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Glycosylation of the calcitonin receptor-like receptor at Asn^{60} or Asn^{112} 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 for functional interaction with CT gene-related peptide (CGRP) or adrenomedullin (ADM), respectively. Three putative *N*-glycosylation sites Asn^{60}, Asn^{112} and Asn^{117} 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 [^{125}I]CGRP and -ADM binding. Similarly, the double mutant myc-hCRLR(N60,112T) exhibited minimal *N*-glycosidase F sensitive glycosylation, presumably at the third Asn^{117}, and the cell surface expression and specific radioligand binding were impaired. Substitution of the Asn^{117} by Thr abolished CGRP and ADM binding in the face of intact *N*-glycosylation and cell surface expression.

Key words: Adrenomedullin; Calcitonin gene-related peptide; Calcitonin receptor-like receptor; Glycosylation; Receptor-activity-modifying protein; Tunicamycin

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]. However, 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 solubilised 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 analysed 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 αCGRP and human amylin were from Bachem AG (Bubendorf, Switzerland), hβCGRP from Peninsula Laboratories (Belmont, CA, USA), and hADM from Peptide Institute (Osaka, Japan). Human CT was provided by E. Felder (Novartis, Basel, Switzerland). Na[^{125}I], enhanced chemiluminescence (ECL)™ Western blot detection reagents and Hybond ECL™ nitrocellulose membranes were from Amersham International (Little Chalfont, UK), and restriction en-

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Abbreviations: ADM, adrenomedullin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; RAMP, human receptor-activity-modifying protein; TSA, SV40 T-antigen transformed human embryonic kidney cells; VIP, vasoactive intestinal polypeptide

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zymes 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 Seamless™ Cloning Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions and [14]. Mutations were A→C substitutions in position 2 of corresponding Asn-encoding triplets within a *Bsu*36I/*Pst*I restriction fragment coding for the hCRLR domain that contains the three predicted *N*-glycosylation sites. *Bsu*36I/*Eam*1104I and *Eam*1104I/*Pst*I DNA fragments carrying the mutations were amplified with cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) by polymerase chain reaction (PCR) for 30 cycles. The products were digested with corresponding restriction enzymes and gel-purified. Matching fragments were ligated into *Bsu*36I/*Pst*I-digested myc-hCRLR yielding myc-hCRLR(N60T), -(N112T) and -(N117T). A double mutant myc-hCRLR(N60, 112T) was obtained as follows: *Not*I/*Xho*I DNA fragments, including the coding sequences of myc-hCRLR(N60T) and -(N112T), were digested with *Bgl*II. The DNA fragment *Not*I/*Bgl*II containing mutation (N60T) and the fragment *Bgl*II/*Xho*I with mutation (N112T) were cloned into *Not*I/*Xho*I-digested pcDNA3 yielding myc-hCRLR(N60, 112T). 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/Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l glucose) medium (1:1) supplemented with 10% foetal calf serum and 400 μg/ml Geneticin. Twenty-four 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 2 days prior to the experiments. Tunicamycin dissolved in dimethyl sulfoxide (DMSO) or DMSO alone were added where indicated. Tunicamycin- and DMSO-treated cells were detached with 0.05% EDTA in phosphate-buffered saline (PBS) and kept in suspension during the experiments.

2.4. Radioligand binding

[^{125}I]-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 [^{125}I]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% bovine serum albumin 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% sodium dodecyl sulphate (SDS). Cells in suspension were collected by centrifugation at 200×*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×*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×*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 Hybond™ ECL™ 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 visualised with horseradish peroxidase (HRP)-labelled monoclonal antibodies to myc (1:5000; Invitrogen, Carlsbad, CA, USA) with the ECL™ technique. Chemiluminescence was visualised 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 μl Ham F12/DMEM (1:1) medium containing 10% foetal calf serum and 4 μg/ml monoclonal HRP-labelled antibodies to myc (Invitrogen) for 90 min at room temperature. The cells were then washed four 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-di-[3-ethylbenz-thiazoline sulfonate (6)] diammonium salt (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 optical density at 405 nm compared to myc-hCRLR controls by Student's *t*-test. The values for half maximal inhibitory concentrations (IC_{50}) 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]hαCGRP 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]hαCGRP 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). The IC_{50} of [^{125}I]CGRP and

Table 1
[^{125}I]hαCGRP and -hADM binding inhibition (IC_{50}) to TSA cells co-expressing myc-hCRLR or receptor mutants with RAMP1 or -2

	[^{125}I]hαCGRP[a] IC_{50} (nM)					[^{125}I]hADM[b] IC_{50} (nM)				
	hαCGRP	hβCGRP	hADM	hAmylin	hCT	hαCGRP	hβCGRP	hADM	hAmylin	hCT
myc-hCRLR	2.2 ± 0.8	1.8 ± 1.0	67 ± 23	65 ± 25	> 1000	> 1000	> 1000	9.7 ± 2.4	> 1000	> 1000
myc-hCRLR(N60T)	5.7 ± 2.9	2.2 ± 0.9	45 ± 22	106 ± 35	> 1000	> 1000	> 1000	33 ± 5	> 1000	> 1000
myc-hCRLR(N112T)	10.7 ± 5.5	1.4 ± 0.8	49 ± 20	78 ± 35	> 1000	> 1000	> 1000	94 ± 14	> 1000	> 1000

Results of IC_{50} are means ± S.E.M. of at least three independent experiments.
[a]TSA cells co-transfected with RAMP1.
[b]TSA cells co-transfected with RAMP2.

-ADM receptor binding of the myc-hCRLR and the EC_{50} of cAMP accumulation with hαCGRP and ADM were those of the non-myc-tagged receptor (not shown). Tunicamycin dose-dependently reduced [^{125}I]hαCGRP 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 μg/ml. Thus, RAMP1 or -2 dependent expression of myc-hCRLR as CGRP- or ADM receptors requires *N*-glycosylation.

3.2. *N-Glycosylation of Asn^{60} and/or Asn^{112} 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(N60, 112T) 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

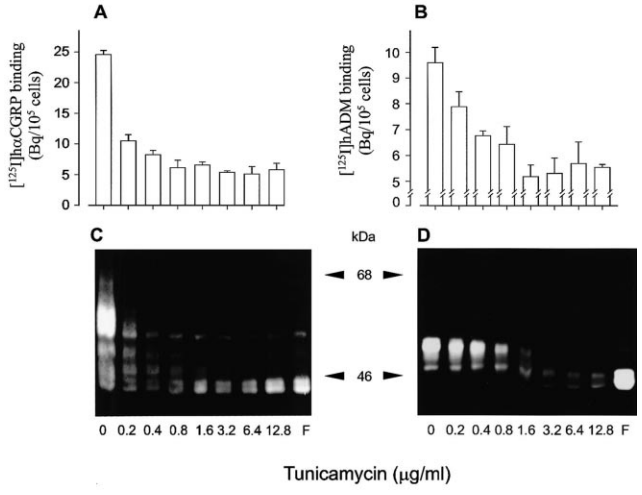

Fig. 1. Radioligand binding and Western blot analysis of myc-hCRLR expressed in tunicamycin-treated TSA cells. The cells were co-transfected with myc-hCRLR and RAMP1 (A, C) or -2 (B, D) expression constructs in the absence (0) and presence of increasing concentrations of tunicamycin. Open bars in (A) and (B) indicate total binding. Non-specific [^{125}I]hαCGRP or [^{125}I]hADM binding in the presence of 1 μM non-labelled hαCGRP or 0.1 μM non-labelled hADM was 3.0 and 3.8 Bq/10^{5}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 visualised by ECL™. Arrows indicate the positions of protein size markers. Representative experiment carried out three times.

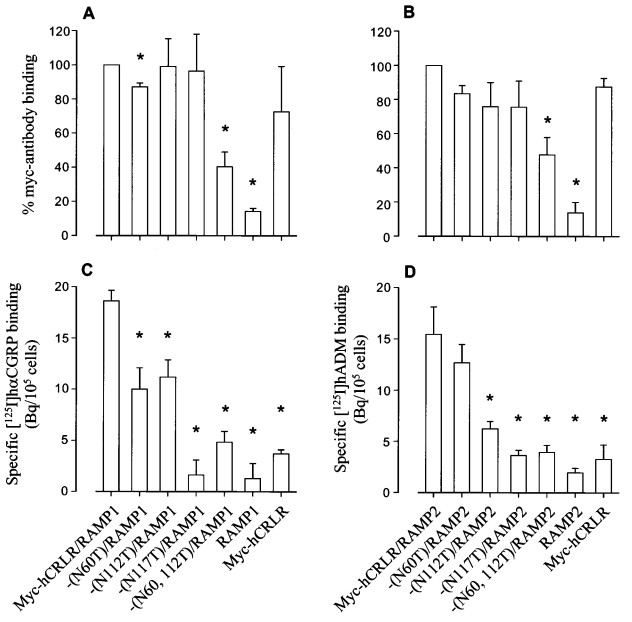

Fig. 2. Cell surface expression and specific radioligand binding of the myc-hCRLR and the mutant receptors. TSA cells were transiently transfected with the indicated combinations of receptors and RAMP1 (A, C) or -2 (B, D) expression constructs. A and B: HRP-labelled myc-antibody binding, normalised to that of TSA cells co-expressing myc-hCRLR and RAMP1 (A) or -2 (B). C and D: Specific radioligand binding. The results are means ± S.E.M. 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.

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(N60, 112T) 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).

3.3. *[^{125}I]hαCGRP and -ADM recognition by myc-hCRLR, -(N60T), -(N112T), -(N60, 112T) and -(N117T)*

In cells expressing myc-hCRLR or the glycosylation mutants (N60T) and (N112T), specific binding of [^{125}I]hα-CGRP and -ADM was maintained in the presence of the corresponding RAMP (Fig. 2). The IC_{50} of hα- and βCGRP, hADM and human amylin of [^{125}I]hαCGRP and -ADM binding reflected the affinities of the expressed receptors (Table 1). Binding inhibition of [^{125}I]hαCGRP by the non-labelled peptides in cells expressing myc-hCRLR and the (N60T) and (N112T) mutants was similar. With [^{125}I]hADM, myc-hCRLR(N60T) and -(N112T) exhibited a 3- and 10-fold higher IC_{50} of hADM as compared to the non-modified myc-hCRLR. Different from the two (N60T) and (N112T) single-site mutant receptors specific [^{125}I]hαCGRP and -ADM binding to the double-mutant (N60, 112T) was below 5% of the added tracers.

In cells expressing myc-hCRLR(N117T) together with the corresponding RAMP specific [^{125}I]hαCGRP and [^{125}I]hADM binding was below 4% of the added tracers. Up to 10^{-7} M

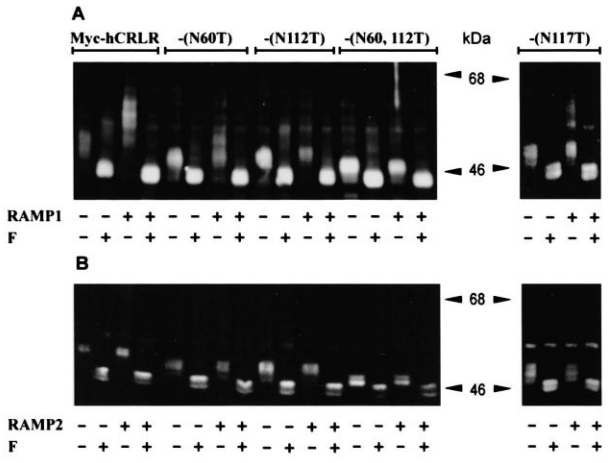

Fig. 3. Analysis of *N*-glycosylation of myc-hCRLR and -(N60T), -(N112T), -(N60, 112T) and -(N117T) in TSA cells on Western blots. Transiently expressed myc-hCRLR and mutant receptors in the absence (−) and presence (+) of RAMP1 (A) or RAMP2 (B). Cell extracts (50 μg protein) incubated in the absence and presence (F) of 80 mU/μl *N*-glycosidase F were subjected to 8% SDS–PAGE. Non-modified and mutant myc-hCRLR on immunoblots were visualised by the ECL™ technique. The positions of protein size markers are indicated by arrows. Representative experiment carried out three times.

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).

3.4. Western blot analysis of myc-hCRLR and -(N60T), -(N112T), -(N60, 112T) 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(N60, 112T) with *N*-glycosidase F treatment indicated minor glycosylation at Asn^{117} that was unaffected by co-expressed RAMP.

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 Asn^{60} or Asn^{112} 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(N60, 112T) 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 Asn^{117} 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 Asn^{117} in myc-hCRLR(N60, 112T) was insufficient for its normal delivery to the cell surface. Thus, Asn^{117} 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 hCT 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. hCT receptors lacking the second or third consensus site exhibited less potent cAMP production. Correspondingly, with the myc-hCRLR(N112T) the IC_{50} of ADM with respect to [^{125}I]ADM binding was somewhat higher than that of the myc-hCRLR. In marked contrast, substitution of the Asn^{117} by Thr inactivated the myc-hCRLR.

In conclusion, hCRLR *N*-glycosylation at Asn^{60} and/or Asn^{112} 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.

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