Respective roles of calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) in cell surface expression of CRLR/RAMP heterodimeric receptors

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running title: cell surface expression of CRLR/RAMP heterodimers
Receptor activity modifying proteins RAMP1, RAMP2 and RAMP3 are responsible for defining affinity to ligands of the calcitonin receptor-like receptor (CRLR). It has also been proposed that RAMPs are molecular chaperones required for CRLR transport to the cell surface. Here, we have studied the respective roles of CRLR and RAMPs in transporting CRLR/RAMPs heterodimers to the plasma membrane by using a highly specific binding assay that allows quantitative detection of cell surface expressed CRLR or RAMPs in the Xenopus oocytes expression system. We show that: (i) heterodimer assembly is not a prerequisite for efficient cell surface expression of CRLR, (ii) N-glycosylated RAMP2 and RAMP3 are expressed at the cell surface and their transport to the plasma membrane requires N-glycans, (iii) RAMP1 is not N-glycosylated and is transported to the plasma membrane only upon formation of heterodimers with CRLR, (vi) introduction of N-glycosylation sites in the RAMP1 sequence (D58N/G60S, Y71N and K103N/P105S) allows cell surface expression of these mutants at levels similar to that of wild-type RAMP1 co-expressed with CRLR. Our data argue against a chaperone function for RAMPs and identify the role of N-glycosylation in targeting these molecules to the cell surface.
Functional properties of G protein-coupled receptors (GPCR) can be altered upon formation of receptor homo- or hetero-dimers (1). In 1998, McLatchie et al. (2) have demonstrated that ligand binding of a GPCR, namely calcitonin receptor-like receptor (CRLR), depends on association with receptor activity modifying proteins (RAMPs). CRLR, originally identified as an orphan GPCR, was shown to form a high-affinity receptor to calcitonin-gene related peptide (CGRP), when associated with RAMP1, or, to specifically bind adrenomedullin (AM), when associated with RAMP2 or RAMP3. RAMPs are type I transmembrane proteins that share ~ 30% of amino acids identity and a common predicted topology with the short cytoplasmic C-termini, one transmembrane domain, and the large extracellular N-termini that are responsible for the acquisition of the RAMP-specific receptor phenotypes (3-5). The discovery of RAMPs has demonstrated that ligand-GPCR interaction may require accessory proteins that are either directly involved in the formation of receptor binding pockets or, participate in the acquisition by their partner receptor of specific conformations required for ligand binding. More recently, a second GPCR, namely calcitonin receptor (CTR), was demonstrated to form heterodimeric complexes with RAMPs. CTR/RAMP1 and CTR/RAMP3 heterodimers revealed the pharmacological profiles of specific receptors to amylin (6,7). This finding, as well as the ubiquitous and abundant RAMPs expression across tissues, suggested that RAMPs might be involved in defining functional properties of different GPCRs. However, the direct evidence for RAMPs interaction with GPCRs other than CRLR and CTR is still lacking.

The molecular mechanisms that guide the processes of assembly, intracellular trafficking and cell surface expression of RAMPs with their partner receptors remain poorly understood. Several studies performed in HEK293T cells suggested that CRLR-RAMP heterodimerization is required for CRLR plasma membrane targeting (2,3). Therefore, it was proposed that RAMPs play a role of molecular chaperones for CRLR cell surface expression.
However, Kuwasako et al. (8) and Hilairet et al. (9) have recently reported that, when expressed alone, a significant number of RAMP3 and CRLR reach the cell surface of HEK293T cells. Buhlmann et al. have demonstrated that in the embryonic kidney TSA cells CRLR is efficiently expressed at the cell surface with or without RAMPs (10). This apparent discrepancy was attributed to endogenous CRLR and RAMPs expression detected in several of the commonly used mammalian expression systems, including HEK293T cells (2,8,9). However, the fact that cell surface expression of CTR, a second established RAMPs partner, does not require RAMPs co-transfection, suggested that the proposed hypothesis requires further confirmation.

In the present study, we assessed the respective roles of CRLR and RAMPs in cell surface expression of CRLR/RAMP receptors by using a binding assay allowing quantitative detection of CRLR or RAMPs at the cell surface. This assay is based on binding of an iodinated antibody of known specific activity to an epitope placed in the extracellular parts of CRLR and RAMPs. For these experiments, we took advantage of the *Xenopus* oocyte expression system in which CRLR/RAMPs heterodimers are expressed as fully functional receptors for CGRP or AM (2). Importantly, in this expression system, a significant interaction with endogenously expressed CRLR and/or RAMPs can be excluded (see below). Using this assay, we show that assembly in heterodimers is not a prerequisite for cell surface expression of CRLR, whereas RAMPs are efficiently transported to the plasma membrane only when N-glycosylated. Transport of non-glycosylated RAMPs can, however, be restored by co-expression with CRLR. This was also true for co-transport to the cell surface of RAMPs with CTR but not with several other GPCR tested, thus providing the evidence: (i) for the primary role of GPCRs in trafficking of GPCR/RAMP heterodimers to the cell surface; ii) for the critical role of N-glycosylation for trafficking RAMPs to the cell surface and, (iii) for a selective association of RAMPs with CRLR and CTR.
EXPERIMENTAL PROCEDURES

cDNA constructs and FLAG epitope insertion. Mouse cDNAs for RAMP1 (GenBank #NP_005846), RAMP2 (GenBank #NP_005845), RAMP3 (GenBank #NP_005847), CRLR (GenBank #NP_061252), PTH/PTHrP-R (receptor to parathyroid hormone and parathyroid hormone-related peptide, IMAGE clone #4238969) and GluR (receptor to glucagon, IMAGE clone #4241407) and rat cDNAs for CTR, V2R (V2 type receptor to vasopressin) and V1aR (V1a type receptor to vasopressin) were used in this study. The corresponding proteins were tagged with the FLAG reporter octapeptide DYKDDDDK that is recognized by the anti-FLAG M2 (M2Ab) mouse monoclonal antibody (Sigma). The sites for FLAG insertion were chosen in the extracellular parts of the proteins, as determined, according to the predicted membrane topology of these proteins (Proscan software, www.expasy.org/tools). For CRLR, RAMP1, RAMP2 and RAMP3, the FLAG epitope was inserted between the residues flanking the predicted signal peptide cleavage sites, so that in the processed proteins the FLAG epitope undertakes the most N-terminal positions of the proteins (SignalIP software: www.cbs.dtu.dk/services/SignalIP). The predicted positions for signal peptide cleavage are flanked by amino acids 26A and 27C for RAMP1, 44A and 45S for RAMP2, 26G and 27C for RAMP3, 24Q and 25A for CTR and 22A and 23E for CRLR. The cleavage of the signal peptides in FLAG-tagged RAMP1 and RAMP3 proteins was tested in in vitro protein translation experiments which were performed in the absence or presence of canine pancreatic microsomal membranes (CPMM, Promega) with the following treatment of the RAMP3 protein with the endoglycosydase F. After separation on SDS-PAGE (13 %), a decrease of 1-2 kDa in molecular weights of RAMP1 and RAMP3 synthesized in the presence of CPMM was
observed, thus indicating that these proteins are correctly processed in the presence of FLAG (data not shown).

*Site-directed mutagenesis*  
FLAG insertion and site-directed mutagenesis were performed by PCR-based approach and all tagged constructions and mutants were confirmed by cDNA sequencing. Four consensus N-glycosylation sites in RAMP2 and RAMP3 were eliminated, using site-directed mutagenesis by mutation of consensus asparagines to serines. The corresponding mutants are: RAMP2-Δ4N (N50S, N58S, N99S, N144S) and RAMP3-Δ4N (N28S, N57S, N70S and N102S), respectively. Three N-glycosylation consensus sites were introduced into RAMP1. The mutants are: RAMP1-D58N/G60S, RAMP1-Y71S and RAMP1-K103N/P105S.

**Binding analysis of CRLR and RAMPs cell surface expression**  
Complementary RNAs for RAMPs and GPCRs were synthesized *in vitro* using SP6 polymerase. Equal amounts of RAMPs and GPCR cRNAs were injected into *Xenopus* oocytes (10 ng of total cRNA/oocyte). Injected oocytes were kept into modified Barth solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.8 mM MgSO₄, 0.3 mM Ca(NO₃)₂, 0.4 mM CaCl₂, 10 mM Hepes-NaOH (pH 7.2). The cell surface expression of CRLR, as well as wild-type and mutant FLAG-tagged RAMPs, was quantitatively determined by specific binding of [¹²⁵I]M₂IgG₁ iodinated anti-FLAG M₂ antibody (¹²⁵I-M₂Ab) (11). M₂Ab was iodinated, using the Iodo-Beads iodination reagent (Pierce) and carrier-free Na¹²⁵I (Hartmann), according to the Pierce protocol. Iodinated antibody had a specific activity of 2-10 x 10¹⁷ cpm/mol. Binding of the iodinated antibody to oocytes expressing the FLAG-tagged CRLR or RAMPs was determined 24-48 hours after the cRNA injection, as described (11). Specific binding was calculated as the difference of the binding between the oocytes injected with FLAG-tagged cRNAs and the noninjected oocytes.
Determination of EC$_{50}$ values of CRLR/RAMPs heterodimers in the Xenopus oocytes

Combinations of wild-type and/or FLAG-tagged RNAs were injected into Xenopus oocytes (10 ng of total cRNA/oocyte). Injected oocytes were kept into MBS for 20 hours. The EC$_{50}$ values were determined by measurements of CGRP- and AM-generated Cl$^-$ currents generated by cystic fibrosis transmembrane regulator (CFTR), a cAMP-activated chloride channel, according to Chraibi et al. (12). Current was measured under two-electrode voltage clamp at a potential oscillating between –40 mV and –80 mV. Chloride conductance was then calculated.

**Immunoprecipitation** Injected with wild-type or mutant FLAG-tagged GPCRs and/or RAMPs, the Xenopus oocytes were incubated overnight in MBS containing 1.0 mCi/ml $[^{35}S]$methionine (NEN). Microsomes were prepared as described by Geering et al. (13) and the immunoprecipitations were performed with anti-FLAG M2 antibody under nondenaturing conditions, resolved by 8-13% SDS-PAGE and revealed by fluography.
RESULTS

To assess the respective roles of CRLR and RAMPs in cell-surface expression of CRLR/RAMPs heterodimers, we have developed a quantitative assay based on the binding of $^{125}$I-labeled M$_2$ anti-FLAG monoclonal antibody (M$_2$Ab) directed against a FLAG reporter epitope introduced into the extracellular N-termini of the mouse CRLR, RAMP1, RAMP2 and RAMP3 proteins (see Experimental Procedures). To ensure that the insertion of a FLAG epitope did not change significantly the function of CRLR/RAMPs heterodimers, we measured the CGRP- and AM-stimulated currents generated by the cystic fibrosis transmembrane regulator (CFTR), a cAMP-activated chloride channel. CFTR was co-injected in the *Xenopus* oocytes with either wild-type or FLAG-tagged CRLR and/or RAMPs and the effector concentrations for half-maximal response (EC$_{50}$) for CGRP and AM were determined. As shown in Fig. 1A, the CRLR-FLAG/RAMP1wt and CRLRwt/RAMP1-FLAG heterodimers have the EC$_{50}$ values of 28±9 pM (n=4) and 56±10 pM (n=5), respectively, not different from that observed for the CRLRwt/RAMP1wt heterodimers (67±17 pM, n=5). Similarly, the presence of FLAG, did not change the EC$_{50}$ values for AM (Fig. 1B) of the CRLRwt/RAMP2-FLAG (47±61 pM, n=4) and CRLRwt/RAMP3-FLAG (65±419 pM, n=3) heterodimers, as compared to the CRLRwt/RAMP2wt (33±47 pM, n=3) and CRLRwt/RAMP3wt (24±151 pM, n=4) heterodimers, respectively. Importantly, the oocytes injected with CRLR alone have no detectable CGRP- or AM-induced Cl$^-$ currents (data not shown), thus ruling out a significant interaction with endogenously expressed RAMPs. Also, the absolute CFTR current values were not significantly different between the combinations of either wild-type or FLAG-tagged CRLR and RAMPs (data not shown), indicating that neither ligand affinities nor efficiency of membrane targeting are modified by FLAG insertion.
The respective roles of CRLR and RAMPs in transport to the cell surface of CRLR/RAMP heterodimers were assessed in two sets of experiments in which we compared the cell surface expression of CRLR, RAMP1, RAMP2 and RAMP3 expressed either individually or in combinations. In the first group, the cell surface expression of FLAG-tagged CRLR expressed with or without wild-type RAMPs was compared. As shown in Fig. 2A, CRLR is efficiently expressed at the cell surface without RAMPs (1.7±0.23 fmol/oocyte, n=85), and co-expression with either RAMP1, RAMP2 or RAMP3 did not change the cell surface expression level of CRLR (1.5±0.22 fmol/oocyte (n=54) for CRLR/RAMP1; 1.9±0.34 fmol/oocyte (n=56) for CRLR/RAMP2 and 1.6±0.22 fmol/oocyte (n=89) for CRLR/RAMP3, not significantly different from CRLR expressed alone). In the second group, the cell surface expression of FLAG-tagged RAMP1, RAMP2 and RAMP3 expressed with or without wild-type CRLR was compared. As shown in Fig. 2B, RAMP2 and RAMP3 are transported to the cell surface without CRLR (0.6±0.16 fmol/oocyte (n=55), and 0.5±0.10 fmol/oocyte (n=86), respectively), whereas RAMP1 cell surface expression was at near undetectable level (0.1±0.19 fmol/oocyte (n=46)). Co-expression of RAMPs with CRLR leads to efficient transport to the plasma membrane of RAMP1 (2.1±0.39 fmol/oocyte (n=37), p<0.001 as compared to RAMP1 expressed alone) and increases to some extent but significantly RAMP2 and RAMP3 cell surface expression (1.3±0.40 fmol/oocyte (n=57), p<0.05 and 0.75±0.07 fmol/oocyte (n=84), p<0.05 as compared to RAMP2 and RAMP3 expressed alone, respectively). These experiments show that (i) assembly in heterodimers is not a prerequisite for efficient cell surface expression of CRLR, (ii) RAMP1 is transported to the plasma membrane only upon formation of heterodimers with CRLR and, (iii) individually expressed RAMP2 and RAMP3 are transported to the plasma membrane, but their maximal cell surface expression requires association with CRLR.
Interestingly, the cell surface expression of individually expressed RAMP2 and RAMP3 correlated with the presence of N-glycosylation consensus sites in known RAMP2 and RAMP3 amino acid sequences from different species (mouse, human, and rat), whereas, for RAMP1, the N-glycosylation consensus sites are not present and the RAMP1 protein is intracellularly retained. Since, the N-glycosylation has been shown to play a variety of roles, including facilitating protein trafficking to the plasma membrane, we hypothesized that N-glycosylation of RAMP2 or RAMP3 was the basis for the observed difference between RAMP1 and RAMP2 or RAMP3. To assess the role of N-glycosylation in RAMP2 and RAMP3 cell surface expression, we eliminated the four N-glycosylation consensus sites present in both RAMP2 and RAMP3 mouse proteins by site-directed mutagenesis (see Experimental Procedures) and compared the cell surface expression of wild-type and mutant RAMP2 and RAMP3. As shown on Fig. 3A, RAMP2-Δ4N N-glycosylation mutant is expressed at the cell surface at near undetectable level (0.1±0.02 fmol/oocyte (n=95) for RAMP2-Δ4N as compared to 1.7±0.14 fmol/oocyte (n=91) for wt RAMP2, p<0.001). Importantly, co-expression of RAMP2-Δ4N with CRLR completely restored the cell surface expression of this mutant (Fig. 3A). Similarly, RAMP3-Δ4N N-glycosylation mutant is not transported to the cell surface (0.09±0.02 fmol/oocyte (n=85) for RAMP3-Δ4N as compared to 1.9±0.16 fmol/oocyte (n=85) for wt RAMP3, p<0.001) and co-expression of this mutant with CRLR partially restored its cell surface expression (Fig. 3B). The cell surface expression of single N-glycosylation mutants of RAMP2 or RAMP3 was significantly decreased by more than 50% (data not shown). Immunoprecipitation of [35S]methionine labeled proteins (Fig. 3C) performed on oocytes injected with either wild-type or mutant RAMPs confirmed that RAMP2 and RAMP3 are N-glycosylated. Importantly, wild-type and mutant RAMPs are expressed at a similar level, thus indicating that the loss of cell surface expression of RAMP2-Δ4N and RAMP3-Δ4N was not due to decreased protein stability or degradation of the
mutants. Thus, these experiments show that: (i) cell surface expression of both wild-type RAMP2 and RAMP3 requires the N-glycosylation, (ii) similarly to RAMP1, RAMP2-Δ4N and RAMP3-Δ4N mutants require CRLR for their cell surface expression, (iii) co-transport to the cell surface expression of RAMP1, RAMP2-Δ4N and RAMP3-Δ4N with their partner receptors can be used as a stringent test for GPCR-RAMPs heterodimerization.

To further assess the possibility that N-glycosylation of RAMP2 and RAMP3 may be the basis of the observed difference between RAMP1 and RAMP2/RAMP3 cell surface expression, we took advantage that 3 out 4 of the N-glycosylation sites of RAMP2 and RAMP3 are within the partially conserved stretches of amino acid that are also present in RAMP1 (Fig. 4A). We therefore introduced N-glycosylation sites in the sequence of RAMP1 (D58N/G60S or Y71N or K103N/P105S) and examined their cell surface expression. As shown in Figure 4B, the three mutants were able to traffic to the cell surface unlike wt RAMP1. For two mutants (RAMP1-Y71N and RAMP1-K103N/P105S), the level of cell surface expression of RAMP was equal or superior to that of the RAMP1/CRLR heterocomplexes. We next verified that the mutated RAMP1s were indeed N-glycosylated. As shown in Figure 4C, wt RAMP1 was, as expected, not glycosylated whereas RAMP1(Y71N) and RAMP1(K103N/P105S) were. Interestingly, the RAMP1-D58N/G60S mutant was only partially N-glycosylated and its cell surface expression level reached only ~40% of that of Y71N and K103N/P105S mutants (Fig. 4B). This experiment confirms the critical importance of N-glycosylation for cell surface expression of the RAMP proteins.

Since the existence of other RAMPs partners has been demonstrated (CTR) or proposed (5,15-17), we were interested to compare the effects of different GPCRs on RAMPs expression at the cell surface. The aim of these experiments was twofold: (i) to check the selectivity of RAMPs association to different GPCRs and, (ii) to compare the role of other RAMPs partners in trafficking to the cell surface of GPCR/RAMP heterodimers. We have
chosen three receptors that share a high degree of amino acids homology with CRLR, namely CTR (~65% homologous), parathyroid hormone and parathyroid hormone-related peptide receptor (PTH/PTHrP-R, ~45% homologous) and glucagon receptor (GluR, ~45% homologous) and two GPCRs that do not exhibit the significant degree of homology with CRLR, namely, vasopressin V1aR and V2R receptors. As shown in Fig. 5A, 5B and 5C, in addition to CRLR, only CTR was able to co-transport to the cell surface the RAMP1, RAMP2-Δ4N and RAMP3-Δ4N, despite the similar levels of protein expression observed for all receptors (data not shown). This data, as well as previous observations that individually expressed PTH/PTHrP-R, GluR, V1aR and V2R are efficiently targeted to the cell surface of the *Xenopus* oocytes (18-21), suggested that RAMPs are not associated with these receptors. As an additional test, we co-immunoprecipitated FLAG-tagged RAMP1 with co-expressed wild-type receptors, using the anti-FLAG M2 antibody. As shown in Fig. 5D, RAMP1 is physically associated only with CRLR and CTR but not with other receptors, thus confirming the data of cell surface binding analysis. These experiments show that among tested receptors RAMPs selectively associates with CRLR and CTR and that, similarly to CRLR, CTR is responsible for the efficient RAMPs trafficking to the cell surface.
DISCUSSION

In 1998, McLatchie et al. have proposed that RAMPs play a role of molecular chaperones required for CRLR cell surface expression and that association with RAMPs results in differential N-glycosylation of CRLR (2). The fully-glycosylated mature form of CRLR in CRLR/RAMP1 heterodimer and the core-glycosylated immature forms of CRLR in CRLR/RAMP2 and CRLR/RAMP3 heterodimers have been proposed as specific CGRP or AM receptors, respectively. However, further studies performed by Aldecoa et al. (22) and by Hilairet et al. (9) have demonstrated that differential N-glycosylation is not a prerequisite for ligand selectivity of CRLR/RAMP heterodimers (9,22) and Hilairet et al. (9) have proposed that rather CRLR/RAMPs protein-protein interactions are responsible for expression of CGRP and AM receptor phenotypes (9). Similarly, several studies in which a significant amount of individually expressed CRLR or RAMPs was detected at the cell surface have indicated that the chaperone function of RAMPs should be reevaluated (8-10).

A new assay to assess cell surface expression of CRLR and RAMPs. In this study, to assess the respective roles of CRLR and RAMPs in cell surface expression of CRLR/RAMP heterodimers, we used a specific binding assay allowing quantitative detection of CRLR and RAMPs at the cell surface. We and others have previously used a similar approach to study the process of assembly and trafficking to the plasma membrane of the α-β-γ subunits of the epithelial sodium channel (ENaC) (11,23) and of the α-β subunits of Na,K-ATPase (24). This binding assay, when performed in the Xenopus oocytes, has the following advantages: First, CRLR/RAMP1 and CRLR/RAMP2 and CRLR/RAMP3 heterodimers are expressed in the Xenopus oocytes as the fully functional CGRP and AM receptors, respectively (2); second,
the number of exogenously expressed plasma membrane proteins reaches as much as ~50 % of the total number of proteins expressed at the oocyte surface, indicating that a significant interaction with endogenously expressed proteins can be excluded (25). For example, in our experiments, CRLR expression level was ~2 fmol/oocyte, whereas Zampighi et al. have estimated the total number of protein particles in the oocyte membrane as ~10 fmol/oocyte (25). This fact, as well as the absence of CGRP- or AM-induced effects in CRLR-injected oocytes, clearly demonstrate that endogenous RAMPs, if present, were not able to significantly influence cell surface expression of exogenously expressed proteins. This favorably compares the *Xenopus* oocytes with most of the mammalian expression systems in which endogenous RAMPs and/or CRLR are expressed at detectable levels (2). Third, the *Xenopus* oocytes are characterized by a stringent cellular quality control system that is responsible for the correct assembly, folding, intracellular trafficking and cell surface expression of oligomeric membrane proteins (for a review, see (26)). In our experiments, the stringency of oocytes quality control was demonstrated by the fact that RAMP1 wt, RAMP2-Δ4N and RAMP3-Δ4N N-glycosylation mutants were not able to reach the cell surface (Fig. 3). It is important to note that Δ4N mutants were not degraded to any extent, suggesting that they folded properly. Our data rather suggest that the non glycosylated mutants were still able to associate properly with their cognate partner i.e. CRLR (presumably in the ER compartment) and that they are able to traffic normally to the membrane.

**Critical importance of RAMPs glycosylation for cell surface expression.** Our data suggest that (i) assembly in heterodimers is not a prerequisite for CRLR, RAMP2 and RAMP3 cell surface expression, whereas RAMP1 trafficking to the plasma membrane requires association with CRLR and (ii) that CRLR is responsible for the maximal expression of CRLR/RAMP heterodimers at the cell surface. Although statistically significant, this effect is relatively
small in absolute terms (~110% for RAMP2 and ~40% for RAMP3). Our findings partially differ from previous observations in HEK293T cells, in which RAMPs have been proposed as molecular chaperones required for CRLR cell surface expression. We can offer the following explanations for such a difference. Previous estimations of CRLR cell surface expression in HEK293T cells were done by analysis of intracellular and submembrane distribution of GFP-tagged CRLR (8) or by an estimation of the fully-glycosylated mature form of CRLR, considered as cell surface expressed receptors (9). These two methods do not allow precise cell surface quantification. Another approach was FACS analysis of Myc-tagged CRLR in HEK293T (2), but a recent report has shown that an important number of CRLR alone can reach the cell surface (9). This finding was attributed to the presence of a significant expression of endogenous RAMP in this cell line, a factor which can be excluded in the present study. Finally, Buhlmann et al., using the Myc-tagged CRLR in the embryonic kidney TCA cells, have obtained a similar CRLR cell surface expression whether the receptor was expressed alone or in combination with RAMP1 or RAMP2 (10).

We also demonstrate that cell surface expression of individually expressed RAMP2 and RAMP3 requires N-glycosylation of the proteins. In agreement with recent data by Gujer et al. (27) that efficient CRLR cell surface expression and ligand binding require receptor N-glycosylation, our results indicate that N-glycosylation may play an important role in CRLR/RAMP heterodimer cell surface expression and receptor function. This is strongly supported by our ability to confer an efficient cell membrane trafficking to RAMP1 by just introducing a single N-glycosylation site in the sequence (Fig. 4). Only the RAMP1-(D58N/G60S) mutant was not fully competent to reach the surface in parallel with poor efficiency of that specific glycosylation site. We do not know the mechanism for this phenotype. It is, however, interesting to observe that the mutation is introduced just after a highly conserved cysteine residue. This is consistent with the finding that localized folding
event on the nascent chain, such as disulfide bond formation, which blocks access to the oligosaccharyl transferase, may be a determinant of glycosylation site usage (28). It will be interesting to examine the disulfide bond formation of RAMPs and its consequence on glycosylation (M. Flahaut et al, ms in preparation).

RAMPs heterodimerize with CRLR and RAMPs but have no affinity for several more distantly related GPCRs. We also tested several other GPCRs as potential partners for RAMPs. The interest in these experiments came from the widely discussed possibility that pharmacological profiles of GPCRs, other than CRLR and CTR, may be dependent on RAMPs association (5,15-17). However, in the absence of information about the protein domains required for CRLR- and CTR-RAMPs heterodimerization, the prediction for the possible partners from hundreds of GPCRs identified in the Human Genome Project (29) is a difficult task. CRLR and CTR, belong to the Family B of GPCRs which also includes receptors for secretin, pituitary adenylate cyclase polypeptide type I (PACAP), vasoactive intestinal peptide (VIP), parathyroid hormone and parathyroid hormone related peptide (PTH/PTHrP), glucagon and glucagon-like peptides (GLP), and receptors for other hormones (for GPCR phylogenic tree see: www.gpcr.org). Thus, together with CRLR and CTR, we tested for RAMPs association the closely related PTH/PTHrP-R and GluR and Family B-unrelated V1aR and V2R. These experiments failed to find any significant interaction between RAMPs and receptors other than CRLR and CTR, thus arguing against a general role of RAMPs in the regulation of GPCRs function. Importantly, similarly to CRLR, CTR was able to co-transport to the cell surface expression the RAMP1 and RAMP2-Δ4N and RAMP3-Δ4N mutants, thus indicating that co-transport of a GPCR with RAMPs to the cell surface can be used as a common criterion for the formation of GPCR/RAMP heterodimers.
Figure legends

**Figure 1.** Flag insertion in Ramp1, Ramp2, Ramp3 or CRLR does not change the EC$_{50}$ value for CGRP or AM. The EC$_{50}$ value for CGRP (A) and AM (B) were determined by measurement of CGRP- and AM-generated Cl$^-$ currents in the oocytes co-injected with CFTR and different combinations of wild-type and FLAG-tagged CRLR and RAMPs. Shown are mean $\pm$ S.E. of four to five oocytes. The data from each oocyte are normalized with their maximal response to CGRP or AM. A. CRLR$^\text{wt}$/RAMP1$^\text{wt}$ (■), CRLR$^\text{wt}$/RAMP1-$\text{FLAG}$ (●), CRLR-$\text{Flag}$/RAMP1$^\text{wt}$ (◆). B. CRLR$^\text{wt}$/RAMP2$^\text{wt}$ (○), CRLR$^\text{wt}$/RAMP3$^\text{wt}$ (Δ), CRLR$^\text{wt}$/RAMP2-$\text{FLAG}$ (○), CRLR$^\text{wt}$/RAMP3-$\text{FLAG}$ (□).

**Figure 2.** Respective roles of CRLR and RAMPs in cell surface expression of CRLR/RAMPs heterodimers. A. *Xenopus* oocytes were injected with 5 ng of FLAG-tagged CRLR with or without wild-type RAMPs (5 ng). The oocytes were then incubated 36 h at 19°C and CRLR cell surface expression was determined by the binding of $^{125}$I-labeled anti-FLAG antibody. Shown are mean $\pm$ S.E. of four to eight experiments performed with 12 oocytes per experimental condition. B. *Xenopus* oocytes were injected with 5 ng of FLAG-tagged RAMPs with or without wild-type CRLR (5 ng). The oocytes were then incubated 36 h at 19°C and CRLR cell surface expression was determined by the binding of $^{125}$I-labeled anti-FLAG antibody. Shown are mean $\pm$ S.E. of four to eight experiments performed with 12 oocytes per experimental condition. Asterisk, denotes statistical significance <0.05.

**Figure 3.** Cell surface expression of RAMP2-$\Delta$4N and RAMP3-$\Delta$4N N-glycosylation mutants requires CRLR co-expression. A. 5 ng of FLAG-tagged RAMP2, RAMP2-$\Delta$4N and wild-type CRLR were injected in the *Xenopus* oocytes. The oocytes were then incubated
36 h at 19°C and CRLR cell surface expression was determined by the binding of ¹²⁵I-labeled anti-FLAG antibody. Shown are mean±S.E. of four to eight experiments performed with 12 oocytes per experimental condition. Two Asterisks denote statistical significance <0.001. B. 5 ng of FLAG-tagged RAMP3, RAMP3-Δ4N and wild-type CRLR were injected in the *Xenopus* oocytes. The oocytes were then incubated 36 h at 19°C and CRLR cell surface expression was determined by binding of ¹²⁵I-labeled anti-FLAG antibody. Shown are mean±S.E. of four to eight experiments performed with 12 oocytes per experimental condition. The Asterisk denotes statistical significance <0.05. C. RAMP1 and wild-type or N-glycosylation mutants of RAMP2 and RAMP3 were immunoprecipitated from the [³⁵S]methionine metabolically labeled *Xenopus* oocytes after 12 h of mRNA injection (5 ng). As predicted from the protein sequence, RAMP1 is expressed with an apparent molecular weight (*Mr*) of ~14 kDa. N-glycosylated RAMP2 and RAMP3 are expressed with apparent *Mr* of 33 kDa and 22, 25 and 27 kDa, respectively. RAMP2-Δ4N and RAMP3-Δ4N are expressed with apparent *Mr* of 17 and 14 kDa, as predicted for apparent *Mr* of nonglycosylated RAMP2 and RAMP3.

**Figure 4. Insertion of N-glycosylation consensus sites promotes RAMP1 cell surface expression.** A. Alignment of RAMP1, RAMP2 and RAMP3 amino acid stretches used for insertion of N-glycosylation consensus sites (N-x-S) into RAMP1. Amino acids changed either to asparagine or to serine are shown in bold. B. Insertion of N-glycosylation consensus sites results in expression of RAMP1 on the cell surface. Shown are mean±S.E. of five experiments performed with 12 oocytes per experimental condition. Asterisk denotes statistical significance <0.05. Two Asterisks denote statistical significance <0.001. C. RAMP1 wild-type and RAMP1-D58N/G60S, RAMP1-Y71S and RAMP1-K103N/P105S mutants were immunoprecipitated from the [³⁵S]methionine metabolically labeled *Xenopus* oocytes after 12 h of mRNA injection (5 ng). Inserted N-glycosylation consensus sites are
partially (RAMP1-D58N/G60S mutant) or completely (RAMP1-Y71S and RAMP1-K103N/P105S mutants) used, as demonstrated by PNGase F treatment. SDS-PAGE molecular weights for non-glycosylated wild-type RAMP1 and glycosylated mutants are indicated as (ng) and (g), respectively.

**Figure 5.**

**RAMPs selectively associate with CRLR and CTR.** 5 ng of FLAG-tagged RAMP1 (A, D), RAMP2-∆4N (B), RAMP3-∆4N (C) were co-injected with 5 ng of either wild-type CRLR, CTR, PTH/PTHrP-R, GluR, V1aR or V2R. The oocytes were then incubated 36 h at 19°C and RAMP1 (A), RAMP2-∆4N (B) or RAMP3-∆4N (C) cell surface expression was determined by the binding of ¹²⁵I-labeled anti-FLAG antibody. Shown are mean ± S.E. of three to four experiments performed with 12 oocytes per experimental condition. Two Asterisks denote statistical significance <0.001. (D) For co-immunoprecipitation the oocytes were [³⁵S]methionine metabolically labeled for 12 h and RAMP1-associated GPCRs were co-immunoprecipitated using anti-FLAG M2 antibody. Two major CRLR forms with apparent Mr of ~ 57 and 62 kDa were co-immunoprecipitated with RAMP1, whereas a single CTR form of Mr ~ 58 kDa was detected.
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Figure 2
Figure 3

A

![Graph showing Ramp2 cell surface expression (fmol/oocyte)]

B

![Graph showing Ramp3 cell surface expression (fmol/oocyte)]

C

![Image of Western blot showing protein bands at 30 kDa and 14 kDa]
Figure 4

A

RAMP1 57C
D
W
G
RAMP2 98CNWTL
RAMP3 56CNLSE

B

![Bar graph showing RAMP1 cell surface expression](image)

RAMP1wt
RAMP1(D58N, G60S)
RAMP1(Y71N)
RAMP1(K103N, P105S)
RAMP1wt + CRLR

C

![Western blot](image)

14 kDa

PNGase F - + - + - + - +
Respective roles of calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) in cell surface expression of CRLR/RAMP heterodimeric receptors

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