Protein-Protein Interaction and Not Glycosylation Determines the Binding Selectivity of Heterodimers between the Calcitonin Receptor-like Receptor and the Receptor Activity-modifying Proteins

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The receptor activity-modifying proteins (RAMPs) and the calcitonin receptor-like receptor (CRLR) are both required to generate adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) receptors. A mature, fully glycosylated, form of CRLR was associated with 125I-CGRP binding, upon co-expression of RAMP1 and CRLR. In contrast, RAMP2 and -3 promoted the expression of smaller, core-glycosylated, CRLR forms, which were linked to AM receptor pharmacology. Since core glycosylation is classically a trademark of immature proteins, we tested the hypothesis that the core-glycosylated CRLR forms the AM receptor. Although significant amounts of core-glycosylated CRLR were produced upon co-expression with RAMP2 or -3, cross-linking experiments revealed that 125I-AM only bound to the fully glycosylated forms. Similarly, 125I-CGRP selectively recognized the mature CRLR species upon co-expression with RAMP1, indicating that the glycosylation does not determine ligand-binding selectivity. Our results also show that the three RAMPs lie close to the peptide binding pocket within the CRLR-RAMP heterodimers, since 125I-AM and 125I-CGRP were incorporated in RAMP2, -3, and -1, respectively. Cross-linking also stabilized the peptide-CRLR-RAMP ternary complexes, with the expected ligand selectivity, indicating that the fully processed heterodimers represent the functional receptors. Overall, the data indicate that direct protein-protein interactions dictate the pharmacological properties of the CRLR-RAMP complexes.

In 1998, McLatchie et al. (1) reported that co-expression of two classes of polypeptides, a seven-transmembrane receptor known as the calcitonin receptor-like receptor (CRLR) and accessory proteins termed receptor activity-modifying proteins (RAMPs), are required to generate functional receptors for calcitonin gene-related peptide (CGRP) and adrenomedullin (AM). The formation of heterodimers between RAMPs and CRLR was found to be essential for the proper cell surface targeting and pharmacological characteristics of both CGRP and AM receptors (2, 3). The RAMP family comprises three members (RAMP1, -2, and -3) that share less than 30% sequence identity but a common topological organization. They are small intrinsic membrane proteins (predicted sizes: M, 14,000–17,000) with a large extracellular N terminus (~100 amino acids), a single transmembrane domain, and a very short intracellular domain (10 amino acids). Co-expression of RAMP1 with CRLR led to the formation of a CGRP receptor, whereas RAMP2 and -3 promoted the expression of an AM receptor (1).

The distinct ligand-binding specificity imposed by the RAMP expressed with CRLR was accompanied by apparent differences in the receptor maturation pathways. The major CRLR species obtained upon co-expression with RAMP1 corresponded to a mature glycoprotein of M, 66,000 containing fully processed complex oligosaccharides. This species was associated with the binding of 125I-hoCGRP to the cell surface. In contrast, the major CRLR product observed with RAMP2 and -3 was an apparently immature M, 58,000 form containing high mannose core oligosaccharides that were sensitive to endoglycosidase H (endo H) treatment (1, 4). These data suggested three ways in which RAMPs might confer CGRP or AM receptor properties: 1) the glycosylation state of CRLR may determine the binding selectivity; 2) RAMPs might contribute directly by contributing or masking binding sites; or 3) RAMPs might enable different binding states of CRLR by allosterically modifying its conformation (5). Although it was implicitly proposed that the M, 66,000 form of CRLR is the CGRP receptor while the immature M, 58,000 species represent the AM receptor, this hypothesis was never directly tested and not entirely satisfactory.

First, the molecular weight of the AM receptor in native tissues has been reported as being significantly larger (6). Second, the idea that an incompletely processed CRLR could represent the AM receptor is somewhat surprising. Indeed, processing of the oligosaccharides from the high mannose to the complex type normally occurs during the transit through the Golgi apparatus, which is a normal prerequisite for cell surface expression of glycoproteins (7). It is not clear how a receptor could bypass this maturation process. More recent data also argued that the difference in the glycosylation state of CRLR is unlikely to be responsible for the distinct pharmacology. Indeed, the transfection of CRLR-RAMP1 or CRLR-
RAMP2 heterodimers in Drosophila Schneider 2 cells led to CRLR moieties that had no apparent differences in their glycosylation status but still displayed distinct CGRP and AM receptor pharmacology, respectively (2). However, whether distinct maturation pathways that did not result in different glycosylation patterns in these insect cells could still be involved in defining their pharmacological properties was not directly assessed. Moreover, the particular glycosylation machinery characteristic of the insect cells (8) makes it difficult to extrapolate to the mammalian cells.

The present study was therefore undertaken to further investigate the maturation of CRLR and its implication in the pharmacological properties of the receptors produced upon co-expression with the three RAMPs in mammalian cells. Our results demonstrate that RAMP2 and RAMP3 and not only RAMP1 can promote the full maturation of CRLR, leading to its terminal glycosylation. Moreover, only the fully processed receptors were found to bind AM and CGRP. Linked to the observation that the radiolabeled peptides could be cross-linked to RAMPs, our data suggest that direct protein-protein interactions between the RAMPs and CRLR and not the glycosylation state of the later determine the binding selectivity of the complex.

### EXPERIMENTAL PROCEDURES

#### Materials

125I-labeled AM and 125I-labeled hCGRP (specific activity: 2000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Human AM and hCGRP were from Bachem AG (Buhlen, Switzerland). Mouse anti-Myc antibodies (9E10 clone) and mouse anti-HA antibodies (12CA5 clone) were produced by our core facility as ascites fluids. Rabbit anti-Myc antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and rat anti-HA antibodies (3F10 clone) were from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated secondary antibodies were from Amersham Pharmacia Biotech. Bis(sulfosuccinimidyl)suberate (BS3) was from Pierce. All other reagents were of analytical grade and were obtained from various commercial suppliers.

#### Methods

**cDNA Constructs**—The expression vectors for RAMP1, RAMP2, RAMP3, Myc-CRLR, and HA-CRLR were constructed as previously described (1). The RAMP1/2, RAMP2/1 chimeras were constructed using the conserved DPP motif, located at the amino-terminal side of the transmembrane domain, as the boundary between the two proteins (4).

**Cell Culture and cDNA Transfection**—Human embryonic kidney 293T cells (HEK293T) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, 100 units/ml streptomycine, at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

Transient transfactions were performed using calcium phosphate co-precipitation (9). Cells in six-well plates or 100-mm Petri dishes were grown to 70% confluence and transfected with 0.1 μg DNA for HA or Myc epitope-tagged CRLR or 0.05 μg DNA for RAMPs per cm2. The total amount of DNA transfected in each condition was kept constant by adding empty pcDNA3 vector when needed. Twenty hours after transfection, the medium was replaced with fresh medium containing 5 mM sodium butyrate. All of the experiments were performed 72 h after transfection to allow correct glycosylation and cell surface targeting of CRLR and RAMP.

**Total and Cell Surface Immunoprecipitation**—Transfected HEK293T cells grown in 100-mm Petri dishes were washed three times in PBS and incubated with blocking buffer (PBS containing 0.2% bovine serum albumin) for 1 h on ice followed by incubation with anti-Myc (9E10) or anti-HA (12CA5) antibodies (1:150 dilution) in blocking buffer on ice for 2 h. After two washes in blocking buffer and two washes in PBS, the cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (Roche Molecular Biochemicals), 0.1 mg/ml bacitracin, 0.1 mg/ml benzamidine, 2.5 μg/ml leupeptin and centrifuged at 12,000 × g for 15 min at 4 °C. Protein concentration was assessed by using Bio-Rad’s DC assay kit with bovine serum albumin as a standard.

For “total” immunoprecipitation, lysates were incubated overnight at 4 °C with additional anti-Myc or anti-HA antibodies (1:500 dilution) before the addition of 30 μl of protein G-Sepharose (Amersham Pharmacia Biotech) for 3 h. For “cell surface” immunoprecipitation, only protein G-Sepharose was added. The same quantity of proteins was used for “total” and “cell surface” immunoprecipitation. For a higher quantity of 100 μg of total proteins, protein samples were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to immunoblotting using rabbit polyclonal anti-Myc antibodies (1:8000 dilution), or rat monoclonal anti-HA antibodies (3F10 clone, 1:3000 dilution). The Renaissance chemiluminescence kit was used for the Western blot development (PerkinElmer Life Sciences).

**Deglycosylation Treatment**—Deglycosylation of immunopurified Myc-CRLR was carried out with endo H or peptide-N-glycosidase F (PNGase F) (Roche Molecular Biochemicals). Protein G-Sepharose/anti-body/Myc- or CRLR complexes were washed with 50 mM sodium phosphate, pH 7.5, and incubated for 16 h at 37 °C in 40 μl of 50 mM sodium phosphate at either pH 5.5 (endo H) or pH 7.5 (PNGase F) containing 0.5 M n-octyldodecyl maltoside, 50 mM EDTA, 0.1% phenylmethylsulfonyl fluoride, 0.1 mg/ml benzamidine, 0.25 μg/ml leupeptin, 1% β-mercaptoethanol supplemented with 50 milliunits/ml endoglycosidase H or 50 units/ml N-glycosidase F. Sample incubation in the absence of either enzyme were carried out as control. Reactions were stopped by the addition of 5 × protein sample buffer (0.3 ml Tris-HCl, pH 6.8, 10% SDS, 500 mM dithiothreitol, 8.5 mM urea) and heating of the samples at 100 °C.

**Binding and Covalent Cross-linking of 125I-labeled AM and 125I-labeled hCGRP**—HEK293T cells in six-well plates were transfected with Myc-CRLR or HA-CRLR in the presence of empty vector or vector encoding RAMPs (RAMP1, -2, and -3) or chimeric constructs (RAMP1/2 and -2/1). Seventy-two hours post-transfection, cells were washed three times in cold PBS and incubated on ice for 3 h in binding buffer (Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin and 20 mM HEPES, pH 7.4) with 125I-labeled AM (80 pM) or with 125I-labeled hCGRP (40 pM) in the absence or presence of 1 μM unlabeled ligand, human AM 1–52 or hCGRP, respectively. After incubation, cells were washed once with binding buffer and twice with PBS and then cross-linked for 1 h with 1 μl BST in PBS. The cross-linking reaction was stopped by incubating cells (3 × 10 min) in PBS containing 25 mM glycine. Cells were then lysed with blocking buffer followed by immunoprecipitation with anti-Myc antibodies (9E10) or mouse anti-HA antibodies (12CA5), and the immunocomplexes were treated or not treated with endo H or PNGase F as described above. Proteins were separated on 10 or 14% SDS-PAGE and transferred to nitrocellulose. Cross-linked 125I-labeled AM and 125I-labeled hCGRP were detected with BioMax MS Eastman Kodak Co. films (Amersham Pharmacia Biotech).

### RESULTS AND DISCUSSION

**RAMP1, RAMP2, and RAMP3 Promote Cell Surface Expression of a Fully Processed Myc-CRLR**—The identity of the CRLR species targeted to the cell surface was initially assessed in HEK293T cells transiently transfected with Myc-tagged CRLR alone or in combination with RAMP1, -2, or -3. In the absence of co-transfected RAMP, a major species of M68,000, which was previously shown to be the precursor core-glycosylated form of CRLR (1), was detected in the total extract (Fig. 1, lane 1). However, this species was not detected upon cell surface immunoprecipitation (lane 2), indicating that it does not reach the plasma membrane. A lower molecular form of M50,000, the nature of which will be discussed later, was detected both in the total extract and at the cell surface. In the presence of RAMP1, only a M66,000 species was observed. This form, previously described as terminally glycosylated (1), was detected both in the total extract (lane 3) and at the cell surface (lane 4), indicating that RAMP1 promoted additional maturation of CRLR and its transport to the cell surface.

In cells co-expressing Myc-CRLR and RAMP2, both the M58,000 and 66,000 forms were detected in the total extract (lane 5).
5), indicating that RAMP2 is less efficient than RAMP1 in promoting the maturation of CRLR to the M₉, 66,000 form. Despite the fact that the M₉, 58,000 form represented the major species in the total extract, only the M₉, 66,000 form was observed at the cell surface (lane 6). As in the absence of co-transfected RAMP, the M₉, 90,000 form was also observed in the total extract and at the cell surface. When expressed with RAMP3, equal amounts of M₉, 58,000 and 64,000 forms were observed, in both total extracts and at the cell surface (lanes 7 and 8).

Taken together, these results suggest that not only RAMP1 but also RAMP2 and -3, albeit to a lesser extent, can promote further processing of the core glycosylated M₉, 58,000 form to M₉, 66,000 and 64,000 species that can be expressed at the cell surface.

In order to confirm that the M₉, 66,000 and 64,000 species represented CRLR mature species that underwent Golgi processing, the glycosylation state of the forms obtained in each co-transfection condition were analyzed. As expected, when Myc-CRLR was expressed alone, the M₉, 58,000 form (Fig. 2, lane 1) was reduced to M₉, 48,000 by treatments with both endo H (lane 2) and PNGase F (lane 3), the endo H sensitivity confirming the lack of Golgi processing and suggesting that this species represents an immature form of the receptor. In contrast, the M₉, 90,000 form, which was detected at the cell surface, was found to be sensitive to PNGase F but resistant to endo H, indicating that it may represent an SDS-resistant complex involving the fully processed form of the receptor. When co-expressed with either RAMP1, -2, or -3, the CRLR species targeted to the cell surface (M₉, 66,000, 66,000 or 64,000, respectively) were found to be sensitive to PNGase F (being reduced to M₉, 48,000) but resistant to the action of endo H (lanes 6 and 5, respectively). The small decrease in mobility observed in all cases following endo H treatment most likely reflects a contaminating mannosidase activity. These data therefore confirm that the three RAMPs can promote Golgi transit and terminal glycosylation of CRLR.

The initial studies that led to the suggestion that RAMP2 and -3 cannot promote terminal glycosylation of CRLR were carried out using an HA-tagged receptor. To test the possibility that the presence of the HA tag may explain the difference observed between these studies and the present report, the expression pattern of the HA-CRLR was also investigated. Consistent with these previous studies, the core-glycosylated M₉, 58,000 form represented the major species found both in the total extract and at the cell surface when HA-CRLR was co-expressed with RAMP2 (Fig. 3, lanes 5 and 6) or RAMP3 (lanes 7 and 8). Interestingly, a significant amount of core glycosylated HA-CRLR was observed even when RAMP1 was co-expressed (lanes 3 and 4), indicating that the choice of epitope tag may be a critical factor. However, in agreement with what was seen when using Myc-CRLR, the terminally glycosylated form (M₉, 66,000 for RAMP1 and -2 and M₉, 64,000 for RAMP3) could also be detected in total extracts (lanes 3, 5, and 7) and at the cell surface (lanes 4, 6, and 8), albeit to a lesser extent. This confirms that the three RAMPs can promote the maturation of CRLR, although RAMP2 and -3 appear to do so less efficiently.

The difference with previous studies (1, 4), in which no M₉, 66,000 species could be detected when total extracts of cells co-expressing HA-CRLR and RAMP2 was examined, is most likely due to the longer transfection time used in the present study (72 versus 48 h) allowing a longer period for the accumulation of the fully processed receptor. The reason for the lower maturation efficiency of the HA-tagged receptor and the appearance, at the cell surface, of an important quantity of core-glycosylated (M₉, 58,000) HA-CRLR is not clear. One could hypothesize that the presence of the HA tag (and not the Myc tag) precludes the efficient trimming of the receptor carbohydrate in the Golgi, resulting in the transit of the RAMP-CRLR complex through the Golgi without the concurrent processing of the sugar. Alternatively, the presence of the HA tag may favor the escape of incompletely folded receptor from the endoplasm-
The presence of endogenous RAMPs may be involved. Consistent with the latter hypothesis, endogenous RAMP1 and RAMP2 mRNAs have previously been detected in HEK293 cells (1). Furthermore, AM binding and signaling was detected in HEK293 cells transfected with CRLR-GFP in the absence of overexpressed RAMP (10), supporting the notion that endogenous RAMP2 is expressed in this cell type. The presence of endogenous RAMP2 is also supported in the present study by the similar pattern of AM binding and signaling was detected in HEK293 cells co-expressing Myc-tagged (A and B) or HA-tagged (C and D) CRLR in the presence of empty vector (pcDNA3) or vectors encoding RAMP1 (R1), RAMP2/1 (R1/2), RAMP2 (R2), RAMP2/1 (R2/1), or RAMP3 (R3). The cross-linking was carried out in the presence (+) and absence (−) of an excess of nonlabeled peptide to define specific labeling. Immunoprecipitation were performed on total lysates using mouse anti-Myc antibodies (A and B) or mouse anti-HA antibodies (C and D), and immunocomplexes were analyzed by SDS-PAGE (10%). The autoradiograms shown are representative of three independent experiments. Open arrows, CRLR; stars, CRLR-RAMP complexes; IB, immunoprecipitation; IB, immunoblotting.

Only the Fully Processed Form of CRLR Generates AM and CGRP Receptors—Experiments were undertaken to determine which CRLR species could bind AM and CGRP. Cross-linking experiments using $^{125}$I-rAM and $^{125}$I-hoCGRP and the cell-impermeable cross-linker BS3 were carried out in cells expressing either HA- or Myc-tagged CRLR in the presence or absence of the three RAMPs. In addition, the role of the amino-terminal domain of RAMPs on the binding properties of CRLR were assessed using the chimeric constructs, RAMP2/1 and RAMP2/1 (see “Experimental Procedures”).

As shown in Fig. 4A, $^{125}$I-rAM labeling was predominantly observed when Myc-CRLR was co-expressed with RAMP2 or the RAMP2/1 chimera. In each case, two AM-labeled bands of $M_r$ ~70,000 and $M_r$ ~95,000 were observed. The strong signal at $M_r$ ~70,000 is consistent with the binding of $^{125}$I-rAM ($M_r$ 6000) to the fully processed CRLR ($M_r$ 66,000), while the band at $M_r$ ~95,000 most likely represents the AM-bound CRLR-RAMP2 complex. Albeit to a lesser extent, labeling was also observed for the RAMP3/Myc-CRLR combination. The slightly faster electrophoretic mobility of the labeled bands is consistent with the observation that the terminally glycosylated species of CRLR promoted by RAMP3 is smaller ($M_r$ 64,000 versus 66,000; see Fig. 1). This most likely results from a differential glycosylation, since PNGase F treatment leads to the expected $M_r$ 48,000 form (Fig. 2, lane 6 of the myc-CRLR + RAMP3 panel). This biochemical difference, however, does not seem to lead to any detectable pharmacological differences, since both RAMP2 and RAMP3 generate AM receptors. The weaker labeling observed with RAMP3 does not indicate a lower affinity for AM but rather a reduced expression level of Myc-CRLR upon co-expression of RAMP3 as assessed by Western blot analysis (data not shown). When Myc-CRLR was expressed with
RAMPs Are Integral Parts of CGRP and Adrenomedullin Receptors

Fig. 5. Glycosylation states of \(^{125}I\)-rAM- and \(^{125}I\)-hCGRP-labeled CRLR and CRLR/RAMP heterodimers. Cross-linking of \(^{125}I\)-rAM (A) or \(^{125}I\)-hCGRP (B) was performed in the presence of 1 mM BS3 on HEK293T cells expressing Myc-tagged CRLR in the presence of empty vector (pcDNA3; lanes 1–3) or vector encoding RAMP1 (R1; lanes 4–6), RAMP1/2 (R1/2; lanes 7–9), RAMP2 (R2; lanes 10–12), RAMP2/1 (R2/1; lanes 13–15), or RAMP3 (R3; lanes 16–18). Immunoprecipitations were performed on total lysates using mouse anti-Myc antibodies, and immunocomplexes were treated with endo H (lanes 2, 5, 8, 11, 14, and 17), PNGase F (lanes 3, 6, 9, 12, 15, and 18) or the vehicle (lanes 1, 4, 7, 10, 13, and 16). Immunocomplexes were analyzed by SDS-PAGE (10%). The autoradiograms shown are representative of three independent experiments. Open arrows, fully processed CRLR; diamonds, deglycosylated CRLR; stars, CRLR-RAMP complexes; IP, immunoprecipitation; IB, immunoblotting.

RAMP1 and RAMP1/2, only a weak labeling was observed (despite equivalent expression of CRLR; data not shown), in agreement with the previously reported (4) low affinity of the Myc-CRLR/RAMP1 combination for AM. The labeling obtained when CRLR is expressed alone most likely reflects the expression of endogenous RAMP2 as discussed above.

When cross-linking experiments were carried out with \(^{125}I\)-haCGRP (Fig. 4B), two major species of \(M_r \sim 70,000\) and \(\sim 85,000\) were labeled only upon co-expression of Myc-CRLR with RAMP1 or RAMP1/2. The \(M_r\) values of these labeled forms are consistent with the size of CGRP (\(M_r 4000\)) incorporated into the terminally glycosylated CRLR (\(M_r 66,000\)) and CRLR-RAMP1 complex (\(M_r 80,000\)). The lack of CGRP binding when RAMP2, RAMP2/1, and RAMP3 were expressed is consistent with the lack of binding affinity of the AM receptor for CRLR (4). The difference in electrophoretic mobility between the AM-labeled CRLR-RAMP2 complex (\(M_r \sim 95,000\); see Fig. 4A) and the CGBP-labeled CRLR-RAMP1 complex (\(M_r \sim 85,000\); see Fig. 4D) results from the difference in theoretical size between the two RAMPs (\(M_r 16,000\) versus \(14,000\)) and the fact that RAMP2 and not RAMP1 is glycosylated (2) (see also Fig. 6). RAMP2/1 mimicked the effect of RAMP2 in promoting intense AM labeling, whereas RAMP1/2 favored CGBP labeling similarly to RAMP1. This suggests, as previously reported in radioligand binding and functional assays (4), that the extracellular domain of the RAMPs determines the binding selectivity of the CRLR species.

Fig. 4, C and D, illustrate the labeling obtained for both \(^{125}I\)-rAM and \(^{125}I\)-haCGRP when the HA-tagged CRLR was used. Although looking somewhat different from the one generated with Myc-tagged CRLR, the pattern of radiolabel incorporation obtained for HA-CRLR shows that the same species (\(M_r \sim 70,000\) and \(\sim 95,000\) for \(^{125}I\)-rAM and \(M_r \sim 70,000\) and \(\sim 85,000\) for \(^{125}I\)-haCGRP) are involved in ligand recognition. The weaker labeling observed with the HA-tagged receptor is consistent with the low amounts of terminally glycosylated receptor at the cell surface (see Fig. 3).

The glycosylation status of the AM- and CGBP-bound receptor was assessed to determine whether or not the immature form of CRLR could bind ligand. As seen in Fig. 5A, the \(M_r \sim 70,000\) bands labeled with \(^{125}I\)-rAM were found to be resistant to the action of endo H (lanes 2, 5, 8, 11, 14, and 17), PNGase F (lanes 3, 6, 9, 12, 15, and 18) or the vehicle (lanes 1, 4, 7, 10, 13, and 16). Such a resistance has also been reported for the labeled CRLR-RAMP complexes as assessed by Western blot analysis (data not shown).

Surprisingly, and in contrast with the \(M_r \sim 70,000\) bands, the \(M_r \sim 95,000\) complex was unaffected by the PNGaseF treatment (Fig. 5A, lanes 12−15, and 18). Such a resistance has also been reported for the AM-bound CRLR-RAMP2 complex expressed in insect cells (2). This indicates that the cross-linking of CRLR and RAMP2 (or RAMP2/1 or RAMP3) with AM leads to a conformation of the receptor in which the glycosylation sites are no longer accessible to the deglycosylation enzyme.

Weaker AM labeling was observed upon RAMP1 (lanes 4−6)
and RAMP1/2 (lanes 7–9) co-expression, as would be predicted by the poor affinity of AM for CGRP receptors. However, as was the case for RAMP2, RAMP2/1, and RAMP3, the labeled Mₘ 70,000 form was found to be endo H-resistant and thus terminally glycosylated. Interestingly, unlike the Mₘ 95,000 CRLR-RAMP2 complex, the Mₘ 85,000 CRLR-RAMP1 or CRLR-RAMP1/2 complexes were sensitive to PNGase F (lanes 6 and 9, respectively), indicating that distinct conformations are promoted by the different RAMPs. Some AM (Fig. 5A, lanes 1–3) but not CGRP (Fig. 5B, lanes 1–3) cross-linking was observed when Myc-CRLR was expressed alone, again consistent with the notion that endogenous RAMP2 is present. Also in agreement with this notion is the relative labeling of the Mₘ 85,000–95,000 complexes versus the Mₘ 70,000 CRLR bands. Indeed, as observed for RAMP2, RAMP2/1, and RAMP3, the labeling of the Mₘ 70,000 bands is favored in the absence of overexpressed RAMP (Fig. 5A, lanes 10, 13, 16, and 1, respectively). This contrasts with the equivalent labeling of the two species occurring in the case of RAMP1 and RAMP1/2 (Fig. 5A, lanes 4 and 7).

As for AM, [125I]-hoCGRP binding was only observed for mature forms of the receptor. Indeed, both the Mₘ 70,000 CRLR and 85,000 CRLR-RAMP complexes observed upon co-expression of Myc-CRLR and RAMP1 (Fig. 5B, lanes 4–6) or RAMP1/2 (lanes 7–9) were resistant to endoH (lanes 5 and 8) but sensitive to PNGase F treatment (lanes 6 and 9). The sensitivity of the complex to PNGase F, which contrasts with the resistance of CRLR-RAMP2 to this treatment, suggests that the distinct conformation (PNGase F-resistant versus -sensitive) imposed by the different RAMPs is independent of the agonist bound.

[125I]-rAM and [125I]-hoCGRP Are Cross-linked to RAMP1, RAMP2, or RAMP3 within the Receptor Complex—In order to determine if the RAMPs are close to the ligand binding site within the CRLR-RAMP heterodimer, the same samples, previously used to show ligand-CRLR binding, were loaded on higher percentage polyacrylamide gels, enabling the co-visualization of CRLR and RAMP proteins. As shown in Fig. 6A, AM was cross-linked to a species of Mₘ 30,000 in cells co-expressing Myc-CRLR and RAMP2 (lanes 10–12) or RAMP2/1 (lanes 13–15). This band, which was not observed with other combinations (pcDNA3, RAMP1, -1/2, or -3), was found to be resistant to endo H treatment (lanes 11 and 14) but reduced to a Mₘ 24,000 form after PNGase F treatment (lanes 12 and 15). This is consistent with the fact that RAMP2 is glycosylated (one potential N-linked glycosylation site) and that it is targeted to the cell surface as a fully processed protein (2). The extent of labeling observed for RAMP is significantly less than that observed for CRLR. For RAMP3 (lanes 16–18), virtually no labeling could be observed in the untreated and endo H-treated sample, but a weak labeling of a Mₘ 19,000 band could be seen following PNGase F treatment (lane 18), probably as a result of the band sharpening after deglycosylation (RAMP3 harbors four potential N-linked glycosylation sites (14)).

This first demonstration that RAMP2 and RAMP3 can be labeled with [125I]-rAM suggests that they lie close to the binding pocket within the CRLR-RAMP2 heterodimer and that they could play a role in determining binding specificity. A weak labeling of a Mₘ 17,000 band was also observed when Myc-CRLR was co-expressed with RAMP1 (lanes 4–6) or RAMP1/2 (lanes 7–9). These bands, corresponding to RAMP1 and RAMP1/2, were found to be unaffected by either endo H (lanes 5 and 8) or PNGase F (lanes 6–9) treatment, consistent with the lack of N-glycosylation site in this protein. Such Mₘ 17,000 bands were strongly labeled by [125I]-hoCGRP when CRLR was co-expressed with RAMP1 or RAMP1/2 (Fig. 6B, lanes 4–6 and 7–9, respectively). As for RAMP2 and RAMP3, these results suggest that RAMP1 is close to the ligand binding site.

Interestingly, [125I]-rAM labeling observed for RAMP2, RAMP2/1, or RAMP3 is very weak in comparison with CRLR, whereas CRLR, RAMP1, and RAMP1/2 were similarly labeled by [125I]-hoCGRP. These differences in labeling intensity cannot be explained by the number of lysines available for the BS3 cross-linking, since CGRP contains two lysines compared with four for AM, and the NH₂ terminus of RAMP1 contains only one lysine versus three for RAMP2 or RAMP3. Thus, the difference in the relative labeling intensity between CRLR and RAMP most likely reflects different conformations for CRLR-RAMP1 versus CRLR-RAMP2 and CRLR-RAMP3 complexes.

Taken together, our results directly demonstrate that the distinct pharmacology conferred by each RAMP is independent of the glycosylation state of the CRLR. Indeed, both AM and CGRP were found to bind only to the endo H-resistant terminally glycosylated form of the receptor. This is consistent with previous results obtained in insect cells in which the distinct pharmacology of the CRLR-RAMP complexes was observed despite similar glycosylation (2). RAMP1 and -3 were also found to confer amylin binding to the calcitonin receptor gene product without affecting its maturation/glycosylation state (15–17).

Since the difference in glycosylation cannot be invoked, the distinct pharmacology acquired by CRLR reflects either a direct participation of the RAMPs to the selective binding pockets of the peptides or an indirect conformational effect on CRLR (5). The observation that the three RAMPs could be cross-linked to either AM or CGRP indicates that the accessory proteins lie close to the binding site. Although not proving it, this would be consistent with a direct contribution of RAMPs to ligand binding. An indirect conformational effect, however, cannot be excluded. In fact, several lines of evidence indicate that the different CRLR-RAMP complexes adopt distinct conformation. For instance, whereas the CRLR-RAMP1 complex is sensitive to the action of PNGase F, both RAMP2- and RAMP3-CRLR heterodimers were resistant to the enzyme. Also, only the RAMP2-CRLR complex could be detected without cross-linking agent (Fig. 1), indicating a relative resistance of this complex to SDS-denaturation. Interestingly, RAMP2 and -3 but not RAMP1 were found to be glycosylated, and thus the presence of these carbohydrate may play a role in imposing different conformation to the complexes. Determining the precise nature of the interactions between CRLR and RAMPs that lead to distinct ligand binding selectivity will require additional structural studies aimed at describing the three-dimensional structure of the heterodimers.

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