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Glycosylation of human CRLR at Asn123 is required for ligand binding and signaling

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Abstract

Calcitonin receptor-like receptor (CRLR) constitutes either a CGRP receptor when complexed with receptor activity-modifying protein 1 (RAMP1) or an adrenomedullin receptor when complexed with RAMP2 or RAMP3. RAMP proteins modify the glycosylation status of CRLR and determine their receptor specificity; when treated with tunicamycin, a glycosylation inhibitor, CHO-K1 cells constitutively expressing both RAMP2 and CRLR lost the capacity to bind adrenomedullin. Similarly, in HEK293 EBNA cells constitutively expressing RAMP1/CRLR receptor complex CGRP binding was remarkably inhibited. Whichever RAMP protein was co-expressing with CRLR, the ligand binding was sensitive to tunicamycin. There are three putative Asn-linked glycosylation sites in the extracellular, amino terminal domain of CRLR at positions 66, 118 and 123. Analysis of CRLR mutants in which Gln was substituted for selected Asn residues showed that glycosylation of Asn123 is required for both the binding of adrenomedullin and the transduction of its signal. Substituting Asn66 or Asn118 had no effect. FACS analysis of cells expressing FLAG-tagged CRLRs showed that disrupting Asn-linked glycosylation severely affected the transport of the CRLR protein to the cell surface on N66/118/123Q mutant, and slightly reduced the level of the cell surface expression of N123Q mutant compared with wild-type CRLR. But other single mutants (N66Q, N118Q) had no effect for other single mutants. Our data shows that glycosylation of Asn66 and Asn118 is not essential for ligand binding, signal transduction and cell surface expression, and Asn123 is important for ligand binding and signal transduction rather than cell surface expression. It thus appears that glycosylation of Asn123 is required for CRLR to assume the appropriate conformation on the cell surface through its interaction with RAMPs.

Keywords: Adrenomedullin; Calcitonin receptor like receptor; Receptor activity modifying protein; *N*-Glycosylation

1. Introduction

Adrenomedullin, which is isolated from human pheochromocytoma, is a member of the calcitonin gene-related peptide (CGRP) family [1]. It is known to be potently hypotensive in rat and to evoke increases in the levels of cyclic AMP (cAMP) within mammalian endothelial, smooth muscle and mesangial cells [1–6]. Recently, McLatchie et al. reported that the calcitonin receptor-like receptor (CRLR) could function as either a CGRP receptor or an adrenomedullin receptor, depending on the type of receptor activity-modifying proteins (RAMPs) – members of a new family of single-transmembrane-

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domain proteins – expressed [7]: CRLR generates a CGRP receptor with RAMP1, whereas it generates an adrenomedullin receptor with either RAMP2 or RAMP3. We observed, for example, that in human vascular endothelial and smooth muscle cells CRLR and RAMP2 generate a functional adrenomedullin receptor [8].

RAMP proteins modify receptor specificity by regulating the glycosylation status of CRLR: RAMP2 and RAMP3 modulate CRLR core glycosylation, while RAMP1 modulates the terminal glycosylation [7]. The amino terminus of CRLR is situated in the extracellular domain and has three potential Asn-linked glycosylation sites (at positions 66, 118, and 123) [9,10]. On the other hand, no putative Asn-linked glycosylation site consists in RAMP1 and a single putative Asn-linked glycosylation site, which is respectively conserved among human, mouse, and rat, is situated in RAMP2 and RAMP3 [7,11]. And Aldecoa et al. demonstrated that RAMP2 was glycosylated in Schneider 2 insect cell [12]. RAMP2 and RAMP3 have putative Asn-linked glycosylation sites and RAMP1 has no putative site. In the present study, in spite of glycosylation our data shows that of RAMP, receptor activity of CRLR/RAMPs complex was inhibited tunicamycin and substitution of Asn123 in CRLR fully disrupted receptor activity. And we determined that the glycosylation of Asn at position 123 is required for generating a functional adrenomedullin receptor.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nihon Seiken (Kyoto, Japan). MEM-α was purchased from Gibco BRL (Grand Island, NY). Bovine serum albumin (BSA) and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Chemical (St. Louis, MO). All other reagents were from Nacalai Tesque (Kyoto, Japan).

2.2. Plasmids

All CRLR-related plasmids were constructed from CRLR cDNA cloned into pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA), as described previously [8]. FLAG epitope-tagged CRLRs were constructed by cloning mature CRLR into pFLAG-CMV1 vector (Kodak, New Haven, CT). Three kinds of RAMP plasmids were cloned into pIRES-puro vector carrying the puromycin-resistance gene (Clontech, Palo Alto, CA) using the *Not*I restriction site.

2.3. Cell culture and cDNA transfection

CHO-K1, HEK293 EBNA and HEK293 cells were cultured in MEM-α and DMEM supplemented with 10% fetal bovine serum, respectively, and maintained at 37°C under an atmosphere of 95% air/5% CO_2. Clones expressing both CRLR and each RAMP or only RAMP2 were selected by culturing cells transfected with plasmids encoding CRLR and/or RAMP2 and a selection marker (pDREF-CRLR-EBNA-Hyg [8] and pRAMPs-IRES-puro) in the presence of hygromycin (Wako Chemicals, Osaka, Japan) or puromycin (Clontech), respectively.

For transient transfection, HEK293 cells were plated to a density of $1–5\times10^5$ cells/well in 24-well plates and transfected with 1 μg plasmid/well using LipofectAmine Plus according to the manufacturer's instructions (Gibco BRL). In cases where intracellular cAMP was measured, the normal culture medium was replaced with serum-free medium 24 h after transfection, and the transfectants incubated for an additional 24 h.

2.4. Preparation of membrane fractions

Harvested cells were washed with ice-cold buffer (20 mM HEPES–NaOH (pH 7.4) containing Complete (Roche Diagnostics, Mannheim, Germany)), suspended in the same buffer, homogenized, and centrifuged at $1000\times g$ for 30 min at 4°C. The supernatant was then centrifuged again at $10\,000\times g$ for 30 min at 4°C, and the resultant pellet (membrane fraction) was resuspended in the buffer. The protein concentration in each sample was measured by the Bradford method (Bio-Rad, Hercules, CA).

2.5. Binding assays

Adrenomedullin or CGRP binding to wild-type

and mutant CRLRs in the membrane fraction of HEK293 transfectants was assessed using a scintillation proximity assay system (SPA, Amersham–Pharmacia, Little Chalfont, UK), as described previously [7,13]. Briefly, samples of the membrane fraction (equivalent to a protein concentration of 10 μg for adrenomedullin binding or 30 μg for CGRP binding), pre-coupled to 1 mg of PVT-WGA-SPA beads, were placed the wells of a 96-well microplate containing 100 μl of assay solution (50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 0.5% BSA and 100 pM [^{125}I]rat adrenomedullin or [^{125}I]human CGRP (Amersham–Pharmacia)) and incubated for 2 h at 4°C for adrenomedullin binding or at room temperature for CGRP binding. Radioactivity in the membrane fraction was then counted using a micro-β counter (Amersham–Pharmacia, Wallac). Non-specific binding was determined by incubating the membrane fraction in the presence of 1 μM unlabeled rat adrenomedullin (Peptide Institute, Osaka, Japan) for adrenomedullin binding or human CGRP (Peptide Institute) for CGRP binding; specific binding was then calculated by subtracting the non-specific from the total binding.

2.6. Measurement of cAMP

Intracellular cAMP was measured as reported previously [14]. Briefly, cells were washed twice with Hank's balanced salt solution containing 20 mM HEPES (pH 7.4), 0.1% bovine serum albumin and 0.5 mM IBMX, and then incubated in the same solution for 15 min in the presence or absence of ligand. Thereafter, the cells were lysed in lysis buffer 1B from the Biotrack cAMP EIA assay kit (Amersham–Pharmacia), and cAMP was measured according to the manufacturer's instructions.

2.7. Flow cytometry

HEK293 cells were transiently transfected with the respective cDNAs encoding FLAG-tagged wild-type CRLR (FLAG-CRLR) or one of four CRLRs containing mutations at the Asn-linked glycosylation sites (FLAG-N66Q, FLAG-N118Q, FLAG-N123Q and FLAG-N66/118/123Q CRLR). After harvesting 2 days later, the cells were washed twice with phosphate-buffered saline (PBS) resuspended in PBS containing 0.1% BSA, and incubated for 1 h on ice with anti-FLAG antibody (M2; 1:20 dilution). The cells were then washed twice with PBS+0.1% BSA, incubated for 1 h in the dark with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Dako, Glostrup, Denmark), washed twice more with PBS+0.1% BSA, and resuspended in the same buffer. Cell sorting was performed using an EPICS-XL (Coulter Corp., Hialeah, FL); 30 000 cells were sorted in each experiment.

2.8. Statistics

Values are expressed as means ± S.D. The statistical significance of differences among cells was evaluated using unpaired analysis of variance and Students' *t*-tests. Values of $P < 0.05$ were considered significant. All experiments were performed independently at least repeated three times.

3. Results

3.1. Both CGRP and adrenomedullin are not bound to membranes treated with tunicamycin

To study the effect of the glycosylation on adrenomedullin receptor biology, we first compared the effects of tunicamycin, a glycosylation inhibitor, on HEK293 EBNA cells transfected with CRLR and RAMP1 (293E/CRLR/RAMP1) and on CHO-K1 cells transfected with CRLR and RAMP2 cDNAs (CHO-K1/CRLR/RAMP2), and on respective untransfected cells. After exposing the cells to a maximally effective concentration of tunicamycin for 48 h and preparing membrane fractions as described in Section 2, we measured the level of CGRP binding to RAMP1/CRLR and the level of adrenomedullin binding to RAMP2/CRLR. As shown in Fig. 1, expression of CRLR and RAMP1 markedly enhanced CGRP binding to 293 EBNA cell membrane and expression of CRLR and RAMP2 does. This effect was completely blocked by tunicamycin, which reduced binding in both 293E/CRLR/RAMP1 and CHO-K1/CRLR/RAMP2 cells to the levels seen in respective control cells. Adrenomedullin binding to HEK293 cells constitutively expressing CRLR/RAMP3 complex was lost by the effect of tunicamy-

cin (data not shown). In 293E/CRLR/RAMP1, tunicamycin severely reduced CGRP binding. Both ligand bindings to membranes extracted from control cells was unaffected by tunicamycin. Because there is no glycosylation site in RAMP1, glycosylation of CRLR thus appears to be essential for both ligand bindings to the CRLR/RAMP receptor complexes.

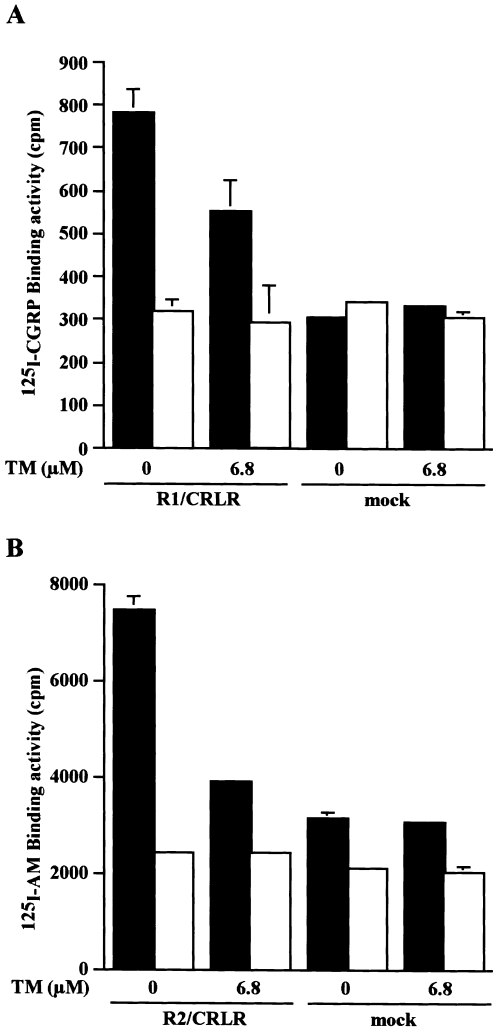

Fig. 1. Effects of tunicamycin on ligand binding to CRLR. (A) [^{125}I]Human CGRP binding to HEK293EBNA cell membranes was assayed. (B) [^{125}I]Rat adrenomedullin binding to CHO-K1 cell membranes was assayed. Membrane fractions were prepared from control and tunicamycin-treated (6.4 μg/ml for 48 h) transfectants constitutively expressing CRLR and each RAMP and from untransfected control cells, as described in Section 2. Closed bar represents total binding and open bar represents non-specific binding.

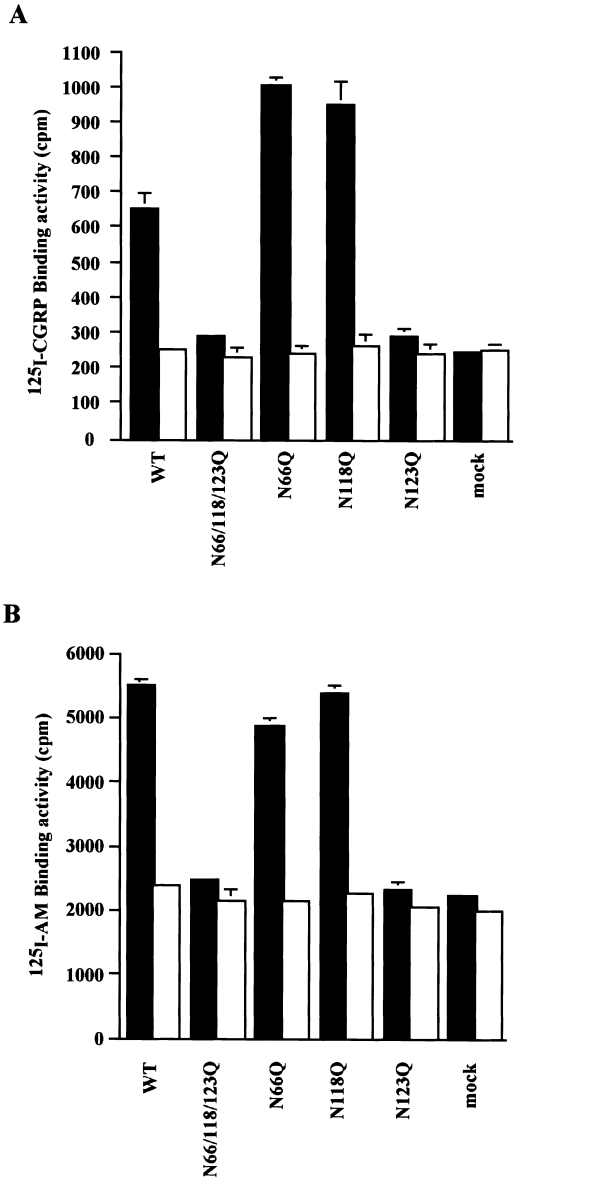

Fig. 2. Ligand binding to CRLRs containing N→Q substitution mutations at Asn-linked glycosylation sites. HEK293 cells were transiently co-transfected with RAMP1 (A) or RAMP2 (B) and wild-type CRLR or one of four mutant CRLRs substituted at Asn66 (N66Q), Asn118 (N118Q), Asn123 (N123Q) or all three (N66/118/123Q). Thereafter, cell membranes were prepared and binding assays were performed as in Fig. 1. Closed bar represents total binding and open bar represents non-specific binding.

3.2. *N-Glycosylation of Asn at position 123 is required for ligand binding*

Three putative Asn-glycosylation sites are situated

in the amino terminal of human CRLR, at positions 66, 118 and 123. To investigate which of these sites is necessary for adrenomedullin binding, a group of mutant CRLRs were constructed by substituting Gln (Q) for Asn (N) at one or all of the glycosylation sites: N66Q, N118Q, N123Q and N66/118/123Q CRLR. HEK293 cells were then transiently co-transfected with RAMP1 or RAMP2 and one of the mutant CRLRs, after which all of the transfectants showed the same level of mRNA expression by northern analysis (data not shown). Membrane fractions were prepared 48 h after transfection, and binding assays were performed. Specific binding to membranes extracted from cells expressing the N123Q and N66/118/123Q CRLR mutants was the same as that to membranes from untransfected cells (Fig. 2). In contrast, CGRP or adrenomedullin binding to membranes extracted from cells expressing either the N66Q or N118Q CRLR mutant was the same as that to membranes from cells expressing wild-type CRLR.

When expressed with RAMP3, the N123Q or N66/118/123Q mutant had similar inhibitory effects on adrenomedullin binding (data not shown).

3.3. N-Glycosylation of Asn123 is important for ligand signaling of cAMP

To assess the importance of glycosylation of Asn123 for adrenomedullin-evoked increases in intracellular cAMP, HEK293 cells were co-transfected for 48 h with RAMP1 or RAMP2 and one of the four CRLR mutants, after which intracellular cAMP was measured. As in untransfected cells, cyclic AMP levels in cells expressing the N66/118/123Q CRLR mutant were unaffected by CGRP or adrenomedullin, even at a concentration of 100 nM (Fig. 3A,B). Expression of the N123Q mutant was also profoundly inhibitory, though a small but significant

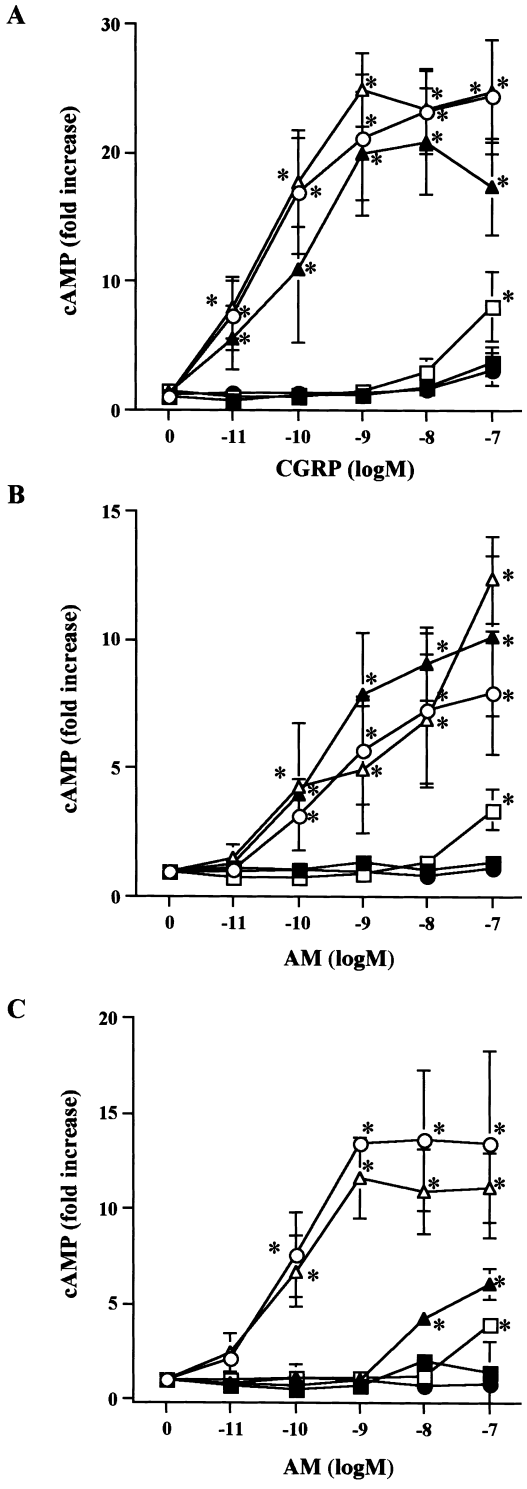

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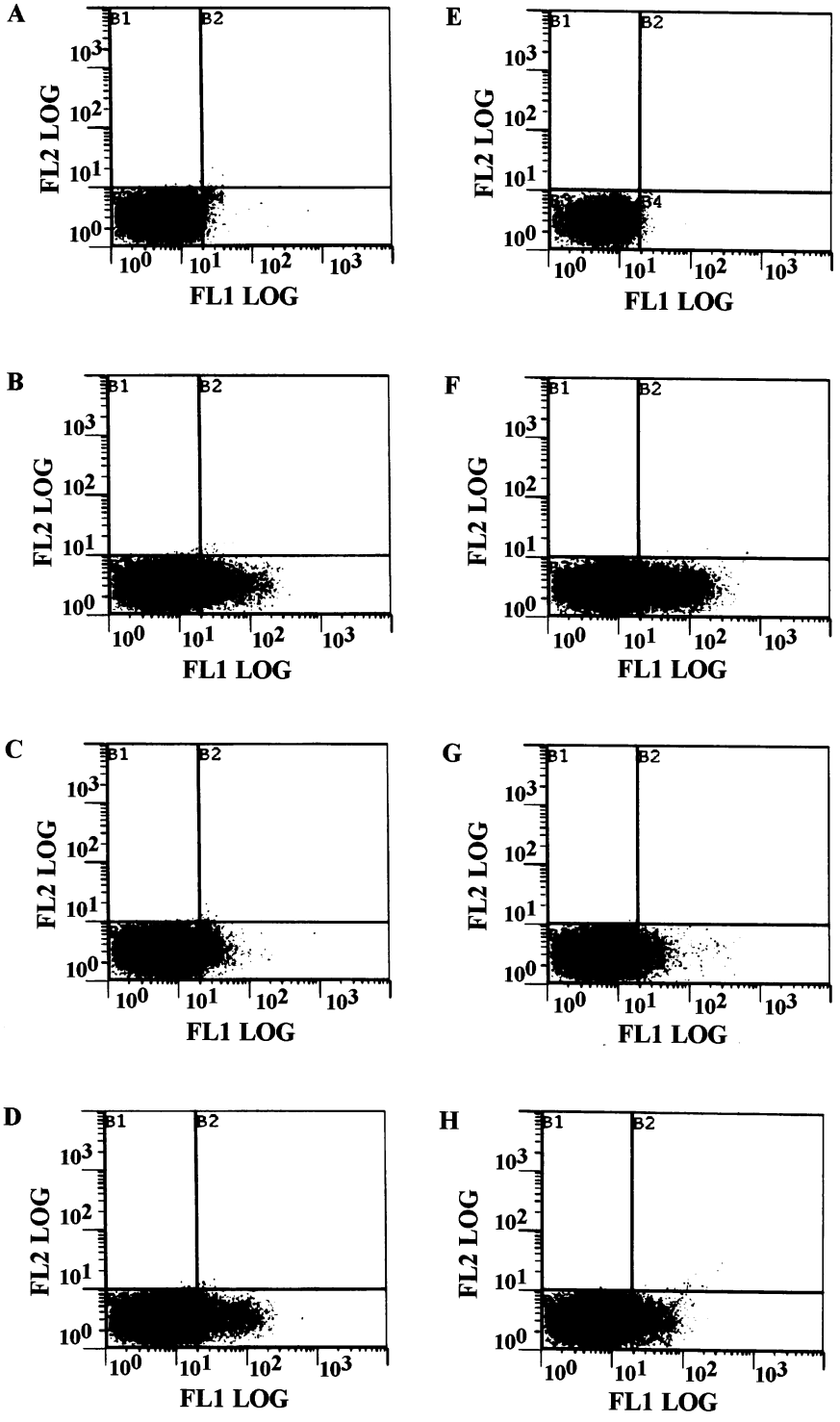
Fig. 3. Effect of ligand on intracellular cAMP levels in HEK 293 cells expressing each RAMP and wild-type or mutant CRLRs. HEK293 cells transiently transfected with RAMP1 and either wild-type (○), N66Q (△), N118Q (▲), N123Q (□), N66/118/123Q (■) CRLR or mock (●) were stimulated with 0–10^{-7} M CGRP (A), with RAMP2 and either wild-type or above CRLR mutants with 0–10^{-7} M adrenomedullin (B), and with RAMP2 and either wild-type (○), N66/118Q (△), N66/123Q (▲), N118/123Q (□), N123Q (■), or mock (●) for 15 min at 37°C. Intracellular cAMP levels were then measured using a cAMP EIA kit. The data are expressed as mean ± S.D. *$P < 0.05$ versus basal levels.



increase in cAMP was elicited by 100 nM adrenomedullin. In contrast, CGRP or adrenomedullin elicited concentration-dependent increases in cAMP in cells expressing the N66Q or N118Q mutant that were indistinguishable from those elicited in cells expressing wild-type CRLR. In these cells, significant increases in cAMP were first seen at an adrenomedullin concentration of 0.1 nM, a concentration 10 000

Fig. 4. Delivery of FLAG-tagged wild-type and mutant CRLRs to the cell surface. HEK293 cells co-transfected with RAMP1 (A–D) or constitutively expressing RAMP2 (E–H) were transiently transfected with cDNA encoding FLAG-tagged wild-type or mutant CRLR, after which the cells were incubated first with mouse monoclonal anti-FLAG antibody (M2) and then with FITC-conjugated anti-mouse IgG. FACS sorting was then performed on more than 30 000 cells in each experiment. Sham-transfected cells showed no significant fluorescence (data not shown). (A,E) Control, HEK293 cells expressing RAMP1 (293/RAMP1) (A) or HEK293 cells expressing RAMP2 (293/RAMP2) (E). (B,F) 293/RAMP1 (B) or 293/RAMP2 (F) cells transiently transfected with FLAG-wild-type CRLR. (C,G) 293/RAMP1 (C) or 293/RAMP2 (G) cells transfected with FLAG-N66/118/123Q CRLR. (D,H) 293/RAMP1 (D) or 293/RAMP2 (H) cells transfected with FLAG-N123Q CRLR.
←

times lower than that required to elicit an effect in cells expressing the N123Q mutant.

Consistent with the binding data, lower concentrations of adrenomedullin had no effect on cAMP levels in cells expressing RAMP3 and either the N123Q or N66/118/123Q CRLR mutant (data not shown).

We attempted to investigate intracellular cAMP increase in further new CRLR mutants: N66/118Q, N66/123Q, N118/123Q, when adrenomedullin evoked. As the results, N66/118Q had no effect and, in contrast, other double mutants were inhibitory (Fig. 3C).

3.4. Delivery of N123Q CRLR to the cell surface

To assess the membrane topology of the CRLR mutants, isoforms containing an amino terminal FLAG epitope were constructed and transfected into HEK293 cells transiently co-transfected RAMP1 or HEK293 cells constitutively expressing RAMP2. No differences in the expression, binding or signaling were observed between untagged and FLAG-tagged CRLR derivatives (data not shown). Moreover, FACS analysis of these cells demonstrated similar levels of antibody binding to FLAG-tagged, wild-type CRLR and to the N66Q and N118Q mutants (data not shown). The antibody binding to N123Q mutant CRLR was lower than wild-type CRLR and N66/118/123Q CRLR was markedly reduced (Fig. 4). N123Q CRLR thus appear to be transported to the cell surface to the some extent; If the efficiencies of transfection were equal to all plasmid constructs, the level of antibody binding in N123Q mutant was 84% (RAMP1/CRLR), 40% (RAMP2/CRLR) and 50% (RAMP1/CRLR) 18% (RAMP2/CRLR) in N66/118/123Q as wild type.

4. Discussion

Most G-protein-coupled receptors (GPCRs) are glycosylated [15], though the specific function of the glycosylation may vary considerably. By inhibiting glycosylation during GPCR synthesis or by enzymatic deglycosylation of expressed receptors, investigators have been able to determine that, in some GPCRs, glycosylation plays a role in receptor transport [16] and function [17–20], while in others glycosylation serves no apparent function [21–25]. Our demonstration that tunicamycin blocks CGRP and adrenomedullin binding confirms that for CRLR glycosylation is required for the receptor to function properly.

It was recently shown that the adrenomedullin receptor exists as a complex of CRLR and RAMP2 or RAMP3 and simultaneously CGRP receptor consists of CRLR and RAMP1, and that the amino terminal of RAMPs is critical to the glycosylation state and ligand binding of CRLR [7,26,27]. When co-expressed with RAMP2 or RAMP3, CRLR is core-glycosylated, whereas CRLR co-expressed with RAMP1 is terminal-glycosylated [7]. Several putative Asn-linked glycosylation sites are situated in RAMP2 and RAMP3 but no site is situated in RAMP1. One nearest to the carboxyl terminus out of these sites is conserved not only among mouse, rat, and human but also between RAMP2 and RAMP3 [7,11]. There are three putative Asn-linked glycosylation sites at positions 66, 118 and 123 in the amino terminus of human CRLR [9,10]; it was unknown which site was most important for the cell surface delivery of CRLR, the binding of CGRP and adrenomedullin and the transduction of its signal. Our analysis of mutant CRLRs showed that Asn66 and Asn118 would be the glycosylation sites not essential and Asn123 is the glycosylation site essential for

binding CGRP and adrenomedullin and transducing its signal. Flow cytometry of cells expressing either RAMP1 or RAMP2 and FLAG-tagged CRLR showed that substitution of a Gln residue for Asn123 somewhat affected transport of the CRLR protein to the cell surface. Taken together, these findings indicate that disruption of cell surface glycosylation at Asn123 of CRLR alters the structure or the receptor, making it unable to bind CGRP or adrenomedullin rather than to deliver to the cell surface.

Human calcitonin receptor (hCTR) generates an amylin receptor with RAMP1 or RAMP3 [28–33]. In addition, RAMP1 confers CGRP binding the calcitonin receptor [33]. Subtypes of hCTR may contain three or four amino-terminal, Asn-linked glycosylation sites in the extracellular domain [34]. Of these subtypes, hCTR3, which is produced by alternative RNA splicing and lacks the first 47 amino acids, including a potential Asn-linked glycosylation site, contains three potential glycosylation sites [35], among which Asn83 is the most important for the binding of calcitonin and the transduction of its signal [20].

It has been proposed that both glycosylation state and the amino termini of RAMP proteins determine the pharmacology of RAMP-coupled receptors, with RAMP determining the receptor specificity either directly or indirectly [30]. Experiments carried out using chimeric RAMP proteins, comprised of the transmembrane and cytosolic domains of RAMP1 and the extracellular amino terminus of RAMP2 (RAMP2/1) and vice versa (RAMP1/2), demonstrated that co-expression of the RAMP2/1 chimera with CRLR produced responses similar to those seen with RAMP2 alone, which suggests that the amino termini of RAMPs are crucial for determining the activity and receptor specificity of CRLRs [26,28]. Furthermore, it was shown that ^{125}I-labeled ligand cross-linked with rCRLR and RAMP protein in Schneider insect cells and RAMP protein appeared at the cell surface in close association with CRLR [12]. It is directly indicated that ligand interacted with both amino-terminus of RAMP and extracellular domain of CRLR. We therefore postulate that CRLR/RAMP complexes are formed principally via the interaction of the transmembrane domain of CRLR and cytosolic carboxyl terminus of RAMP; interaction between the amino terminus of RAMP and the extracellular domain of CRLR determines receptor specificity. Perhaps glycosylation of Asn123 produces a conformational change in CRLR enabling its interaction with RAMP, after which the amino terminus of RAMP may interact with the Asn-glycosylated amino terminal of CRLR, and thereby was concerned with creating a peptide binding-pocket.

Very recently, Bühlmann et al. reported that glycosylation of two CRLR at Asn66 or Asn118 is important for cell surface expression [36], although CRLR protein from a CRLR cDNA used in their report is six amino acid residues shorter than that of our report. In their report it was shown that a single substitution of either Asn66, Asn118, or Asn123 caused remarkable reduction of CGRP or adrenomedullin binding but did not affect transport to the cell surface. Also, the double mutant of Asn66 and Asn118 inhibited the binding and cell surface expression. According to analysis of Asn-linked glycosylation of myc-CRLR and mutant on western blotting in the absence or presence of RAMPs or *N*-glycosides F, it is suggested that Asn123 of myc-CRLR is required for ligand recognition in the presence of RAMPs and not required for Asn-linked glycosylation and cell surface delivery of the hCRLR. Our data showed that single substitution of Asn66 or Asn118 did not affect cell surface expression nor receptor function, whichever RAMP was expressed, and that both Asn66 and Asn118 were disrupted, signal transduction of adrenomedullin receptor being apparently normal, comparable to wild type. In contrast, disruption of Asn123 in our data led to some reduction of transport to the cell surface, and completely inhibited ligand and signal transduction of CRLR. The reason why this difference/discrepancy between these two results exists is unknown. It may be due to substitution of Asn to Gln or Thr, due to plasmids used (for example promoter, transfection efficiency and so on), or due to host cells for transfection. However, Asn123 was important in recognizing ligand in the presence of RAMPs corresponding with our data. In the future, further investigation is required to deepen the understanding of the relation between RAMP function and glycosylation of CRLR.

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