ways. Some need them for structure and stability during folding and some continue to rely on them throughout their existence. Finally, there are numerous proteins that do not require their N-linked sugars during folding [53]. Mutagenesis of the Asn amino acid in the consensus sequence Asn-X-Ser/Thr is sufficient to prevent transfer of carbohydrate moieties to proteins. This approach makes it possible to investigate the contribution of single carbohydrate moieties in multiglycosylated proteins [54].

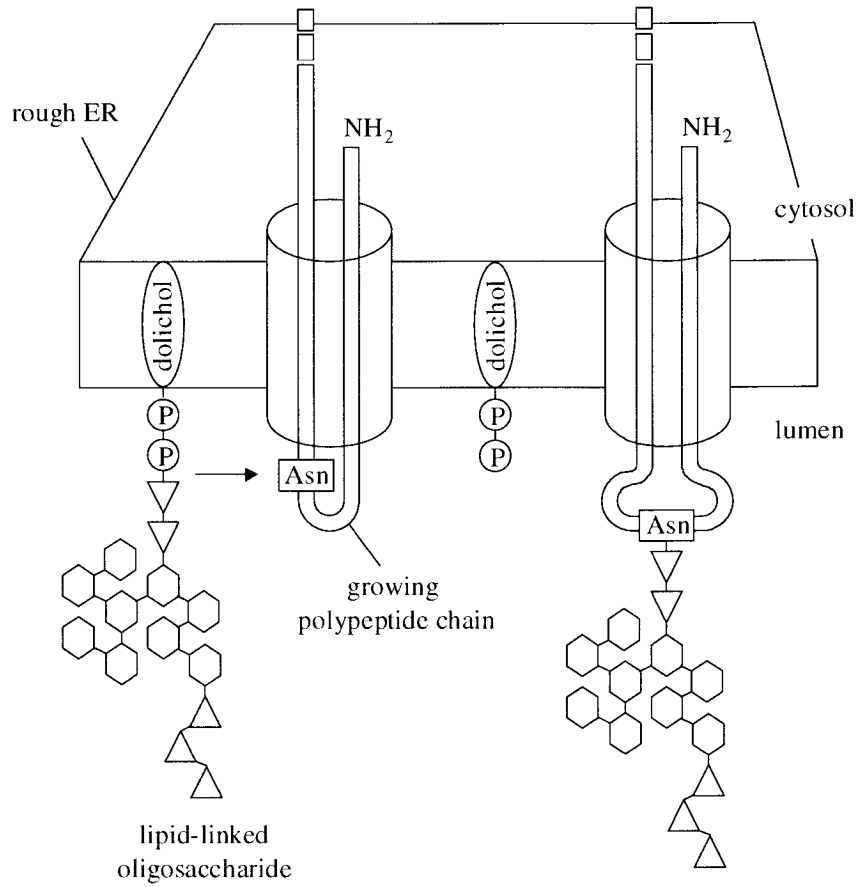


Fig. 6. **Protein glycosylation in the rough endoplasmic reticulum.** As soon as a polypeptide chain enters the ER lumen, a core unit of 14 saccharides is transferred from dolichol pyrophosphate to the target asparagine as an intact unit in a reaction catalysed by a membrane-bound oligosaccharyl transferase enzyme. Modified after [52].

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Aims of the dissertation

With the discovery of RAMPs novel mechanisms defining ligand receptor specificity have been established. Originally cloned orphan rat and human CRLR became functional CGRP or ADM receptors when co-expressed with RAMP1 or -2, respectively.

In mammalian cells, RAMP1 presents CRLR at the cell surface as a terminally glycosylated, mature glycoprotein, whereas RAMP2 presents CRLR as an immature, core-glycosylated receptor. The differential glycosylation of CRLR as well as direct CRLR-RAMP-ligand interactions on the cell surface have been investigated as possible mechanisms to define the specificity of CGRP and ADM receptors in *Drosophila Schneider 2* cells (*report I*).

Within the B family of GPCRs the functional relevance of N-glycosylation varies among receptors. Three putative N-glycosylation sites at Asn⁶⁰, Asn¹¹² and Asn¹¹⁷ are present in the amino-terminal extracellular domain of the human CRLR. The aim of a second study was to investigate the functional significance of N-glycosylation of hCRLR with regard to cell surface expression and [¹²⁵I]CGRP or -ADM binding in the presence of RAMP1 or -2 by using tunicamycin and through Asn to Thr site-directed mutagenesis (*report II*).

Additional mutations of the hCRLR with substitution of the N-glycosylation site Asn¹¹⁷ by Asp, Ala, Gln and Pro were generated and their CGRP receptor function was analysed in the presence of RAMP1. Moreover, the association of the individual Asn¹¹⁷ mutants with RAMP1 at the cell surface was examined (*report III*).

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Report I

**Mammalian calcitonin receptor-like
receptor/receptor-activity-modifying protein
complexes define calcitonin gene-related peptide
and adrenomedullin receptors in *Drosophila*
Schneider 2 cells**

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Abstract

Differential glycosylation of human (h) and rat (r) calcitonin (CT) receptor-like receptors (CRLR) as a result of interactions with accessory receptor activity-modifying proteins (RAMP)1 or -2 was considered to define CT gene-related peptide (CGRP) or adrenomedullin (ADM) receptors in mammalian cells. Here, *Drosophila* Schneider (S2) cells stably co-expressed rCRLR and RAMP1 or -2 as functional CGRP- or ADM receptors. Different from mammalian cells, rCRLR expressed in S2 cells are uniformly glycosylated proteins independent of RAMP1 or RAMP2. Bis(sulphosuccinimidyl)suberate cross-linking revealed receptor components with the size of rCRLR, increased by the molecular weights of the corresponding RAMPs and [¹²⁵I]CGRP or [¹²⁵I]ADM. In conclusion, [¹²⁵I]CGRP/rCRLR/RAMP1 and [¹²⁵I]ADM/rCRLR/RAMP2 complexes have been recognized in *Drosophila* S2 cells.

1. Introduction

Calcitonin (CT) gene-related peptide (CGRP), adrenomedullin (ADM), CT and amylin are homologous peptides with 6- or 7-amino acid ring structures and amidated carboxy-termini required for biological activity [1]. CGRP and ADM are potent vasorelaxant and hypotensive peptides with positive inotropic action on the heart. Along these lines, CGRP was shown to have therapeutic potential in cardiovascular disorders [1]. An enhanced survival rate of ADM-overexpressing transgenic mice in response to lipopolysaccharide administration suggested a protective action of the peptide in septic shock preventing severe hypotension [2]. Therapeutic use of CGRP and ADM awaits the development of corresponding low molecular weight non-peptide mimics with suitable bioavailability. This requires so far non-existing high throughput in vitro screening systems, using cell lines that express a high density of CGRP and ADM receptors.

Initially orphan human (h) and rat (r) CT receptor-like receptors (CRLR) of the B family of G protein-coupled receptors are CGRP or ADM receptors when co-expressed with human receptor activity modifying protein RAMP1 or RAMP2, respectively [3, 4]. RAMP1 and RAMP2 have been shown to direct CRLR to the cell surface. Mature glycosylation of CRLR occurred in the presence of RAMP1 and core-glycosylation was observed when CRLR was expressed alone or together with RAMP2. Differential glycosylation was considered as a mechanism to define the specificity of CGRP and ADM receptors.

Drosophila cell lines, in particular the baculovirus/Sf9 cell system, are powerful and versatile expression systems for heterologous proteins that require posttranslational modification for proper function. But Sf9 cells die from baculovirus infection which is not the case with *Drosophila* Schneider 2 (S2) cells [5]. The latter are suitable for the continuous production in suspension culture at high cell density and room temperature of a variety of stably transfected heterologous proteins such as the glucagon receptor [6].

Here, *Drosophila* S2 cell lines have been developed that stably express CGRP or ADM receptors as rCRLR/RAMP1 or rCRLR/RAMP2 complexes, recognized for the first time, at the cell surface.

2. Materials and methods

2.1. Materials

Rat and human α CGRP(1-37) and rat amylin were purchased from Bachem AG (Bubendorf, Switzerland); r β CGRP(1-37), r α CGRP(8-37) and rCT from Peninsula Laboratories (Belmont, CA, USA); rADM(1-50) from Peptide Institute (Osaka, Japan); and rADM(20-50) from Phoenix Pharmaceuticals (Mountain View, CA, USA). Culture media and FCS were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Hygromycin B was from Life Technologies (Gaithersburg, MD, USA). N-glycosidase F was supplied by Boehringer Mannheim (Mannheim, Germany). Na[¹²⁵I] and ECL Western blot detection reagents were obtained from Amersham International (Little Chalfont, UK), and anti-V5 horseradish peroxidase (HRP)-labeled antibody was from Invitrogen (Carlsbad, CA, USA). The membrane impermeable, water-soluble crosslinker bis(sulphosuccinimidyl) suberate (BS3) was supplied by Pierce (Rockford, IL, USA). Chemicals and other reagents were purchased from Sigma (St. Louis, MO, USA) and E. Merck (Darmstadt, Germany) at the highest grade available.

2.2. Expression of rCRLR and RAMP1 and RAMP2 in *Drosophila* S2 cells

The vector pAc5.1/V5-His (Invitrogen, Carlsbad, CA, USA) was used for constitutive expression of rCRLR and RAMP1 and RAMP2 fused to carboxy-terminal V5- and His₆-epitopes in *Drosophila* S2 cells. Briefly, coding sequences of RAMP1 and RAMP2 with the translational stop sites removed, were amplified by PCR from cloned cDNA (provided by S. Foord, GlaxoWellcome, Stevenage, UK). Oligonucleotide primers were designed with 5' *Kpn*I and 3' *Xba*I (RAMP1) and with 5' *Eco*RI and 3' *Not*I (RAMP2) restriction sites for unidirectional cloning into pAc5.1/V5-His in frame with vector-encoded V5-His₆-epitopes. Similarly, a DNA fragment spanning the coding sequence of rCRLR inbetween a unique internal *Hind*III restriction site and the translation stop codon was amplified from cloned rCRLR encoding cDNA (provided by M.G. Rosenfeld, University of California, San Diego, CA, USA). Cloning of this PCR product carrying 5' *Hind*III and 3' *Xho*I restriction sites, and of a *Not*I / *Hind*III restriction fragment encoding the remaining amino-terminal portion of rCRLR into *Not*I / *Xho*I digested pAc5.1/V5-His yielded the expression construct for a rCRLR-V5-His₆ fusion protein. All constructs were verified by sequencing before the transformation of insect cells.

The cells were maintained in suspension culture at 23°C in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal calf

serum. 10^7 S2 cells were co-transfected by CaPO_4 precipitation (Invitrogen, Carlsbad, CA, USA) with 19 μg of the vector pAc5.1/V5-His (mock transfections) or rCRLR, hRAMP1 or -2 expression constructs in pAc5.1/V5-His or combinations thereof, and with 1 μg of the hygromycin resistance plasmid pCoHYGRO (Invitrogen, Carlsbad, CA, USA). 24 h after transfection 300 $\mu\text{g}/\text{ml}$ hygromycin-B was added to the medium. The medium was changed every 4 days. Stably transfected, polyclonal hygromycin-resistant cell lines were obtained after 3-4 weeks and subcultured (1:5 dilution) every 3-4 days.

2.3. Radioligand binding and cAMP accumulation

$[^{125}\text{I}]$ -labeled h α CGRP and $[^{125}\text{I}]$ rADM were prepared as previously described [4]. Aliquots of 0.5×10^6 S2 cells were incubated for 1 h at 4°C with 1700 Bq $[^{125}\text{I}]$ h α CGRP or $[^{125}\text{I}]$ rADM in the absence and presence of non-labeled peptides in a total volume of 200 μl DMEM/Ham F12 (1:1) supplemented with 0.1% BSA (binding medium). Subsequently, the cells were collected by centrifugation at $14'000 \times g$ and 4°C for 5 min, and the supernatant was aspirated. The tip of the tube containing the cell pellet was cut off and bound radioligand was estimated in a γ -counter (MR252, Kontron, Zurich, Switzerland). Radioactivity in the pellet of cells that were incubated in the presence of 0.1 μM non-labeled peptides was considered as non-specific binding. Cyclic AMP accumulation was measured in 2×10^6 S2 cells incubated at room temperature for 15 min in 200 μl Schneider's *Drosophila* medium containing 1 mM isobutylmethylxanthine (IBMX) in the absence and presence of the indicated peptides. The cells were collected by centrifugation at $1000 \times g$ and room temperature for 5 min and cAMP was measured as described [4].

2.4. Cross-linking of cell surface proteins, deglycosylation and Western blot analysis

For the cross-linking analysis 2.5×10^6 S2 cells, stably expressing CGRP or ADM receptors, were incubated for 1 h at 4°C with 6700 Bq $[^{125}\text{I}]$ h α CGRP or $[^{125}\text{I}]$ rADM in the absence and presence of non-labeled CGRP and ADM in 400 μl binding medium. Subsequently, the cells were collected by centrifugation at $14'000 \times g$ and 4°C for 5 min. The cells were resuspended in 400 μl 0.1 M PBS and incubated for 40 min with 1 mM membrane-impermeable cross-linker BS3. The reaction was quenched with 1 M Tris-HCl, pH 7.5. The cells were collected by centrifugation at $14'000 \times g$ at room temperature for 5 min and resuspended in 50 mM Tris-HCl, pH

7.8, 150 mM NaCl, 1% Nonidet P-40. The cell lysates were cleared by centrifugation at $14'000 \times g$ at room temperature for 5 min and the supernatants were kept at -20°C for further analysis. Deglycosylation of proteins in lysates of S2 cells was carried out with N-glycosidase F. Aliquots, equivalent to $2 - 2.5 \times 10^6$ cells, were incubated for 18 h at 37°C in 50 μl 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 0.5% octylglucopyranosid, 1% β -mercaptoethanol in the absence and presence of 4 U N-glycosidase F. The incubations were stopped with protein gel loading buffer (0.25 M Tris-HCl, pH 6.8, 0.4% bromophenol blue, 8% β -mercaptoethanol, 8% SDS, 40% glycerol). The proteins were separated by 15% Tris-glycine SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose HybondTM ECLTM (Amersham International, Little Chalfont, UK) in 14.4 g/l glycine, 3.03 g/l Tris, 20% methanol in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA, USA) at 25V and 4°C overnight. V5- and His₆-tagged rCRLR and RAMPs were visualized with peroxidase-labeled anti-V5 (1:5000 final dilution) monoclonal antibodies according to the protocol of the ECL Kit (Amersham International, Little Chalfont, UK). Biotinylated protein size markers were visualized by ECL according to the manufacturer's instructions. Cross-linked [¹²⁵I]h α CGRP and [¹²⁵I]rADM were detected with HyperfilmTM MP film (Amersham International, Little Chalfont, UK).

2.5. Statistics

The values for half-maximal inhibitory concentrations (IC₅₀) and for half-maximal effective concentrations (EC₅₀) were calculated by non-linear regression analysis using Fig. P 6.0 software (Biosoft, Cambridge, UK).

3. Results and discussion

3.1. CGRP and ADM receptor function in *Drosophila* S2 cells

Rat CRLR and RAMP1 and RAMP2 with carboxy-terminal V5- and His₆-epitope tags were stably expressed in S2 cells. In control transfected cells and in cells transfected with individual RAMP1, RAMP2 and rCRLR encoding cDNA alone, specific binding of [¹²⁵I]h α CGRP and [¹²⁵I]rADM was not detected (Fig. 1). Specific [¹²⁵I]h α CGRP binding was 17% of added radioligand in the cells co-expressing rCRLR and RAMP1, but not with RAMP2. Rat CRLR and RAMP2 co-expressing cells, on the other hand, exhibited 16% specific [¹²⁵I]rADM, but no [¹²⁵I]h α CGRP binding.

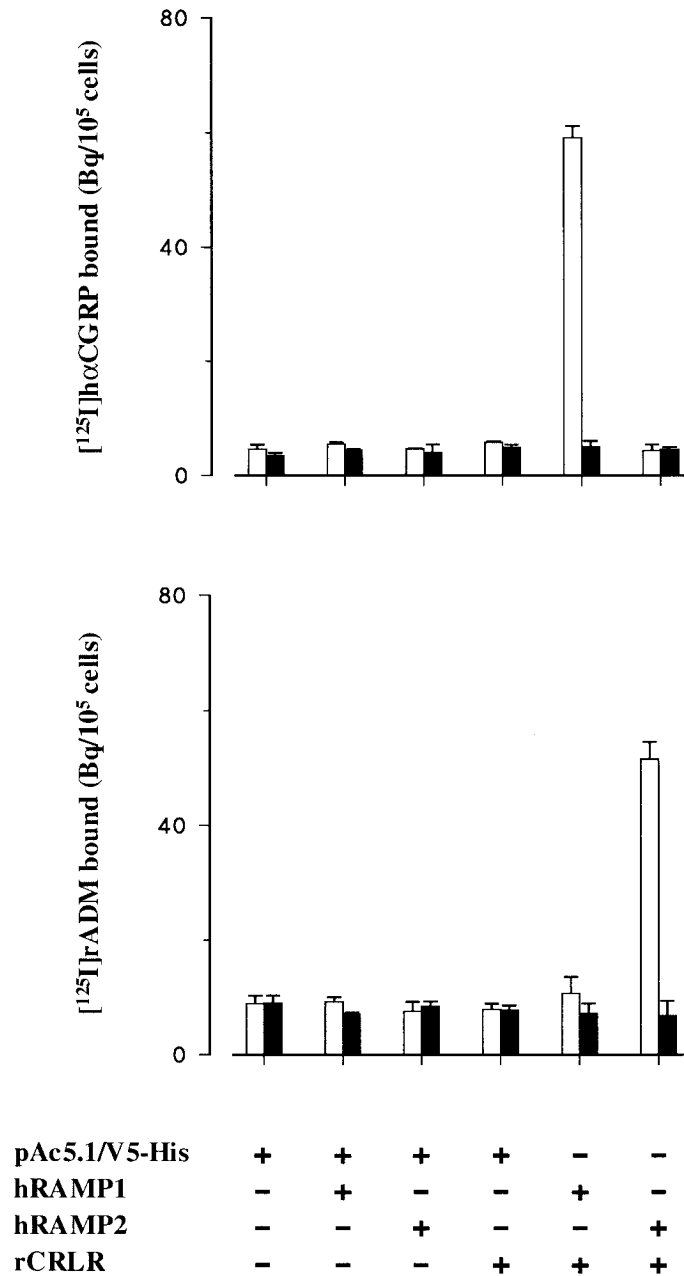


Fig. 1. Binding of [¹²⁵I]hαCGRP (top) and [¹²⁵I]rADM (bottom) to Drosophila S2 cells stably expressing combinations of rCRLR and RAMPs. Open bars indicate total binding and closed bars non-specific binding in the presence of 10⁻⁶ M non-labeled corresponding peptides. The empty Drosophila expression vector pAc5.1/V5-His was used for mock transfections and to equalize the total amounts of DNA in individual transfections. Binding conditions are described in Section 2. The results are mean values ± SEM of triplicate determinations of experiments carried out at least three times.

Table 1.
 $[^{125}\text{I}]\text{h}\alpha\text{CGRP}$ and $[^{125}\text{I}]\text{rADM}$ binding inhibition (IC_{50}) and cAMP accumulation (EC_{50}) in *Drosophila* S2 cells transfected with rCRLR together with RAMP1 or RAMP2.

	rCRLR + RAMP1		rCRLR + RAMP2	
	IC_{50}	EC_{50}	IC_{50}	EC_{50}
	nM			
r αCGRP (1-37)	6.5 ± 2.2	10.7 ± 3.2	225 ± 35	> 1000
r βCGRP (1-37)	4.7 ± 0.72	5.1 ± 0.3	190 ± 46	> 1000
r αCGRP (8-37)	1.7 ± 0.36	> 1000	97 ± 19	> 1000
rADM(1-50)	118 ± 19	> 1000	0.87 ± 0.2	7.7 ± 3.6
rADM(20-50)	> 1000	> 1000	14 ± 8.6	> 1000
Rat amylin	> 500	> 1000	> 1000	> 1000
rCT	> 1000	> 1000	> 1000	> 1000

Results are means ± SEM of three independent experiments

In cells co-expressing rCRLR and RAMP1 $[^{125}\text{I}]\text{h}\alpha\text{CGRP}$ binding was displaced by r αCGRP (1-37), r βCGRP (1-37) and the CGRP antagonist r αCGRP (8-37) with similar half-maximal inhibitory concentrations (IC_{50}), and rADM(1-50) was 18-fold less potent than r αCGRP (1-37) (Table 1). The ADM antagonist rADM(20-50), rat amylin and rCT did not affect $[^{125}\text{I}]\text{h}\alpha\text{CGRP}$ binding at up to 0.5 μM . In the S2 cells co-expressing rCRLR and RAMP2 $[^{125}\text{I}]\text{rADM}$ binding was most potently inhibited by rADM(1-50), and rADM(20-50), r αCGRP (1-37), r βCGRP (1-37) and r αCGRP (8-37) were 16- to 260-fold less effective than rADM(1-50). $[^{125}\text{I}]\text{rADM}$ binding was unaffected by up to 1 μM rat amylin and rCT. The IC_{50} of r αCGRP (1-37), r βCGRP (1-37) and of rADM(1-50) of $[^{125}\text{I}]\text{h}\alpha\text{CGRP}$ and rADM receptor binding corresponded to the half-maximal effective concentration (EC_{50}) of cAMP accumulation. Taken together, CGRP and ADM receptors expressed in S2 cells co-transfected with rCRLR and RAMP1 or RAMP2 encoding cDNA exhibited the pharmacological profile and coupling to cAMP production indistinguishable from that of the receptors expressed in mammalian cells [3, 4]. The results also indicate that *Drosophila* S2 cells do not express

recognizable amounts of endogenous homologues of rCRLR, RAMP1 and RAMP2 interfering with the functional expression of mammalian CGRP or ADM receptors.

3.2. Glycosylation of rCRLR and RAMPs in *Drosophila* S2 cells

Glycosylation of V5- and His₆-epitope tagged rCRLR and RAMP1 and RAMP2, stably expressed in S2 cells, was analysed on Western blots in the absence and presence of N-glycosidase F (Fig. 2).

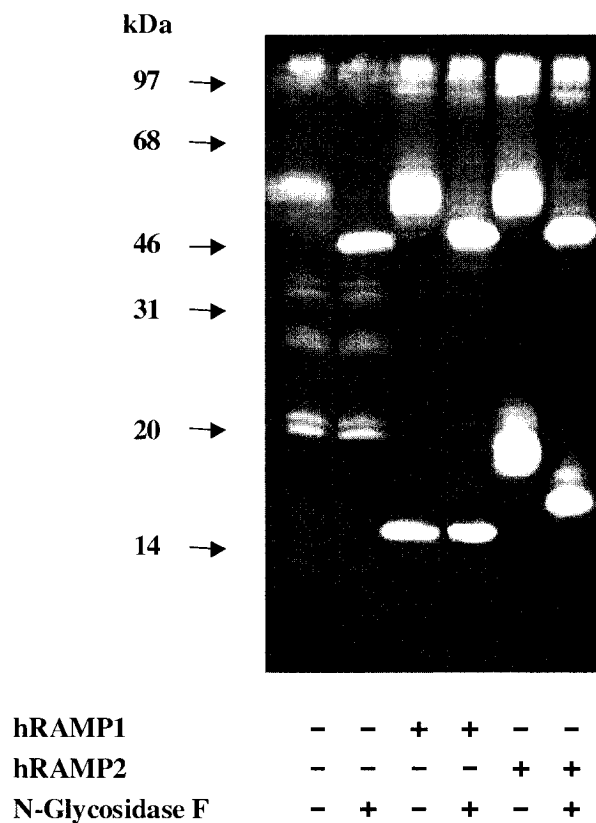


Fig. 2. Glycosylation of rCRLR and RAMP1 and RAMP2 revealed by Western blot analysis. Extracts of S2 cells, stably expressing V5- and His₆-epitope tagged rCRLR and RAMP1 or RAMP2 in indicated combinations, were subjected to SDS polyacrylamide (15%) gel electrophoresis with and without N-glycosidase F treatment. The proteins were visualized on Western blots with monoclonal V5-antibody and the ECL technique (see Section 2). Representative experiment carried out at least three times.

Rat CRLR alone or together with RAMP1 or RAMP2 had indistinguishable apparent M_r of 55-59 kDa reduced to 48 kDa through treatment with N-glycosidase F. The latter corresponds to the protein backbone of rCRLR. In S2 cells glycosylated rCRLR is similar in size to the hCRLR [3] and rCRLR (not shown) in human embryonic kidney (HEK) cells alone and with RAMP2. In HEK cells RAMP1 brought about more extensive glycosylation of hCRLR [3] and rCRLR (not shown) to 66 and 74 kDa components, respectively, not recognized in *Drosophila* S2 cells. RAMP1 expressed in *Drosophila* S2 cells had an apparent M_r of 15 kDa, as predicted from the amino acid sequence, unaltered by N-glycosidase F. RAMP2, on the other hand, had a predominant 18 kDa M_r reduced by N-glycosidase F to a major 16 kDa protein in agreement with the calculated M_r of non-glycosylated RAMP2. This demonstrates glycosylation of RAMP2, but not of RAMP1. The findings are consistent with a single N-glycosylation site predicted from the amino acid sequence of RAMP2 not conserved in RAMP1 [3]. Taken together, the results demonstrate glycosylation of rCRLR and RAMP2 in *Drosophila* S2 cells. Moreover, glycosylation of the rCRLR in S2 cells is independent of the presence of RAMP1 or RAMP2 and does not indicate CGRP or ADM specificity.

3.3. Characterization of [125 I]h α CGRP and [125 I]rADM-binding protein components in S2 cells

The [125 I]h α CGRP was cross-linked with BS3 to *Drosophila* S2 cells co-expressing V5- and His₆-epitope tagged rCRLR and RAMP1. Polyacrylamide gel electrophoresis of cell extracts and autoradiography of Western blots revealed major specifically [125 I]-labeled proteins between 63 and 71 kDa and a minor 18 kDa component (Fig. 3, top). The radiolabeled proteins were not observed when the cross-linker was omitted. Treatment of cross-linked samples with N-glycosidase F reduced the size of the large 63-71 to 51-68 kDa proteins, but the 18 kDa radiolabeled component remained unchanged. Similarly, cross-linking of [125 I]rADM to S2 cells co-expressing V5- and His₆-epitope tagged rCRLR and RAMP2 revealed two specifically labeled components with apparent M_r of 72-76 and 64-67 kDa. The latter were reduced with N-glycosidase F to 54-58 kDa. Subsequent analysis of the same Western blot with V5-epitope specific antibodies was carried out to further characterize [125 I]h α CGRP- and [125 I]rADM-binding proteins. In extracts of rCRLR and RAMP1 or RAMP2 co-expressing S2 cells not treated with cross-linker, glycosylated rCRLR and RAMP2, and RAMP1 were observed (Fig. 3, bottom).

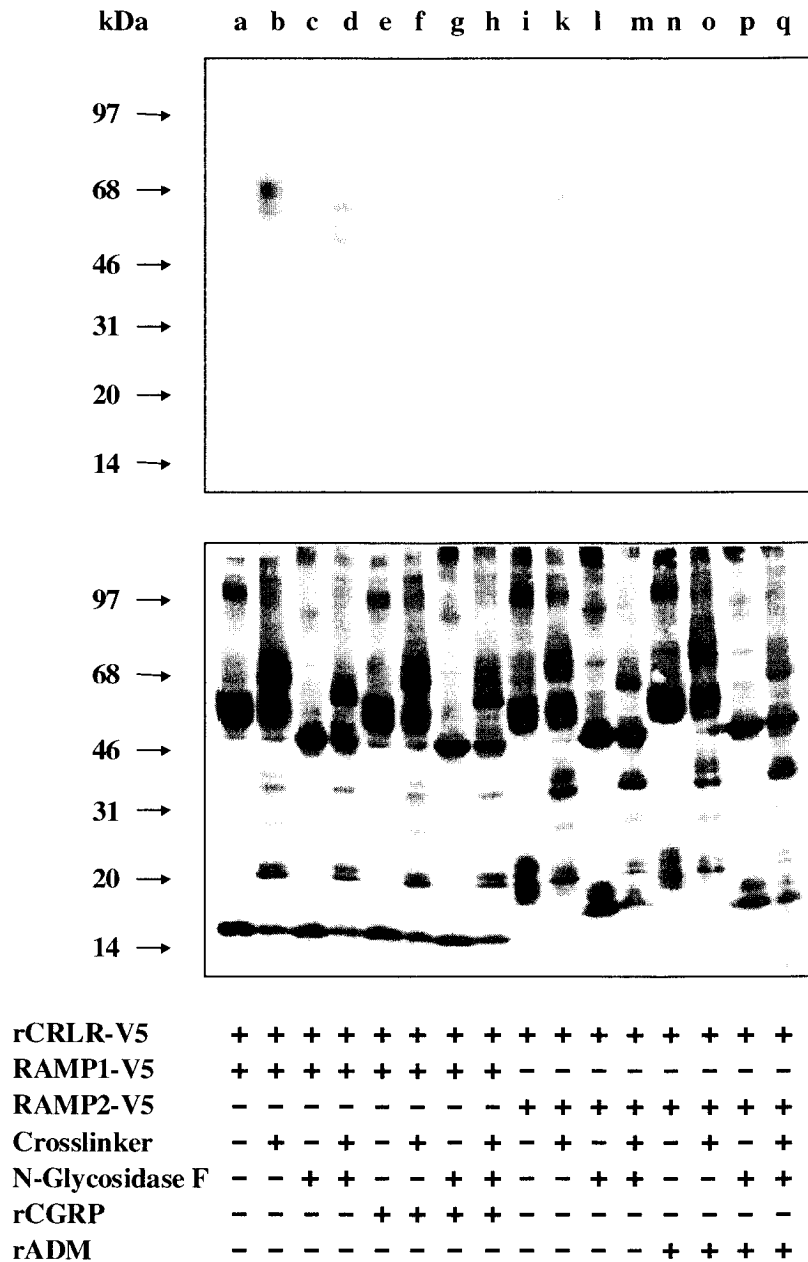


Fig. 3. BS3-cross-linking of [125 I]h α CGRP to *Drosophila* S2 cells that express rCRLR together with RAMP1 (lanes a-h) or of [125 I]rADM to cells that co-express rCRLR and RAMP2 (lanes i-q). Cross-linker and nonlabeled peptides were added and the cell extracts treated with N-glycosidase F as indicated. After SDS polyacrylamide gel electrophoresis of cell extracts, Western blots were autoradiographed (top) and analysed with V5-antibody (bottom). Representative experiment carried out at least three times.

In cell extracts treated with BS3 additional major protein components were detected in the position of [125 I]h α CGRP- and [125 I]rADM-binding components, identified by autoradiography. They were only recognized when RAMP1 or RAMP2 were co-expressed with rCRLR and the M_r were consistent with those of glycosylated rCRLR cross-linked to RAMP1 or to glycosylated RAMP2. Moreover, decreased amounts of free RAMP1 and RAMP2 were observed in cross-linked as compared to non-cross-linked samples, consistent with a shift of a fraction of the two RAMPs into larger molecular weight cross-linking products like rCRLR/RAMP complexes. Additional minor non-identified components, recognized by the V5-antibody, with apparent M_r of 20-46 kDa were also observed. These minor cross-linking products in extracts of rCRLR/RAMP1 co-expressing cells had not a M_r of 18 kDa expected for [125 I]h α CGRP cross-linked to RAMP1. But the weak autoradiographic signal at 18 kDa indicated that the amounts of this cross-linking product were likely below the detection limit of the V5-antibodies and represented a minor fraction of [125 I]h α CGRP cross-linked to RAMP1 alone slightly larger than RAMP1. Interaction on the cell surface of [125 I]h α CGRP with RAMP1 alone cannot be excluded. Evidence for RAMP2 cross-linked to [125 I]rADM has not been obtained.

Deglycosylated proteins were recognized in S2 cells following treatment with N-glycosidase F. In S2 cells co-expressing rCRLR and RAMP1 or RAMP2 and treated with cross-linker major protein components differed in size from the 48 kDa rCRLR by the M_r predicted for RAMP1 and non-glycosylated RAMP2, respectively. In the same samples receptor autoradiography indicated cross-linking of [125 I]h α CGRP to a deglycosylated rCRLR/RAMP1 complex and to rCRLR alone. In rCRLR and RAMP2 co-expressing cells corresponding [125 I]rADM/rCRLR/RAMP2 and [125 I]rADM/rCRLR complexes were also observed. But the larger [125 I]rADM-labeled component was unaltered following N-glycosidase F treatment, indicating that a rCRLR/RAMP2 complex was resistant to N-glycosidase-F when bound to [125 I]rADM.

In a parallel study carried out in mammalian cells (in preparation), myc-tagged RAMP1 was similarly cross-linked to [125 I]h α CGRP-binding V5- and His₆-tagged rCRLR. The results presented here and the observations obtained in mammalian cells demonstrate that the predicted co-transport of CRLR and RAMPs to the plasma membrane [3] results in the formation of CRLR/RAMP1 or CRLR/RAMP2 heterodimers on the cell surface that function as specific CGRP or ADM receptors in *Drosophila* and mammalian cells. This and the identification of amino-terminal putative extracellular domains of RAMP1/RAMP2 chimeras as the critical determinants of ligand specificity [7], imply direct interactions of

extracellular domains of RAMP1 and RAMP2 with CRLR defining the ligand binding site. The here established polyclonal *Drosophila* S2 cell lines, stably co-expressing V5- and His₆-epitope tagged rCRLR and RAMP1 or RAMP2, are suitable for large scale suspension culture.

Acknowledgements

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*Report II***Glycosylation of the Calcitonin Receptor-Like Receptor at Asn⁶⁰ or Asn¹¹² is important for cell surface expression**

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Abstract

The human calcitonin (CT) receptor-like receptor (hCRLR) of the B family of G protein-coupled receptors is N-glycosylated and associates with receptor-activity-modifying proteins (RAMP) for functional interaction with CT gene-related peptide (CGRP) or adrenomedullin (ADM), respectively. Three putative N-glycosylation sites Asn⁶⁰, Asn¹¹² and Asn¹¹⁷ are present in the amino-terminal extracellular domain of the hCRLR. Tunicamycin dose-dependently inhibited the glycosylation of a myc-tagged hCRLR and in parallel specific [¹²⁵I]CGRP and -ADM binding. Similarly, the double mutant myc-hCRLR(N60T/N112T) exhibited minimal N-glycosidase F sensitive glycosylation, presumably at the third Asn¹¹⁷, and the cell surface expression and specific radioligand binding were impaired. Substitution of the Asn¹¹⁷ by Thr abolished CGRP and ADM binding in the face of intact N-glycosylation and cell surface expression.

1. Introduction

The initially orphan calcitonin (CT) receptor-like receptor (CRLR) belongs to the B family of G protein-coupled receptors that includes the CT receptors (CTR) with 60% amino acid sequence homology and more distantly related receptors for parathyroid hormone (PTH), PTH-related protein (PTHrP), secretin and vasoactive intestinal polypeptide (VIP) [1, 2]. Novel receptor-activity-modifying proteins (RAMP) are required for the functional expression of human (h) and rat (r) CRLR, both coupled to cAMP production [3, 4]. The three so far identified RAMP define the specificity of the CRLR and CTR isotype 2 for adrenomedullin (ADM), CT gene-related peptide (CGRP), CT and amylin, all belonging to the CT family of peptides [3-5].

The peptides of the CT family exhibit overlapping physiological actions. Common 6- or 7- amino acid ring structures, linked by disulfide bonds between cysteine residues, and amidated carboxyl-termini are essential for biological activity [6]. CGRP and ADM, the peptides studied here, are potent vasodilators [7, 8].

Mature glycosylation of hCRLR in the presence of RAMP1 and core-glycosylation with RAMP2 were thought to define CGRP and ADM recognition [3]. But, co-expression of rCRLR and RAMP1 or -2 in *Drosophila* Schneider 2 cells revealed RAMP-independent and uniform less extensive mature glycosylation of both the CGRP and ADM receptors, functionally indistinguishable from those in mammalian cells [9]. Within the B family of G protein-coupled receptors the functional relevance of N-linked glycosylation varies among receptors. The VIP receptor needs minimal N-glycosylation for correct delivery to the plasma membrane, but high affinity binding is maintained in solubilized non-glycosylated receptor homogenates [10]. The secretin receptor and the human CTR, on the other hand, are readily transported to the cell surface, but they require N-glycosylation for the interaction with their ligands [11, 12]. Moreover, the non-glycosylated human PTH/PTHrP receptor of tunicamycin-treated cells is expressed at the cell surface and remains fully active [13].

Here, cell surface expression of a myc epitope-tagged hCRLR (myc-hCRLR) and the functional roles of its three putative N-glycosylation sites within the N-terminal extracellular domain have been analyzed through suppression of N-glycosylation with tunicamycin and site-directed mutagenesis. Substitution of the two N-terminal Asn by Thr reduced cell surface delivery and ligand binding. Asn to Thr replacement in the third N-glycosylation consensus sequence inactivated the myc-hCRLR, but the cell surface expression was indistinguishable from that of the non-modified myc-hCRLR.

2. Materials and methods

2.1. Materials

Human α CGRP and human amylin were from Bachem AG (Bubendorf, Switzerland), h β CGRP from Peninsula Laboratories (Belmont, CA, USA), and hADM from Peptide Institute (Osaka, Japan). Human CT was provided by E. Felder (Novartis, Basel, Switzerland). Na[¹²⁵I], enhanced chemiluminescence (ECL)TM Western blot detection reagents and Hybond ECLTM nitrocellulose membranes were from Amersham International (Little Chalfont, UK), and restriction enzymes from Promega (Madison, WI, USA). Tissue culture supplies were from Biological Industries (Kibbutz Beit Haemek, Israel), and Geneticin, LipofectAMINE and OptiMEM medium for transfections were from Life Technologies (Gaithersburg, MD, USA). Other reagents unless indicated were from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. cDNA and mutagenesis

Constructs for expression of myc-hCRLR, RAMP1 and -2 were provided by S. Foord (GlaxoWellcome, Stevenage, UK).

Site-directed mutagenesis, replacing asparagine 60, 112 and 117 by threonine in N-glycosylation consensus sequences of the hCRLR, was carried out with the SeamlessTM Cloning Kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions and ref. 14. Mutations were A→C substitutions in position 2 of corresponding Asn-encoding triplets within a Bsu36I/PstI restriction fragment coding for the hCRLR domain that contains the three predicted N-glycosylation sites. Bsu36I/Eam1104I and Eam1104I/PstI DNA fragments carrying the mutations were amplified with cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA) by PCR for 30 cycles. The products were digested with corresponding restriction enzymes and gel-purified. Matching fragments were ligated into Bsu36I/PstI digested myc-hCRLR yielding myc-hCRLR(N60T), -(N112T) and -(N117T). A double mutant myc-hCRLR(N60T/N112T) was obtained as follows: NotI/XhoI DNA fragments, including the coding sequences of myc-hCRLR(N60T) and -(N112T), were digested with BglII. The DNA fragment NotI/BglII containing mutation (N60T) and the fragment BglII/XhoI with mutation (N112T) were cloned into NotI/XhoI digested pcDNA3 yielding myc-hCRLR(N60T/N112T). All the mutations were verified by sequencing the PCR amplified DNA fragments including the cloning sites in individual pcDNA3 expression constructs in both directions.

2.3. Cell culture, transfection and tunicamycin treatment

SV40 T-antigen transformed human embryonic kidney (TSA) cells were cultured in Ham's F12/DMEM (4.5 g/l glucose) medium (1:1) supplemented with 10% fetal calf serum and 400 µg/ml Geneticin. 24 or 48 h before transient transfection 2.5 or 1×10^5 TSA cells per cm^2 , respectively, were seeded into 24- or 48-well plates coated with 0.1% gelatine. The cells were transfected for 4 h at 37°C in 150 µl OptiMEM medium per cm^2 containing 0.6 µl LipofectAMINE and 100 ng of indicated receptor and of RAMP1 or -2 expression constructs or of pcDNA3 to keep the amount of DNA constant. The transfected cells were kept in tissue culture medium for two days prior to the experiments. Tunicamycin dissolved in DMSO or DMSO alone were added where indicated. Tunicamycin- and DMSO-treated cells were detached with 0.05% EDTA in PBS and kept in suspension during the experiments.

2.4. Radioligand binding

[¹²⁵I]-labelled hαCGRP and hADM were prepared as described [4, 15]. Adherent cells in 24- or 48-well plates and 10^6 or 2×10^6 tunicamycin-treated cells in suspension were incubated with 1700 Bq [¹²⁵I]hαCGRP or -ADM in the absence (total binding) or presence (non-specific binding) of 1 µM non-labelled hαCGRP or hADM in 0.5 ml Ham F12 and DMEM (1:1) supplemented with 0.1% BSA for 2 h at 15°C. Subsequently, adherent cells were washed with ligand-free medium, pre-cooled to 15°C, and lysed with 0.5% SDS. Cells in suspension were collected by centrifugation at $200 \times g$ for 5 min at 4°C and the supernatants were aspirated. The tips of the tubes containing the cell pellets were cut off. Radioactivity in cell lysates and in cell pellets was measured in a MR 252 γ-counter (Kontron, Zurich, Switzerland). Specific binding is defined as the difference between total binding and non-specific binding.

2.5. Western blotting

TSA cells were detached with 0.05% EDTA in PBS and collected by centrifugation at $200 \times g$ for 5 min at 4°C. The cells were lysed in 50 mM Tris (pH 7.8), 150 mM NaCl and 1% Nonidet[®] P-40 for 30 min at 4°C (10 µl/ 10^6 cells), and the lysates cleared by centrifugation at $10,000 \times g$ for 10 min at 4°C. Protein content was measured with the Bio-Rad DC Protein Assay. Aliquots of cell lysates, equivalent to 50-100 µg protein, were treated with 80 mU/µl N-glycosidase F (Boehringer-Mannheim,

Mannheim, Germany) in 10 mM Tris (pH 7.5), 10 mM EDTA, 1% β -mercaptoethanol, 0.1% SDS and 0.5% n-octyl β -D-glucopyranoside for 18 h at 37°C. The reactions were stopped with protein gel loading buffer. Proteins were separated on a 8% SDS-polyacrylamide gel and electro-transferred to HybondTMECLTM nitrocellulose membranes in 14.4 g/l glycine, 3.03 g/l Tris, 20% methanol using a Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA, USA). Myc-tagged receptor proteins were visualized with horseradish peroxidase (HRP)-labelled monoclonal antibodies to myc (1:5000; Invitrogen, Carlsbad, CA, USA) with the ECLTM technique. Chemiluminescence was visualized with a liquid nitrogen-cooled slow scan CCD camera (AstroCam).

2.6. Enzyme-linked immunoassay for cell surface-localised myc

Transfected TSA cells in 48-well plates were incubated in 75 μ l Ham F12/ DMEM (1:1) medium containing 10% fetal calf serum and 4 μ g/ml monoclonal HRP-labelled antibodies to myc (Invitrogen) for 90 min at room temperature. The cells were then washed 4 times with 0.5 ml of the same medium without antibodies. The colour reaction was started with 0.4 ml 100 mM sodium acetate/50 mM sodium phosphate buffer (pH 4.2) containing 1 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS)[®] (Boehringer-Mannheim, Mannheim, Germany) and hydrogen peroxide (1:3500). The reaction was stopped by adding SDS to a final concentration of 0.2%. Absorbance of the supernatant was measured at 405 nm with a Beckman DU68 spectrophotometer.

2.7. Statistical analysis

Differences between mean values of specific binding were analysed by ANOVA and of percent OD at 405 nm compared to myc-hCRLR controls by Student's t-test. The values for half maximal inhibitory concentrations (IC₅₀) were calculated by non-linear regression analysis using FigP 6.0 (Biosoft, Cambridge, UK).

3. Results

3.1. Inhibition of myc-hCRLR N-glycosylation by tunicamycin results in a dose-dependent loss of specific [125 I]h α CGRP and [125 I]hADM binding

A myc epitope on the N-terminus of the hCRLR (myc-hCRLR) was used to identify the protein on immunoblots and to estimate cell surface expression. In TSA cells co-expressing myc-hCRLR and RAMP1 or -2, [125 I]h α CGRP and [125 I]hADM binding amounted to 15% and 12% of added tracer and predominant myc-hCRLR had apparent sizes of 65 and 58 kDa, respectively (Fig. 1).

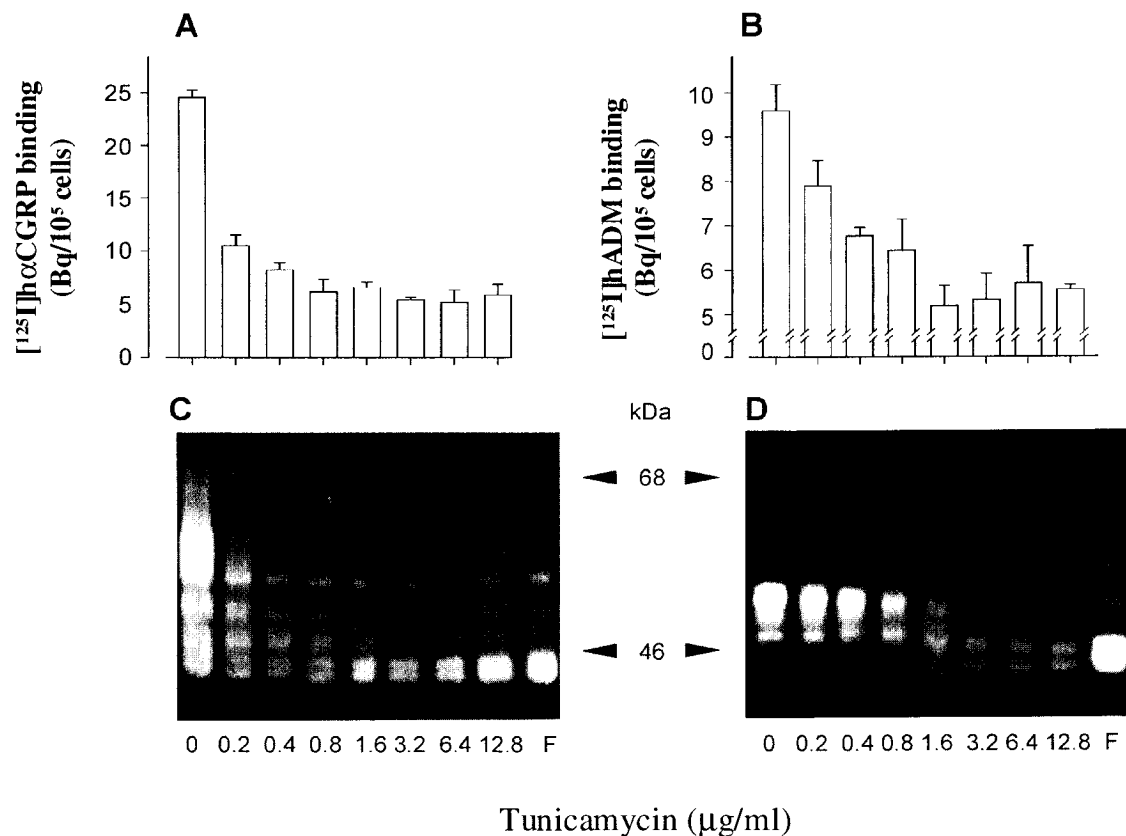


Fig. 1. Radioligand binding and Western Blot analysis of myc-hCRLR expressed in tunicamycin-treated TSA cells. The cells were co-transfected with myc-hCRLR and RAMP1 (A, C) or -2 (B, D) expression constructs in the absence (0) and presence of increasing concentrations of tunicamycin. Open bars in A and B indicate total binding. Non-specific [125 I]h α CGRP or [125 I]hADM binding in the presence of 1 μ M non-labelled h α CGRP or 0.1 μ M non-labelled hADM was 3.0 and 3.8 Bq/10⁵ cells, respectively (not shown). Proteins in cell extracts treated with tunicamycin or with N-glycosidase F (F) were separated by 8% SDS-PAGE. Myc-hCRLR on immunoblots (C, D) was visualized by ECLTM. Arrows indicate the positions of protein size markers. Representative experiment carried out three times.

The IC_{50} of [^{125}I]CGRP and -ADM receptor binding of the myc-hCRLR and the EC_{50} of cAMP accumulation with h α CGRP and ADM were those of the non myc-tagged receptor (not shown). Tunicamycin dose-dependently reduced [^{125}I]h α CGRP and [^{125}I]hADM binding to those observed in the presence of the corresponding non-labelled peptides at 10^{-7} M or in non-transfected cells defined as non-specific binding (not shown). In parallel to the loss of radioligand binding the sizes of the 65 and 58 kDa glycosylated myc-hCRLR were decreased to a protein doublet also observed for N-glycosidase F-treated myc-hCRLR and consistent in size with the calculated molecular weight of the myc-hCRLR protein backbone. Incomplete denaturation, e.g. reduction of presumed disulfide bonds between cysteine residues by β -mercaptoethanol, may explain the migration of deglycosylated myc-hCRLR as a protein doublet. Two intermediate size myc-hCRLR components in cells co-expressing RAMP1 were also gradually reduced at tunicamycin concentrations of between 0.2 and 1.6 μ g/ml. Thus, RAMP1 or -2 dependent expression of myc-hCRLR as CGRP- or ADM receptors requires N-glycosylation.

3.2. N-glycosylation of Asn⁶⁰ and/or Asn¹¹² are required for myc-hCRLR cell surface expression

The hCRLR has three Asn-X-Thr consensus N-glycosylation sites in the N-terminal extracellular domain. With Asn to Thr site-directed mutagenesis, myc-hCRLR(N60T), -(N112T), -(N117T) and the double mutant myc-hCRLR(N60T/N112T) were obtained. Myc-hCRLR and the mutant receptors were expressed in TSA cells in the absence and presence of RAMP1 or -2.

In TSA cells co-expressing myc-hCRLR or individual N-glycosylation site mutants together with RAMP1 or -2, myc-epitope cell surface presentation was used as a measure of plasma membrane localisation (Fig. 2). HRP-labelled myc-antibodies detected myc-hCRLR and all single-site mutant receptors at comparable levels at the cell surface. Different from the single-site mutant receptors cell surface localisation of the double mutant myc-hCRLR(N60T/N112T) in the presence of the RAMP was lowered to between 40% and 50% of the non-modified myc-hCRLR. The expression levels of myc-hCRLR and the mutants, as revealed by Western blot analysis of cell extracts, were comparable (Fig. 3).

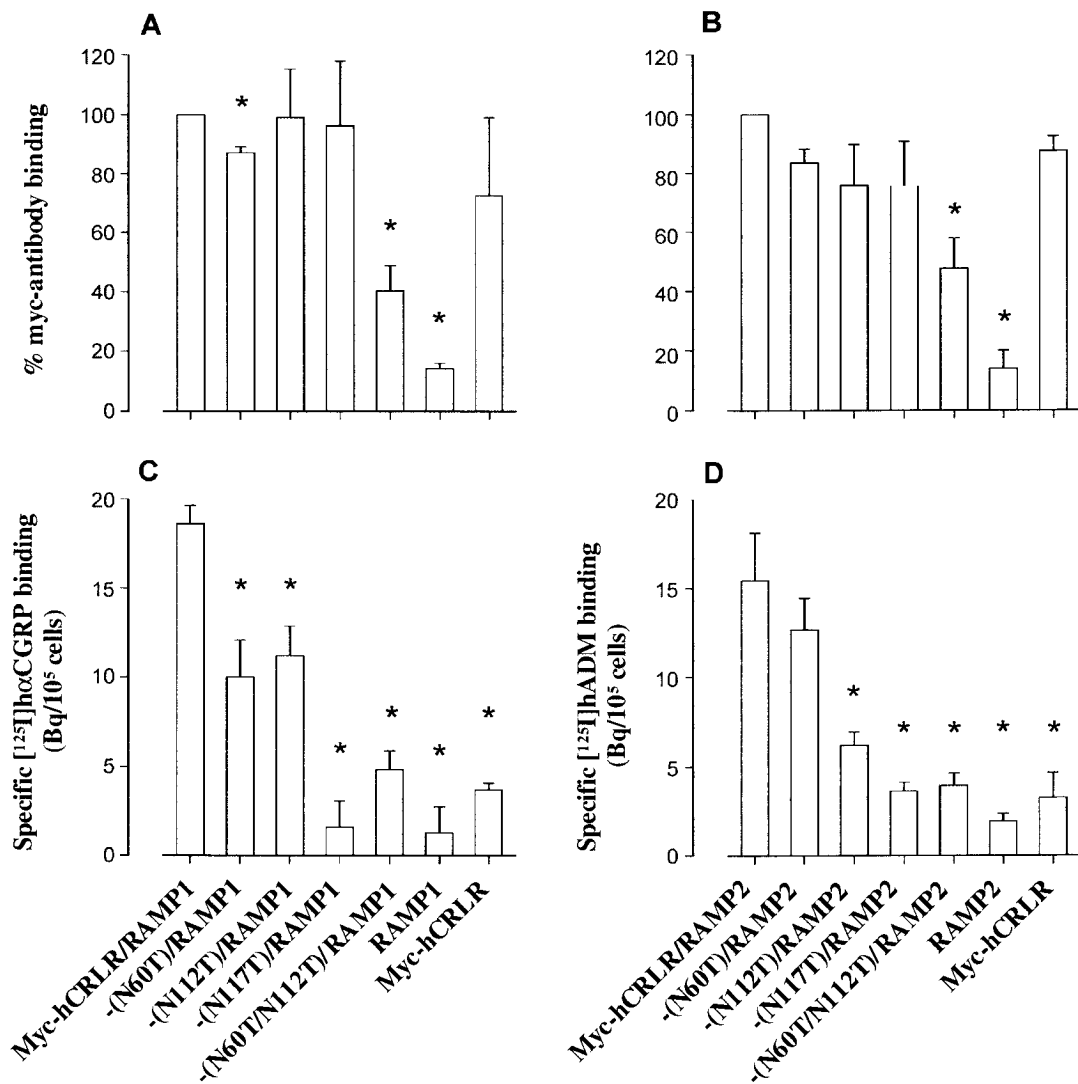


Fig. 2. Cell surface expression and specific radioligand binding of myc-hCRLR and the mutant receptors. TSA cells were transiently transfected with the indicated combinations of receptors and RAMP1 (A, C) or -2 (B, D) expression constructs. A and B, HRP-labelled myc-antibody binding, normalized to that of TSA cells co-expressing myc-hCRLR and RAMP1 (A) or -2 (B). C and D, specific radioligand binding. The results are means \pm SEM of four independent experiments. Statistically significant differences ($P < 0.02$) between means of percent myc-antibody binding and specific radioligand binding compared to the myc-hCRLR/RAMP1 or -2 are indicated with an asterisk.

3.3. [¹²⁵I]hαCGRP and -ADM recognition by myc-hCRLR, -(N60T), -(N112T), -(N60T/N112T) and -(N117T)

In cells expressing myc-hCRLR or the glycosylation mutants (N60T) and (N112T), specific binding of [¹²⁵I]hαCGRP and -ADM was maintained in the presence of the corresponding RAMP (Fig. 2). The IC₅₀ of hα- and βCGRP, hADM and human amylin of [¹²⁵I]hαCGRP and -ADM binding reflected the affinities of the expressed receptors (Table 1). Binding inhibition of [¹²⁵I]hαCGRP by the non-labelled peptides in cells expressing myc-hCRLR and the (N60T) and (N112T) mutants was similar. With [¹²⁵I]hADM, myc-hCRLR(N60T) and -(N112T) exhibited a three- and ten-fold higher IC₅₀ of hADM as compared to the non-modified myc-hCRLR. Different from the two (N60T) and (N112T) single-site mutant receptors specific [¹²⁵I]hαCGRP and -ADM binding to the double-mutant (N60T/N112T) was below 5% of the added tracers.

In cells expressing myc-hCRLR(N117T) together with the corresponding RAMP specific [¹²⁵I]hαCGRP and [¹²⁵I]hADM binding was below 4% of the added tracers. Up to 10⁻⁷ M hαCGRP and hADM failed to stimulate cAMP production in COS-7, TSA and human embryonic kidney 293 cells co-expressing myc-hCRLR(N117T) and RAMP1 or -2 (not shown).

Table 1.
[¹²⁵I]hαCGRP and -hADM binding inhibition (IC₅₀) to TSA cells co-expressing myc-hCRLR or receptor mutants with RAMP1 or -2.

	[¹²⁵ I]hαCGRP ^a					[¹²⁵ I]hADM ^b				
	hαCGRP	hβCGRP	hADM	hAmylin	hCT	hαCGRP	hβCGRP	hADM	hAmylin	hCT
	IC ₅₀ (nM)									
myc-hCRLR	2.2 ± 0.8	1.8 ± 1.0	67 ± 23	65 ± 25	> 10 ³	> 10 ³	> 10 ³	9.7 ± 2.4	> 10 ³	> 10 ³
-(N60T)	5.7 ± 2.9	2.2 ± 0.9	45 ± 22	106 ± 35	> 10 ³	> 10 ³	> 10 ³	33 ± 5	> 10 ³	> 10 ³
-(N112T)	10.7 ± 5.5	1.4 ± 0.8	49 ± 20	78 ± 35	> 10 ³	> 10 ³	> 10 ³	94 ± 14	> 10 ³	> 10 ³

Results of IC₅₀ are means ± SEM of at least three independent experiments

^a TSA cells co-transfected with RAMP1

^b TSA cells co-transfected with RAMP2

3.4. Western blot analysis of myc-hCRLR and -(N60T), -(N112T), -(N60T/N112T) and -(N117T) in TSA cells

N-glycosylation of myc-hCRLR and of the mutant receptors was analysed on Western blots through estimation of relative size differences of receptors before and after N-glycosidase F-treatment of cell extracts (Fig. 3). Myc-hCRLR had an apparent size of 55 to 58 kDa in the absence of the RAMP. The size increased by approximately 10 kDa when RAMP1 was co-expressed, but was not affected by RAMP2. N-glycosidase F-treatment reduced the size of myc-hCRLR independent of co-expressed RAMP to a protein doublet of approximately 45 kDa also observed in the tunicamycin-treated cells. In the absence of the RAMP the three mutants myc-hCRLR(N60T), -(N112T) and -(N117T) were smaller than the non-modified myc-hCRLR. This size remained unchanged when RAMP2 was co-expressed and it was also reduced by N-glycosidase F to that of deglycosylated myc-hCRLR. The size of myc-hCRLR(N112T) was unaltered when RAMP1 was co-expressed. A minor fraction of myc-hCRLR(N60T) was further glycosylated when co-expressed with RAMP1, but the maximal size was lower than that of the myc-hCRLR. Glycosylation of myc-hCRLR(N117T) in the presence of RAMP1 occurred less efficiently but the achieved size was that of the myc-hCRLR. The reduction of the size of the double mutant myc-hCRLR(N60T/N112T) with N-glycosidase F-treatment indicated minor glycosylation at Asn¹¹⁷ that was unaffected by co-expressed RAMP.

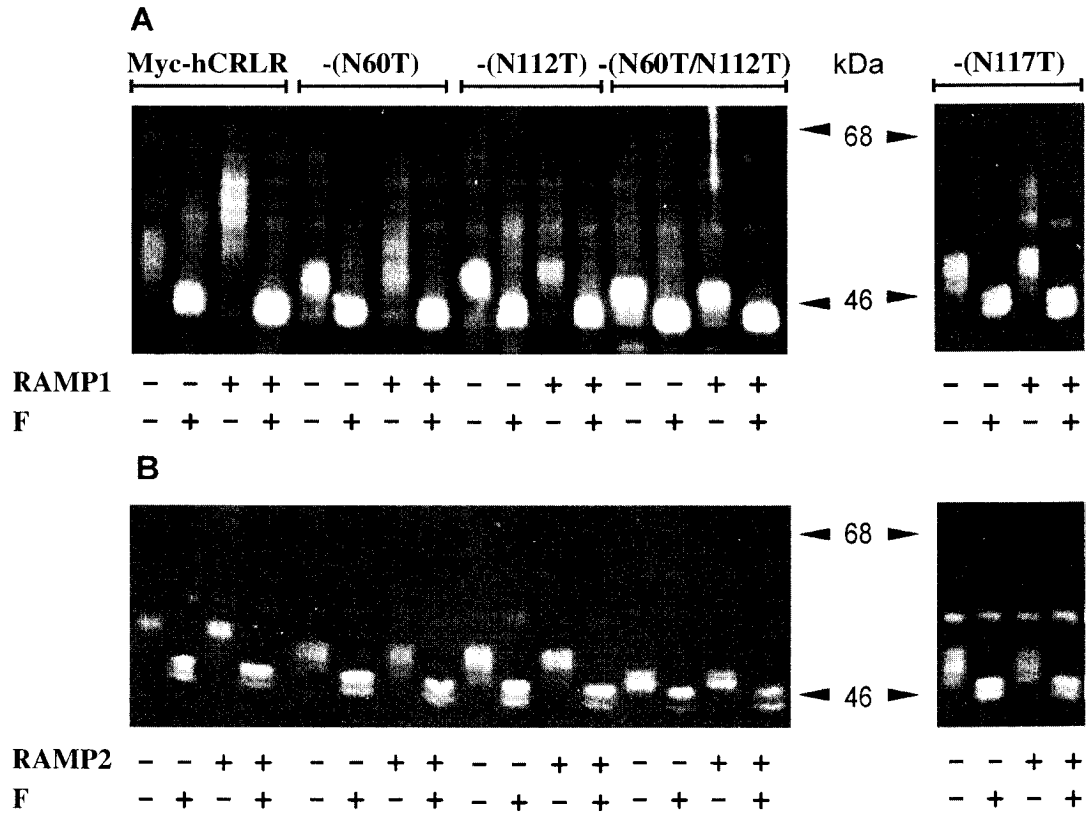


Fig. 3. Analysis of N-glycosylation of myc-hCRLR and -(N60T), -(N112T), -(N60T/N112T) and -(N117T) in TSA cells on Western blots. Transiently expressed myc-hCRLR and mutant receptors in the absence (-) and presence (+) of RAMP1 (A) or RAMP2 (B). Cell extracts (50 μ g protein) incubated in the absence and presence (F) of 80 mU/ μ l N-glycosidase F were subjected to 8% SDS-PAGE. Non-modified and mutant myc-hCRLR on immunoblots were visualized by the ECLTM technique. The positions of protein size markers are indicated by arrows. Representative experiment carried out three times.

4. Discussion

N-glycosylation of hCRLR was demonstrated by treatment with N-glycosidase F that reduced its size to that calculated for the protein backbone [3]. Mature-glycosylation of h- and rCRLR as a result of the interaction with RAMP1 and core-glycosylation in the presence of RAMP2 was thought to define the specificity of the CRLR for CGRP and ADM [3, unpublished]. In *Drosophila* Schneider 2 cells, on the other hand, rCRLR

glycosylation was independent of co-expressed RAMP1 and -2, but CGRP- and ADM receptor function was that of rCRLR in mammalian cells [4, 9].

In the present study, the functional significance of N-glycosylation of hCRLR on the cell surface expression and [125 I]CGRP or -ADM binding in the presence of RAMP1 or -2 was investigated in cells treated with tunicamycin and through Asn to Thr site-directed mutagenesis of three N-terminal N-glycosylation consensus sites. Treatment with tunicamycin reduced the size of myc-hCRLR to that of N-glycosidase F treated myc-hCRLR, indicating total suppression of N-glycosylation. In parallel, [125 I]CGRP and -ADM binding were lowered. This is in line with other receptor proteins located at the plasma membrane where inhibition of N-glycosylation by tunicamycin impaired their transport to the cell surface and as a result ligand binding [10, 16].

Site-directed mutagenesis of Asn⁶⁰ or Asn¹¹² of myc-hCRLR to Thr revealed less extensive N-glycosylation in the absence and presence of RAMP1 and -2, but cell surface expression was largely unchanged. In the double mutant myc-hCRLR(N60T/N112T) the extent of N-glycosylation was further reduced and cell surface expression was now decreased. As a result, only minimal specific [125 I]CGRP and -ADM binding was observed. These results were consistent with those obtained in tunicamycin-treated cells where dose-dependent inhibition of N-glycosylation also impaired [125 I]CGRP and -ADM binding. Interestingly, mutation of Asn¹¹⁷ to Thr did not affect N-glycosylation and expression of the receptor at the cell surface to any great extent, but specific [125 I]CGRP and -ADM binding was obliterated. Apparently, minimal N-glycosylation of Asn¹¹⁷ in myc-hCRLR(N60T/N112T) was insufficient for its normal delivery to the cell surface. Thus, Asn¹¹⁷ of myc-hCRLR is required for CGRP or ADM recognition in the presence of RAMP1 or -2, respectively. It is, however, not required for N-glycosylation and cell surface delivery of the hCRLR.

The functional role of N-glycosylation of the related hCTR receptors, in particular of the hCTR type 3 with approximately 60% homology to the CRLR and also three N-terminal N-glycosylation consensus sites has been reported [12]. Much like with the hCRLR, site-directed mutagenesis of the first consensus site did not affect high-affinity salmon CT binding. hCTR receptors lacking the second or third consensus site exhibited less potent cAMP production. Correspondingly, with the myc-hCRLR(N112T) the IC₅₀ of ADM with respect to [125 I]ADM binding was somewhat higher than that of the myc-hCRLR. In marked contrast, substitution of the Asn¹¹⁷ by Thr inactivated the myc-hCRLR.

In conclusion, hCRLR N-glycosylation at Asn⁶⁰ and/or Asn¹¹² is required for cell surface expression and [125 I]CGRP or -ADM binding in the

presence of RAMP1 or -2. The myc-hCRLR(N117T), on the other hand, is delivered to the cell surface, but the mutated hCRLR is inactive.

Acknowledgements

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Report III

**Mutations of the Asparagine¹¹⁷ Residue of a
Receptor-activity-modifying protein 1 (RAMP1)-
dependent Human Calcitonin Gene-Related
Peptide (CGRP) Receptor Result in Selective
Loss of Function[†]**

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ABSTRACT

The initially orphan human calcitonin (CT) receptor-like receptor (hCRLR) interacts with a novel accessory receptor-activity-modifying protein 1 (RAMP1) to reveal a functional CT gene-related peptide (CGRP) receptor. In mammalian cells, RAMP1 is required for mature N-glycosylation of the hCRLR predicted to occur at Asn⁶⁰, Asn¹¹² and/or Asn¹¹⁷ in the amino-terminal extracellular domain. Here we have shown that the substitution of Asn¹¹⁷ by Ala, Gln, Thr or Pro abolished CGRP-evoked cAMP formation which was left unchanged when the Asn¹¹⁷ was replaced by Asp. Moreover, the hCRLR and the Asn¹¹⁷ mutants exhibited comparable N-glycosylation, cell surface expression and association with RAMP1. In contrast, the hCRLR Asn^{60,112} to Thr double mutant showed defective RAMP1-dependent N-glycosylation, and impaired cell surface expression and CGRP receptor function. Unlike Asn⁶⁰ and Asn¹¹², Asn¹¹⁷ is presumably not involved in N-glycosylation, but essential for CGRP binding to the hCRLR/RAMP1 complex.

The α -calcitonin (CT) gene-related peptide (α CGRP) is a 37 amino acid neuropeptide encoded by an alternative mRNA splicing product of the CT gene (1). β -CGRP is the product of a second gene and it differs in man and rat in 3 and 1 amino acid residues from α CGRP, respectively (2). α - and β -CGRP, and adrenomedullin (ADM) are potent vasodilators. They belong to the CT family of peptides, which includes CT and amylin (3). The peptides have in common N-terminal ring structures of 6- or 7-amino acids linked by disulfide bonds between cysteine residues and amidated C-termini, both required for biological activity. α - and β -CGRP are widely expressed in the central and peripheral nervous system and elicit their biological effects through interaction with G protein-coupled receptors predominantly linked to cAMP production. Initially orphan rat and human (h) CT receptor-like receptors (CRLR) of the B family of G protein-coupled receptors require novel accessory receptor-activity-modifying proteins (RAMP) for functional expression at the cell surface (4-6). Human and mouse RAMP1, -2 and -3 define the specificity of CRLR for CGRP or ADM (6-8). The rat CRLR and the human CT receptor isotype 2 (hCTR2) with 60% amino acid sequence homology associate with RAMP1 at the cell surface as two different CGRP receptor subtypes (9). hCTR2 co-expressed with RAMP1 or -3 revealed two different amylin receptor isotypes (10, 11).

Here we have investigated association with RAMP1 and CGRP receptor function of the hCRLR mutated at the three predicted Asn⁶⁰, Asn¹¹² and Asn¹¹⁷ N-glycosylation consensus sites that are conserved in all so far identified CRLR. The results indicate that Asn⁶⁰ and/or Asn¹¹² unlike Asn¹¹⁷ are required for cell surface expression. With the Asn¹¹⁷ to Ala, Gln, Thr and Pro mutants N-glycosylation, cell surface expression and association with RAMP1 are maintained, but ligand binding was abolished.

MATERIALS AND METHODS

Materials. Human α CGRP(1-37) was purchased from Bachem. The membrane-impermeable cross-linker bis(sulfosuccinimidyl)suberate (BS3) and ImmunoPure® Immobilized Protein G were supplied by Pierce. N-glycosidase F was from Boehringer Mannheim and restriction enzymes were purchased from Promega. Na[¹²⁵I], ECL™ Western blot detection reagents and Hybond™ ECL™ nitrocellulose membranes were obtained from Amersham Pharmacia Biotech. Tissue culture media and fetal calf serum were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Geneticin 418, LipofectAMINE or LipofectAMINE 2000 and OptiMEM medium were supplied by Life Technologies. Other chemicals and reagents were purchased from Sigma and Merck at the highest grade available.

Plasmids and Construction of hCRLR Mutants. The cDNA encoding the hCRLR with an N-terminal myc epitope (EQKLISEEDLL) (myc-hCRLR) in the mammalian expression vector pcDNA3 (Invitrogen) was provided by S. Foord (GlaxoWellcome). The construct for expression of the hCRLR with C-terminal myc and (His)₆ epitope-tags (hCRLR-myc) was obtained as follows: A DNA fragment encoding the C-terminus of hCRLR between a unique *Pst*I restriction site and the translational stop codon was amplified by PCR using a 3' oligonucleotide that introduced a *Hind*III recognition sequence in place of the hCRLR translation stop codon. The PCR product was digested with *Hind*III, releasing a *Hind*III DNA fragment that encoded the C-terminus of hCRLR downstream of a unique *Hind*III restriction site in the hCRLR coding sequence with the stop codon removed. This DNA fragment together with a *Bam*HI-*Hind*III restriction fragment encoding the remaining N-terminal portion of hCRLR were cloned into a modified *Bam*HI/*Hind*III digested pcDNA3 expression vector encoding myc and (His)₆ downstream of the *Hind*III restriction site. This reconstructed the hCRLR coding sequence in frame with the sequence encoding myc and (His)₆ at the C-terminus. The cDNA encoding RAMP1 in frame with C-terminal V5(GKPIP NPLLGLDST)- and (His)₆-epitope tags was removed by *Kpn*I/*Pme*I digestion from a previously described construct in the *Drosophila* expression vector pAc5.1/V5His (Invitrogen) (12) and subcloned into *Kpn*I/*Eco*RV digested pcDNA3. The three N-glycosylation consensus sequences Asn-X-Ser/Thr within the N-terminal extracellular domain of hCRLR were modified as indicated in Figure 1 through site-directed mutagenesis with the Seamless™ Cloning Kit (Stratagene). The nucleotide sequence between 5' *Bsu*36I and 3' *Pst*I restriction site encodes

the subdomain of the hCRLR that contains all three potential N-glycosylation sites. Pairs of primers were designed to carry out PCR amplification with cloned *Pfu* DNA polymerase (Stratagene) of *Bsu36I/Eam1104I* and *Eam1104I/PstI* DNA fragments with the nucleotide substitutions required to create the mutations. The products obtained after 30 cycles were digested with *Eam1104I* and *Bsu36I* or *PstI* and gel-purified. Ligation of matching fragments into *Bsu36I/PstI* digested myc-hCRLR reconstituted the coding sequence for the myc-hCRLR(N60T), -(N112T), -(N117D), -(N117A), -(N117Q), -(N117T) and -(N117P) mutants. The double mutant myc-hCRLR(N60T/N112T) was generated by *BglIII* digestion of *NotI/XhoI* DNA fragments of myc-hCRLR(N60T) and of myc-hCRLR(N112T) and religation of the *NotI/BglIII* fragment containing the mutation N60T and the *BglIII/XhoI* fragment with the mutation N112T into *NotI/XhoI* digested pcDNA3. The nucleotide sequence between the *Bsu36I* and the *PstI* restriction sites in the final hCRLR mutant expression constructs was verified by sequencing in both directions.

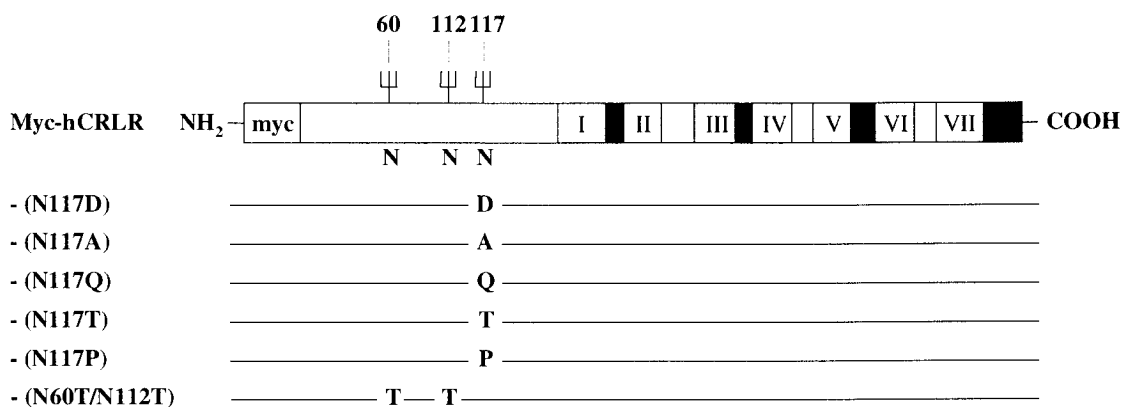


Figure 1. **Schematic representation of non-modified and mutant myc-hCRLR.** The N-terminal myc-epitope, extracellular- (white boxes), transmembrane- (grey boxes with roman numbers) and intracellular domains (black boxes) are shown. Asparagine (N) residues at positions 60, 112 and 117 are N-glycosylation consensus sites of myc-hCRLR that were substituted by aspartic acid (D), alanine (A), glutamine (Q), threonine (T), and proline (P) residues as indicated.

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

Cross-linking, Immunoprecipitation and Deglycosylation. Transiently transfected TSA cells in 100 mm dishes were incubated with 8×10^4 Bq [125 I]h α CGRP in 4.8 ml HamF12/DMEM (1:1) supplemented with 0.1% BSA (binding medium) for 2 h at 15 °C. Subsequently, the cells were washed with PBS and incubated for 1 h at room temperature in 4.8 ml 0.1 M PBS containing 1 mM cross-linker BS3. Cross-linking was quenched by adding Tris-HCl to a final concentration of 15 mM, pH 7.5. The cells were lysed in 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 -D-maltoside and 0.2 mg/ml cholesterohemisuccinate. The cell lysates were cleared by centrifugation for 3 min at 10'000 x g. Myc-tagged receptors were immunoprecipitated from cleared lysates at 4 °C by sequential incubation with 50 µl ImmunoPure® Immobilized Protein G for 1 h, 2 µg of myc-antibodies for 2 h and 100 µl ImmunoPure® Immobilized Protein G overnight on an end-over-end rotator. The precipitates were then collected by centrifugation for 5 min at 10'000 x g. The pellets were washed three times 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 and 0.25 mg/ml dodecyl- β -D-maltoside. The immunoextracted proteins were eluted from the pellets by incubation in 80 µl SDS-PAGE loading buffer for 15 min at 50 °C. [125 I]CGRP in the immunoprecipitates was measured in a γ -counter (Kontron). Deglycosylation of proteins in aliquots of cell lysates was carried out with 4 U of N-glycosidase F in 50 µl 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 0.5% octylglucopyranoside, 1% β -mercaptoethanol for 18 h at 37 °C. The incubations were stopped with SDS-PAGE loading buffer.

Western Blot Analysis. Proteins in cell lysates and immunoextracts were separated by SDS-PAGE and electrotransferred to nitrocellulose Hybond™ ECL™ membranes in a Trans-Blot cell (BioRad Laboratories) at 25 V and 4 °C overnight. Immunoblots were blocked with 5% low-fat milk and the epitope-tagged proteins were visualized by enhanced chemiluminescence with horseradish peroxidase-labeled monoclonal myc- and V5-antibodies (1:2000 final dilution) (Invitrogen). 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 horseradish peroxidase-conjugated sheep antibodies to mouse immunoglobulins (1:5000 final dilution) (Amersham). Cross-linked [¹²⁵I]hαCGRP was recognized by autoradiography with Hyperfilm™ MP film (Amersham).

Immunohistochemistry. TSA cells cultured in tissue culture medium supplemented with 400 µg/ml geneticin 418 were transiently transfected in 3 ml OptiMEM medium containing 12 µl LipofectAMINE 2000 and 2.4 µg indicated receptor and RAMP1-V5 expression constructs. 48 h later the cells were detached with 0.05% EDTA in PBS. Cell surface expression of myc epitope-tagged receptors was estimated by myc immunofluorescence-staining of intact cells in suspension. The cells were incubated with mouse monoclonal antibodies to myc (Invitrogen) diluted 1:600 in tissue culture medium for 1 h at room temperature. The same medium was used to wash the cells twice and for the incubation with TRITC-labeled polyclonal rabbit anti-mouse immunoglobulin (1:150 final dilution) (Dako Diagnostics) for 30 min at room temperature. The cells were again washed twice with tissue culture medium and once with PBS. The cells were then fixed with 3% formaldehyde in PBS for 20 min. TRITC-fluorescence of equal aliquots of cells was measured in a SpectraMAX GeminiXS fluorescence reader (Molecular Devices) with excitation at 530 nm, emission at 590 nm and cutoff at 570 nm. Background fluorescence of the cells transfected with pcDNA3 was subtracted. Fluorescence-staining of cells was also visualized by fluorescence microscopy. Aliquots of cells were dried on a slide and mounted with Immu-Mount (Shandon Scientific). The cells were viewed with an Eclipse E600 Nikon microscope with a G2-A filter and 50-fold magnification. Photographs taken from individual slides at constant exposure time were scanned with an LS-2000 Nikon scanner with the parameters adjusted to minimize background fluorescence.

Statistical Analysis. The values for half-maximal effective concentrations (EC_{50}) were calculated by non-linear regression analysis using Fig. P 6.0 software (Biosoft, Cambridge, UK) and differences between mean values were analysed by ANOVA. Differences between mean values of percent cell surface expression of individual myc-hCRLR mutants compared to non-modified myc-hCRLR controls were analysed by Student's test. $P < 0.05$ were considered statistically significant.

RESULTS

Stimulation of cAMP production and cell surface expression of myc-hCRLR and mutants thereof co-expressed with RAMP1-V5. Cyclic AMP accumulation in response to CGRP was estimated in transiently transfected COS-7 cells (Figure 2). In cells expressing the myc-hCRLR and RAMP1-V5, cAMP production was stimulated 36-fold by 10^{-6} M h α CGRP with an EC_{50} of 3.8 ± 1.1 nM ($n = 8$). In cells expressing myc-hCRLR(N60T) or -(N112T) together with RAMP1-V5 the cAMP response was that of the non-mutated myc-hCRLR (not shown) and these mutants were not further investigated. But with the double mutant myc-hCRLR(N60T/N112T) cAMP production by 10^{-6} M h α CGRP was lowered to 30% of that of the myc-hCRLR, and the EC_{50} was increased 9-fold. The various amino acid substitutions at Asn¹¹⁷ in the myc-hCRLR affected CGRP-evoked stimulation of cAMP formation differently. The cAMP response was similar in cells expressing non-modified myc-hCRLR or the mutant myc-hCRLR(N117D) both together with RAMP1-V5. But the cAMP production remained at basal levels in cells co-transfected with myc-hCRLR(N117T) or -(N117P) and RAMP1-V5 expression constructs. In the cells expressing myc-hCRLR(N117A) and -(N117Q) h α CGRP stimulated the cAMP production with an EC_{50} of over 100 nM.

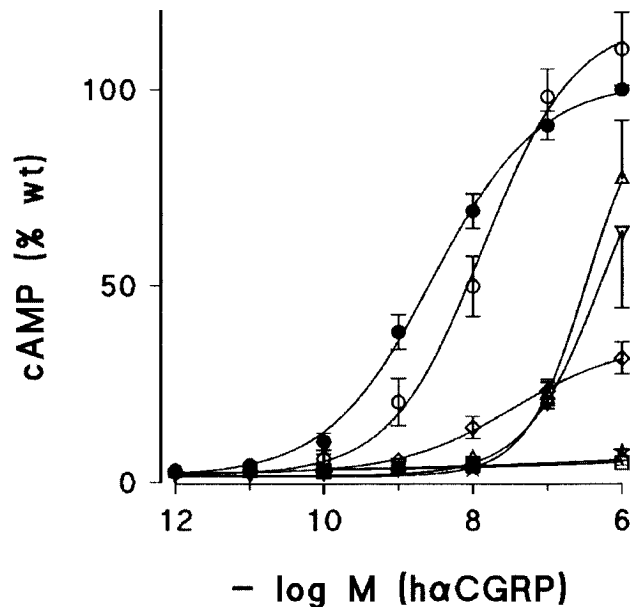


Figure 2. **Stimulation of cAMP production in COS-7 cells by CGRP.** The cells were grown in 24-well plates to 50% confluency. For transfection the cells were incubated for 4 h at 37 °C in 250 μ l/well OptiMEM medium containing 200 ng of myc-hCRLR (●), myc-hCRLR(N60T/N112T) (◇), -(N117D) (O), -(N117Q) (Δ), -(N117A) (∇), -(N117P) (\star) or -(N117T) (\square) and 200 ng of RAMP1-V5 expression constructs and 1 μ l LipofectAMINE 2000. 48 h after transfection, the cells were incubated with the indicated concentrations of h α CGRP for 15 min at 37 °C. The results are means \pm SEM of three independent experiments.

Myc immunofluorescent-staining of intact cells, reflecting receptor cell surface expression, was comparable in TSA cells transiently expressing myc-hCRLR or the -(N117D), -(N117A), -(N117Q), -(N117T) or -(N117P) mutants together with RAMP1-V5, but it was hardly visible in cells expressing myc-hCRLR(N60T/N112T) (Figure 3). The hCRLR with a C-terminal, intracellular myc epitope-tag and normal function served as a negative control. Quantitative analysis revealed between 50% and 80% cell surface expression of myc-hCRLR(N117D), -(N117A), -(N117Q), -(N117T) or -(N117P) as compared to the myc-hCRLR, and that of the myc-hCRLR(N60T/N112T) was below 25% (Figure 4). Western blot analysis of total cell extracts showed comparable expression levels of myc-hCRLR and its mutants in the presence of RAMP1-V5 (Figure 5).

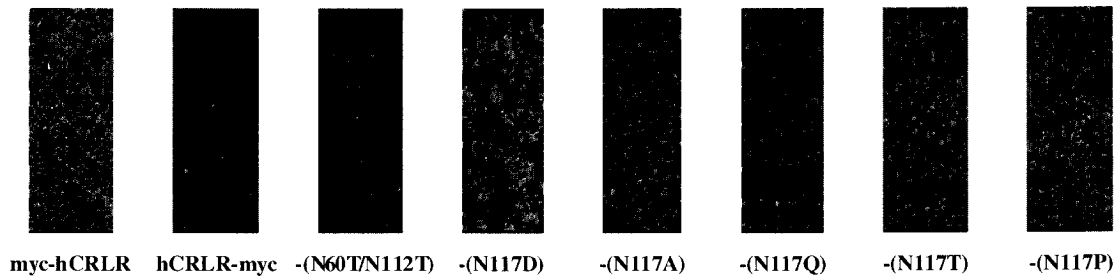


Figure 3. **Myc immunofluorescent-staining of intact TSA cells.** The cells were transfected with expression constructs of the RAMP1-V5 and of the hCRLR with myc at the N-terminus (myc-hCRLR) or indicated mutants, or of the hCRLR with myc at the C-terminus (hCRLR-myc) as a negative control. Mouse antibodies to myc and TRITC-labeled rabbit 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 under Materials and Methods.

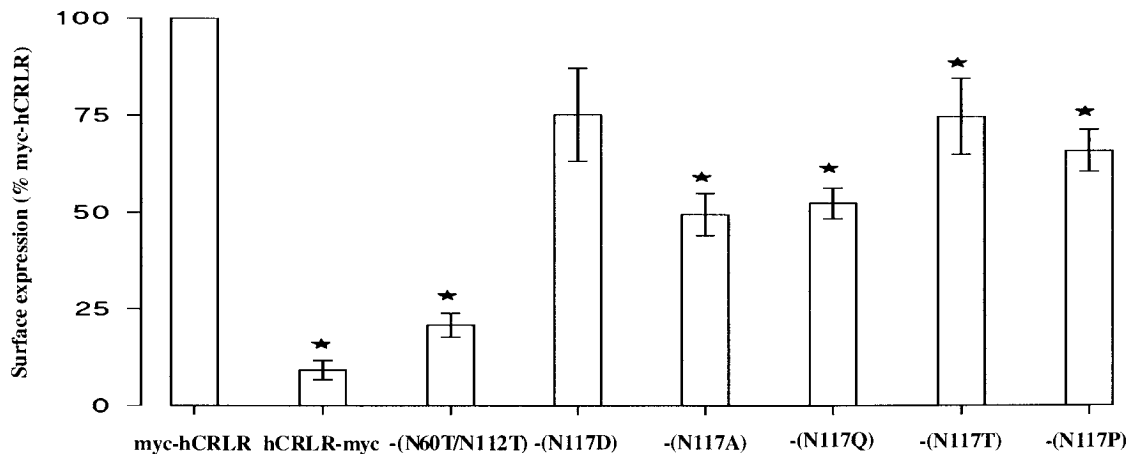


Figure 4. **Quantitative analysis of myc immunofluorescent-staining at the cell surface of intact TSA cells.** The cells were transfected and immunostained and prepared for analysis in a fluorescence microplate reader as described under Materials and Methods. Myc-immunofluorescence as a measure of receptor cell surface expression was normalized to that of myc-hCRLR. The results are means \pm SEM of four independent experiments. * $P < 0.05$.

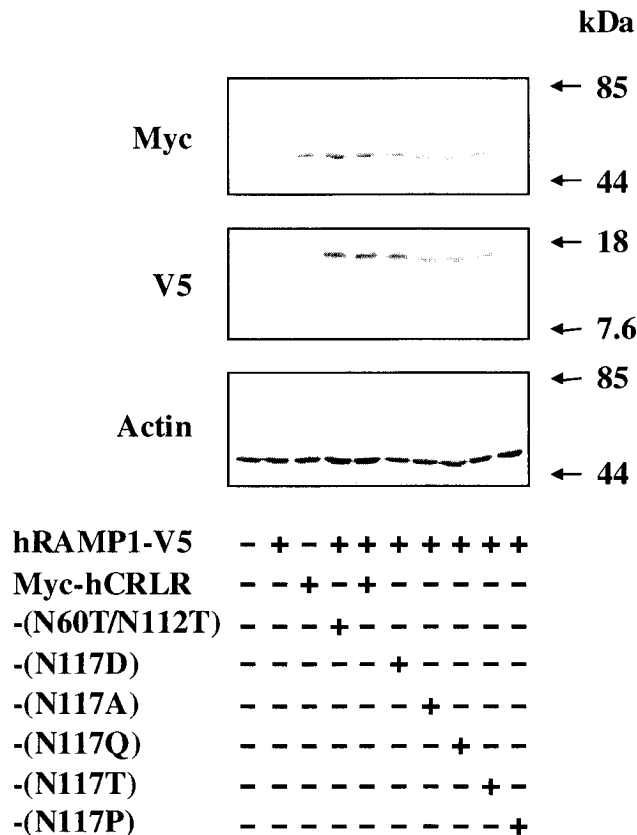


Figure 5. Expression levels of myc-hCRLR and its mutants and of co-expressed RAMP1-V5. TSA cells were grown in 6-well plates to 80% confluency and transfected by incubation for 4 h at 37 °C in 2 ml/well OptiMEM medium containing 4.8 µl LipofectAMINE and indicated combinations of 67 ng RAMP1-V5 and 67 ng myc-hCRLR or -mutant expression constructs. The expression vector pcDNA3 was used for mock transfections and to equalize the total amount of DNA in individual transfections. Two days later, aliquots of cell extracts were treated with 4 U N-glycosidase F and subjected to 15% SDS-PAGE. Myc-tagged receptors and RAMP1-V5 were visualized on Western blots with HRP-conjugated myc- and V5-antibodies. Actin as a reference to control for protein loading was detected with mouse monoclonal antibodies to actin and secondary horseradish peroxidase-conjugated sheep antibodies to mouse immunoglobulins. Representative experiment carried out at least three times.

Taken together, impaired cell surface delivery of myc-hCRLR (N60T/N112T) as compared to non-modified myc-hCRLR in the presence of RAMP1-V5 resulted in a reduced CGRP-evoked cAMP response. In contrast, the myc-hCRLR(N117A), -(N117Q), -(N117T) and -(N117P) lost CGRP receptor function in the face of cell surface expression levels comparable to those of the active myc-hCRLR and its -(N117D) mutant.

Cell-surface association of RAMP1-V5 with myc-hCRLR and the Asn¹¹⁷ mutants and [¹²⁵I]hαCGRP-binding in TSA cells. Mock-transfected cells and cells transiently expressing RAMP1-V5 alone or together with myc-hCRLR or the individual mutants were incubated with [¹²⁵I]hαCGRP and subsequently treated with the cross-linker BS3. Protein components carrying myc epitope-tags were immunoprecipitated from cell homogenates with corresponding antibodies. Co-precipitated [¹²⁵I]hαCGRP was estimated in a γ-counter and [¹²⁵I]hαCGRP-binding protein components were characterized on Western blots by autoradiography and with antibodies to the myc-hCRLR and the RAMP1-V5 (Figure 6). Autoradiography revealed [¹²⁵I]hαCGRP-labeled proteins of between 66-70 and 81-86 kDa in TSA cells expressing myc-hCRLR or -(N117D) together with RAMP1-V5. In immunoprecipitates obtained from mock-transfected cells or from cells expressing RAMP1-V5 alone or with myc-hCRLR(N117A), -(N117Q), -(N117T) or -(N117P) [¹²⁵I]hαCGRP-labeled proteins were undetectable. In cells expressing myc-hCRLR(N60T/N112T) with RAMP1-V5 the size of [¹²⁵I]hαCGRP-labeled proteins was between 61-63 and 68-70 kDa.

On the same Western blots myc-immunoreactive protein components were undetectable in extracts of mock-transfected cells or cells expressing RAMP1-V5 alone. A myc-immunoreactive protein doublet at between 56 and 60 kDa was observed in extracts of cells expressing myc-hCRLR(N60T/N112T) and RAMP1-V5. This size corresponded to that of the myc-hCRLR deglycosylated with N-glycosidase F (Figure 5), indicating impaired N-glycosylation of myc-hCRLR(N60T/N112T). Protein doublets of the same size were also recognized in extracts of cells expressing RAMP1-V5 together with myc-hCRLR and the various Asn¹¹⁷ mutants. But all these samples contained also myc-tagged proteins with the size of the 66-70 and 81-86 kDa [¹²⁵I]hαCGRP-labeled components detected in extracts of cells expressing myc-hCRLR that is known to undergo mature N-glycosylation in the presence of RAMP1 (6). The size difference of the 66-70 and 81-86 kDa protein components corresponded to the molecular weight calculated for RAMP1-V5.

The same Western blots were subsequently analyzed with V5-antibodies. In myc-immunoextracts of cells expressing myc-hCRLR(N60T/N112T) together with RAMP1-V5 a V5-tagged 70 kDa protein was observed. This protein component was undetectable in mock- or RAMP1-V5 transfected cells and its size was consistent with non-glycosylated myc-hCRLR(N60T/N112T) crosslinked with RAMP1-V5.

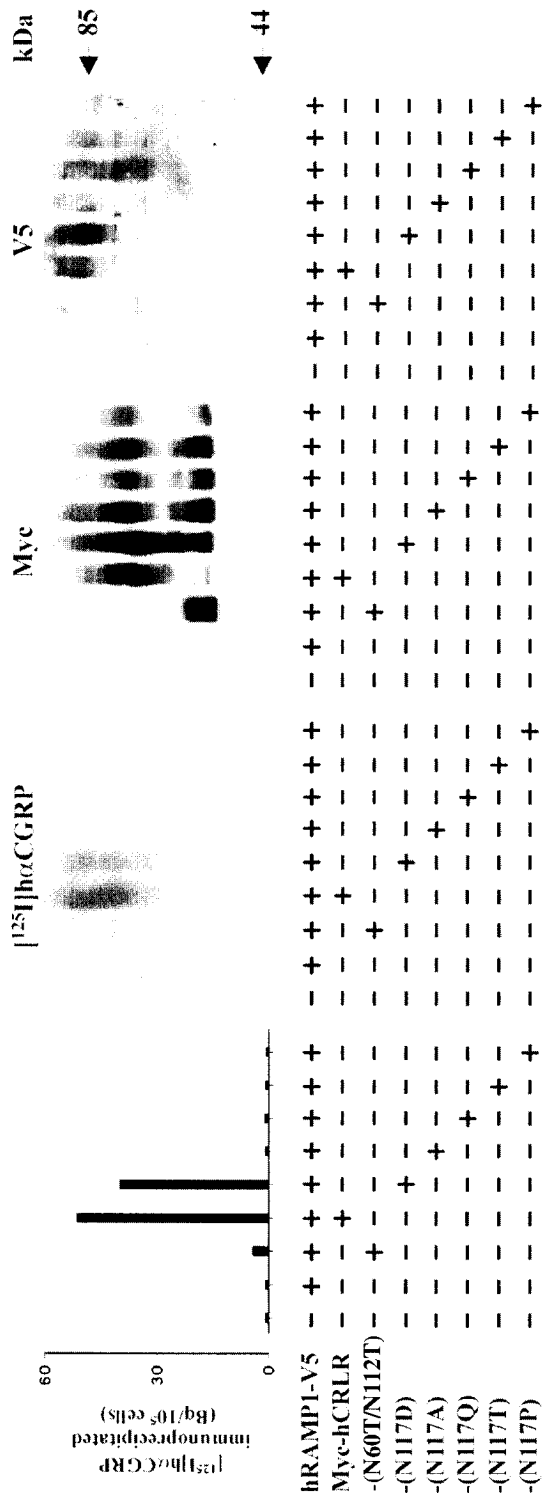


Figure 6. $[^{125}\text{I}]\text{h}\alpha\text{CGRP}$ autoradiography and Western blot analysis of proteins immunoprecipitated with myc-antibodies from TSA cells. The cells were grown in 100 mm dishes to 80% confluency. They were then transfected in 12 ml/dish OptiMEM medium containing 29 μl LipofectAMINE and 800 ng pcDNA3 (mock transfection) or 400 ng RAMP1-V5 expression construct together with 400 ng pcDNA3 or together with 400 ng myc-hCRLR or the indicated mutant myc-hCRLR expression constructs. Two days after transfection $[^{125}\text{I}]\text{h}\alpha\text{CGRP}$ -binding, protein cell surface cross-linking, immunoprecipitations and autoradiography and Western blot analysis of immunoprecipitated proteins subjected to 10% SDS-PAGE were carried out as described under Materials and Methods. Representative experiment carried out three times.

In cells expressing non-modified myc-hCRLR or the myc-hCRLR Asn¹¹⁷ mutants together with RAMP1-V5, V5-immunoreactive protein components extracted with myc-antibodies varied between 81 and 86 kDa equal to the largest proteins recognized with myc-antibodies and by autoradiography. Extracts of cells expressing RAMP1-V5 together with myc-hCRLR(N117A), -(N117Q) or -(N117T) contained additional V5-tagged proteins similar to the 70 kDa V5-immunoreactive component in extracts of myc-hCRLR(N60T/N112T) and RAMP1-V5 co-expressing cells.

Taken together, the results indicate co-immunoprecipitation of RAMP1-V5 cross-linked to mutant- and non-modified myc-hCRLR at the cell surface. But, [¹²⁵I]hαCGRP-binding occurred only in cells expressing myc-hCRLR or -(N117D) together with RAMP1-V5 and it was lower in cells exhibiting impaired cell surface expression and N-glycosylation of myc-hCRLR(N60T/N112T) in the presence of RAMP1-V5 (Figures 3 and 4). The difference in size between the 66-70 and 81-86 kDa radiolabeled protein components in cells co-expressing myc-hCRLR or -(N117D) and RAMP1-V5 and the fact that the 66-70 kDa components were recognized by the myc- but not by the V5-antibodies indicated cross-linking of [¹²⁵I]hαhCGRP to the receptors alone and to the 81-86 kDa receptor/RAMP1-V5 complexes visualized by myc- and V5-antibodies.

Co-immunoprecipitation of hRAMP-V5 with myc-hCRLR and its Asn¹¹⁷ mutants in the absence of cross-linker. Myc-epitope carrying protein components were immunoextracted from TSA cell homogenates and characterized on Western blots with myc- and V5-antibodies (Figure 7). In extracts of mock-transfected cells or of cells expressing RAMP1-V5 alone, myc- and V5-immunoreactive proteins remained undetectable. Immunoextracts of cells expressing myc-hCRLR alone or of cells co-expressing myc-hCRLR(N60T/N112T) and RAMP1-V5 contained myc-hCRLR with an apparent M_r of 56-60'000. In TSA cells co-expressing myc-hCRLR or its different Asn¹¹⁷ mutants and RAMP1-V5 major protein components were between 66 and 70 kDa. This indicated RAMP1-dependent N-glycosylation of the hCRLR in mammalian cells that was reversed when the cell extracts were treated with N-glycosidase F (Figure 5). RAMP1-V5 co-immunoprecipitated with wild-type and all mutant myc-hCRLR, and was recognized with V5-antibodies at its predicted size of 14 kDa. In the face of comparable amounts of myc-hCRLR and individual mutants recognized on Western blots the amounts of co-precipitated RAMP1-V5 varied between the different extracts. They were higher in extracts of cells expressing the functional myc-hCRLR and -(N117D)

CGRP receptors. In total cell homogenates, on the other hand, the RAMP1-V5 expression levels paralleled those of deglycosylated myc-hCRLR and its mutants (Figure 5). In extracts of cells expressing RAMP1-V5 alone the amounts were lower than in cells co-expressing myc-hCRLR or its mutants.

Taken together, the results indicate association of RAMP1-V5 with non-modified myc-hCRLR and all the mutants. In cells expressing the functionally defective myc-hCRLR mutants the fractions of RAMP1-V5 associated with the receptors in immunoprecipitable complexes were smaller than in extracts of cells expressing the functional myc-hCRLR or its -(N117D) mutant.

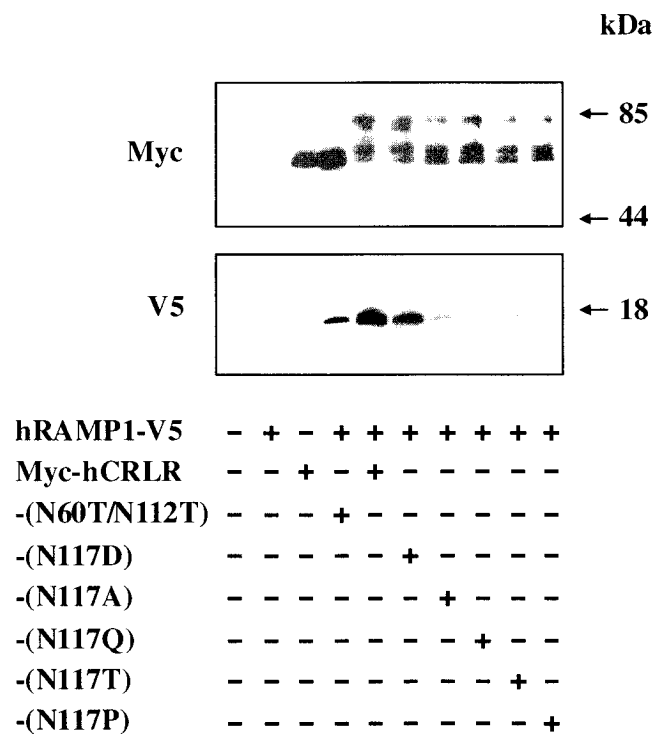


Figure 7. **Co-immunoprecipitation of RAMP1-V5 with myc-hCRLR and the indicated mutants.** TSA cells were grown and transfected as described in Figure 6. Proteins immunoprecipitated with myc-antibodies from cell lysates were subjected to 15% SDS-PAGE and Western blots were analyzed with HRP-conjugated myc- and V5-antibodies. Representative experiment carried out three times.

DISCUSSION

The recognition sequence for N-linked glycosylation is Asn-X-Ser/Thr. Substitution of Asn by a different amino acid eliminates N-glycosylation at a particular consensus site. Mutations of the Asn residues are therefore used to dissect the functional importance of individual N-glycosylation sites.

Here, the three predicted N-glycosylation sites, Asn⁶⁰, Asn¹¹² and Asn¹¹⁷, of the hCRLR have been mutated and CGRP receptor function analyzed in the presence of RAMP1. Our study focused on CGRP-evoked cAMP production, surface expression and the association with RAMP1 in cells expressing individual Asn mutants together with RAMP1. The Asn⁶⁰ or Asn¹¹² to Thr mutants exhibited normal CGRP receptor function and were not further investigated. Adenylyl cyclase activation by CGRP, on the other hand, was impaired by Asn¹¹⁷ to Ala or Gln substitutions and abolished when Asn¹¹⁷ was replaced by Thr or Pro. Importantly, Asp in the place of Asn¹¹⁷ did not affect CGRP receptor activity of the myc-hCRLR. Moreover, all Asn¹¹⁷ hCRLR mutants maintained wild-type N-glycosylation and over 50% cell surface expression similar to the non-modified myc-hCRLR. The findings demonstrate that Asn¹¹⁷ in the hCRLR is not used for N-glycosylation to any great extent. Since the Asn¹¹⁷ to Asp substitution does not affect myc-hCRLR function the introduction of a negative charge in an otherwise unaltered side chain of the amino acid residue in position 117 does not disturb the active conformation. The hCRLR/RAMP1 association was also not affected.

Substitution of Asn¹¹⁷ by Ala, Gln, Thr or Pro, however, resulted in an inactive receptor. Here, the subfraction of RAMP1-V5 that co-immunoprecipitated with the defective myc-hCRLR mutants was lower than that in cells expressing the functionally active myc-hCRLR or its myc-hCRLR(N117D) mutant. The findings are consistent with impaired formation or stability of the inactive mutant receptor/RAMP1 complexes.

The amino acid sequence flanking Asn¹¹⁷ of hCRLR is highly conserved among CRLR, CTR and parathyroid hormone receptors of different species all belonging to the B family of G protein-coupled receptors. Analysis of the N-terminal extracellular domain of myc-hCRLR with the GOR IV secondary structure prediction method (13) revealed limited 5 to 7 amino acid β -sheet and extensive random coil structure flanking Asn¹¹⁷ with no obvious alterations by the Asn¹¹⁷ substitutions. Thus, an interpretation of the present findings awaits more detailed analysis of functional alterations by mutations in the amino acid sequences adjacent and distant from Asn¹¹⁷.

The Asn^{60/112} to Thr double mutant of myc-hCRLR revealed functional defects different from those of the Asn¹¹⁷ mutants. Here, N-glycosylation and therefore cell surface expression were impaired. But, the myc-

hCRLR(N60T/N112T) that reached the cell surface revealed specific [¹²⁵I]hαCGRP binding and adenylyl cyclase activation by CGRP with an EC₅₀ similar to that of non-modified myc-hCRLR. Limited N-glycosylation at Asn¹¹⁷ may explain the reduced cell surface expression of the myc-hCRLR(N60T/N112T). Similar to the inactive myc-hCRLR Asn¹¹⁷ mutants, the subfraction of RAMP1 associated with myc-hCRLR(N60T/N112T), as revealed by co-immunoprecipitation, was diminished as compared to the functional myc-hCRLR and myc-hCRLR(N117D). Together the results indicate that N-glycosylation of the hCRLR is required for transport to the cell surface. But defective N-glycosylation through mutations of both Asn⁶⁰ and Asn¹¹² does not preclude close to normal interaction of hCRLR with CGRP and RAMP1.

In conclusion, impaired or abolished CGRP receptor function caused by substitutions of Asn residues in the three N-glycosylation consensus sequences of hCRLR occurs through two distinct mechanisms. Low level CGRP receptor activity in cells expressing the hCRLR Asn^{60,112} to Thr mutant with defective N-glycosylation is due to deficient cell surface delivery. The Asn¹¹⁷ to Ala, Gln, Thr or Pro mutations of the hCRLR, on the other hand, did not greatly impair cell surface expression, but abolished the functional interaction with CGRP.

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Conclusions and outlook

With the discovery of RAMPs, accumulating evidence indicates that the two seven transmembrane domain G protein-coupled receptors, the CRLR and the CT receptor (CTR) form the basis of the receptors for CGRP, CT, ADM and amylin, all belonging to the CT family of peptides [1, 2]. Co-expression of CRLR with RAMP1 or -2 creates novel CGRP or ADM receptors, respectively. An amylin receptor is revealed following co-transfection of hCTR with RAMP1 or -3, whereas hCTR already functions as CT receptor when expressed without RAMP [3].

Different mechanisms of the RAMP-modulated receptor specificity have been considered. Mature glycosylation of h- and rCRLR as a result of the interaction with RAMP1 and core glycosylation in the presence of RAMP2 were thought to define the specificity of the CRLR for CGRP or ADM in mammalian cells. But, rCRLR expressed in *Drosophila* Schneider S2 cells was shown to be uniformly glycosylated independent of co-expressed RAMP1 or -2, but CGRP- and ADM receptor function was that of rCRLR in mammalian cells. Therefore, the pattern of glycosylation of the CRLR does not define its ligand specificity, at least in *Drosophila* S2 cells.

Besides glycosylation, receptor-RAMP-ligand interactions on the cell surface have been considered as a possible mechanism of RAMP activity. RAMPs modulate receptor specificity through interaction with the receptor. Chemical crosslinking, co-immunoprecipitation and confocal microscopy identified complexes of CRLR or CTR associated with RAMP1 on the cell surface [4]. Physical interactions between CRLR or CTR and RAMPs on the cell surface are required to form ligand-specific binding epitopes, and association of individual RAMPs with CRLR or CTR defines ligand specificity. The existence of RAMPs indicates a new mechanism whereby cells and tissues change their responsiveness to different neuropeptides. It remains to be elucidated whether RAMPs regulate other receptors beyond CRLR and CTR.

Suppression of N-glycosylation with tunicamycin and site-directed mutagenesis of Asn⁶⁰ and Asn¹¹² by Thr reduced cell surface delivery of the myc-hCRLR and its ligand binding. Substitution of Asn¹¹⁷ by Ala, Gln, Thr or Pro abolished CGRP binding, but N-glycosylation, cell surface expression and association with RAMP1 was maintained. In contrast, CGRP receptor activity was left unchanged when Asn¹¹⁷ of the myc-hCRLR was replaced by Asp. Thus, N-glycosylation at Asn⁶⁰ and/or Asn¹¹²

of the myc-hCRLR is required for delivery to the cell surface and subsequent CGRP or ADM binding in the presence of RAMP1 or -2. Asn¹¹⁷, on the other hand, is not involved in N-glycosylation, but essential for ligand binding to the CRLR/RAMP complex.

CGRP and ADM are potent vasorelaxant and hypotensive peptides with concomitant cardiostimulatory activities. Therapeutic use of CGRP and ADM awaits the development of corresponding low molecular weight nonpeptide mimics with suitable bioavailability. Thus, the identification of receptors for CGRP and ADM facilitates the screening for such analogues.

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