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Functional Expression of Heteromeric Calcitonin Gene-related Peptide and Adrenomedullin Receptors in Yeast*

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The ability of G protein-coupled receptors (GPCRs) to form homo- and heteromeric complexes has important implications for the regulation of cellular events. A notable example of heteromer formation is the interaction of the calcitonin receptor-like receptor (CRLR) with different members of the receptor activity modifying protein (RAMP) family, which results in the formation of two different receptors, a calcitonin gene-related peptide (CGRP) receptor and an adrenomedullin receptor. To analyze the role of RAMPs in determining ligand specificity, we have co-expressed CRLR and RAMP proteins in the yeast *Saccharomyces cerevisiae*, which provides a null system to study the function of mammalian receptors. Co-expression of RAMP1 and CRLR reconstituted a CGRP receptor that was able to activate the pheromone-signaling pathway with pharmacological properties similar to those observed previously in mammalian cells. Co-expression of CRLR with RAMP2 or RAMP3 resulted in a response with the pharmacological properties of an adrenomedullin receptor. These data indicate that RAMPs are necessary and sufficient to determine ligand specificity of CRLR. Contrary to observations in mammalian cells, the glycosylation of CRLR was not affected by the presence of RAMPs in yeast, indicating that glycosylation of CRLR is not the prime determinant of ligand specificity. The first functional reconstitution of a heteromeric seven transmembrane receptor in yeast suggests this organism as a useful research tool to study the molecular nature of other heteromeric receptors.

G protein-coupled receptors (GPCRs)[1] represent the largest family of cell-surface receptors. As key controllers of diverse physiological processes, they are of considerable biological and therapeutic interest. Although this class of receptors was originally thought to act as monomers that mediate their effects through stimulation of heterotrimeric G proteins, recent studies indicate that they can also act as multimeric complexes that, besides G proteins, can directly regulate a variety of other downstream effectors, including mitogen-activated protein kinase cascades and transcription factors (1). Several early lines of evidence have suggested that certain families of GPCRs can form homo-oligomers (2, 3). More recent evidence (3–8) has confirmed this and has indicated that GPCRs can also form heteromeric complexes, either with related GPCRs or with members of distinct families of GPCRs. Moreover, some receptors appear to require interaction with additional accessory factors for proper function (9–12). GPCRs therefore seem to function in a rather complex molecular environment. One striking example of the complexity of some GPCRs is provided by receptors for adrenomedullin (ADM) and calcitonin gene-related peptide (CGRP), two members of the calcitonin family of peptides. In this case, a seven-transmembrane protein, the calcitonin receptor-like receptor (CRLR), has been reported to require two different types of associated proteins, receptor activity modifying proteins (RAMPs) and receptor component protein (RCP), for full activity (13, 14).

CGRP is a potent vasoactive neuropeptide, which has been implicated in vasodilation, migraine, and chronic pain, whereas ADM is a multifunctional regulatory peptide with a wide range of biological actions, including vasodilation, cell growth, natriuresis, and certain antimicrobial effects (15, 16). Despite the physiological importance and clinical implications of these two peptides, the identification and characterization of their receptors have been difficult for some time. A seven-transmembrane protein with 55% homology to the calcitonin receptor (CRLR) provided CGRP receptor function only after transfection into specific cellular backgrounds (17, 18). The lack of a CGRP response in other cell lines made positive identification of this protein as a CGRP receptor problematic and suggested that CRLR might require cell type-specific accessory factors to become CGRP-responsive. A novel accessory protein, RAMP1, was identified that generated a functional CGRP receptor upon co-transfection with CRLR into cell culture (19). Confirming the idea that cell type-specific co-factors are required for CGRP responsiveness, transfection of CRLR into cell lines only yielded a functional CGRP receptor when the cell line endogenously expresses RAMP1. Conversely, transfection with RAMP1 only resulted in CGRP receptor function in cells containing CRLR (18, 19). RAMP1 belongs to a family of single transmembrane proteins, which currently consists of three members as follows: RAMP1, RAMP2, and RAMP3. Surprisingly, co-expression of CRLR with either RAMP2 or RAMP3 resulted in formation of a receptor with different ligand specificity, an adrenomedullin receptor (19). The mechanism by which RAMPs affect CRLR signaling is not completely understood; to date three functions have been associated with these accessory proteins. RAMPs can 1) act as a chaperone, facilitating the transport of CRLR from the Golgi to the plasma membrane; 2) they may determine receptor speci-

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[1] The abbreviations used are: GPCRs, G protein-coupled receptors; CGRP, calcitonin gene-related peptide; RAMP, receptor activity modifying protein; CRLR, calcitonin receptor-like receptor; ADM, adrenomedullin; RCP, receptor component protein; PIPES, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin.

ficity by being part of the ligand binding site and/or by modifying CRLR conformation; and 3) they may define the glycosylation status of the receptor and thus determine ligand specificity (13, 19). In addition to RAMPs, another accessory protein was found to confer CGRP receptor function in *Xenopus laevis* oocytes (20). However, in contrast to RAMPs, this receptor component protein (RCP) did not restore CGRP receptor function after co-transfection with CRLR in mammalian cells. Because RCP is ubiquitously expressed and was present in all of the cellular background where CRLR/RAMPs were co-transfected, it was possible that the RCP protein, although by itself not sufficient to confer CGRP responsiveness, was nevertheless part of the receptor complex. Indeed, recent co-immunoprecipitation and antisense studies indicate that RCP is part of the CRLR-RAMP receptor complexes and that it has a role in modulating signal transduction properties of these receptors (14). In retrospect, the fact that different cell lines endogenously express various components that affect CGRP and ADM signaling has complicated the identification of the molecular nature of CGRP and ADM receptors. A heterologous expression system that lacks endogenous expression of both RCPs and RAMPs might be useful to study the contribution of the various accessory proteins to the pharmacology of the CGRP and ADM receptors.

The budding yeast *Saccharomyces cerevisiae* has been used for the heterologous expression of many G protein-coupled receptors. Cells in which receptors are functionally coupled to the mating factor response pathway (21–23) provide a system in which molecular manipulation is easy and in which receptor activity is simply monitored by means of mating pathway-responsive reporter constructs. Because the heterologous mammalian receptor is the only GPCR that is functionally coupled to the readout and because of the absence of potential mammalian-specific accessory factors, yeast cells are essentially a null system for the reconstitution and study of GPCRs. In this study we have tested whether the yeast system might also be useful in the study of heteromeric GPCRs, allowing for the dissection of the exact contribution of various components to the pharmacological properties of these receptors. We have utilized yeast for the co-expression of CRLR and RAMPs and show functional reconstitution of CGRP and ADM receptors.

MATERIALS AND METHODS

Reagents and Media—The following ligands were purchased from Bachem: human βCGRP (H-6730), human αCGRP (H-1470), human ADM (H-2932), rat ADM (H-2934), calcitonin (H-2250), amylin (H-7905), human CGRP-(8–37) (H-9895), and human ADM-(22–52) (H-4144). The human Cys(Acm)^{2,7} CGRP, human ADM-(13–52), and rat ADM-(11–50) were purchased from Peninsula Laboratories. Yeast cells were grown at 30 °C in synthetic media (yeast nitrogen base without amino acids, Difco) with the appropriate nutritional supplements and 2% glucose as a carbon source. The medium was buffered at pH 7.2 with 25 mM PIPES.

Yeast Strains—Strain CY16463 (*FUS1p-HIS3 GPA1p-GαsD229S can1 far1Δ his3 leu2 lys2 ste14::trp1::LYS2 ste18γ26 ste3Δ tbt-1 trp1 ura3*), for the purposes of this study is referred to as wild type, contains a rat GαsD229S under the control of the yeast *GPA1* promoter and a hybrid Gγ gene that consists of Ste18p residues 1–88, human γ2 residues 60–67, and Ste18p residues 106–110 expressed from the yeast *STE18* promoter. The genetic features of this strain, including the rat GαsD229S and the chimeric Gγ subunits, have been described previously (24). Strain CY19888 is an *stp22*-deficient derivative of CY16463 made by standard yeast molecular biological techniques.

Cloning of CRLR and RAMPs—CRLR and RAMPs were cloned from a heart cDNA library (CLONTECH Quick clone 7121-1). PCRs were performed with Advantage-GC cDNA PCR kit (CLONTECH K1907-1), and the primers were as follows: RAMP1, forward CAGTGGTACCACTCGGCACCGCTGTGCACCATGGCC and reverse CAGTTCTAGACTACACAATGCCCTCAGTGCGCTTGCCTC; RAMP2, forward GATCTTGGTACCATGGCCTCGCTCCGGGTG and reverse GATCTTTCTAGACTAGGCCTGGGCCTCACTGTC; RAMP3, forward CAGTGGTACCATGGAGACTGGAGCGCTGCGG and reverse CAGTTCTAGATCACAGCAGCGTGTCGGTGCG; and CRLR, forward GGGTACCCCACCATGGAGAAAAAGTGTACCTCGTAT and reverse CGGGATCCCGCAAACAGTGAGACAACCATCCTTCTA. We first cloned all the genes into mammalian expression vectors (pcDNA3.1) using the *Kpn*I/*Xba*I sites introduced in the primers. All constructs were functional when expressed in mammalian cells. CRLR was subcloned into *Nco*I/*Xba*I sites of yeast expression vectors CP1289 (*2μ ori AmpR LEU2 REP3 PGK*-promoter) to create CP5899 and CP4258 (*2μ ori AmpR LEU2 REP3 PGK*-promoter-*MFα1*-(*1–89*)) to create CP5900. RAMPs were subcloned into *Spe*I/*Hin*dIII sites of yeast expression vector p426 ADH (*2μ ori AmpR URA3 ADH* promoter) (25), leading to the creation of plasmids pRAMP1 (CP6746), pRAMP2 (CP6747), and pRAMP3 (CP6748). RAMPs were also cloned into the *Hin*dIII/*Bgl*II sites of CP1625 (26) (*2μ ori AmpR URA3 ADH* promoter-*MFα1*-(*1–89*)), resulting in pMFL-RAMP1 (CP6280), pMFL-RAMP2 (CP6450), and pMFL-RAMP3 (CP6761).

Determination of Receptor Activity—Receptor activation was determined by measuring the induction of β-galactosidase activity under the control of the *FUS1* pheromone-inducible promoter (expressed from plasmid CP1584 (24), *2μ ori AmpR TRP1 FUS1p*-LacZ). This assay was essentially performed as described previously (24) with some minor modifications. In short, yeast cells were grown overnight to exponential phase and diluted to an A_{600} of 0.2. BSA and Bactopeptone were added to a final concentration of 0.1% each. The yeast cells were seeded in 96-well plates in 100 μl final volume and treated with ligands for 4 h at 30 °C. β-Galactosidase activity was determined by the addition of 20 μl of substrate/lysis solution (0.5 mM fluorescein di-β-D-galactopyranoside (Molecular Probes), 2.5% Triton X-100, and 125 mM PIPES, pH 7.2). Plates were incubated for 1 h at 37 °C, and the reactions were stopped by the addition of 20 μl of 1 M Na_2CO_3. Fluorescence was read at an excitation wavelength of 485λ and an emission wavelength of 535λ at optimal gain. Duplicate samples were analyzed and the results shown in the figures are representative of at least two independent experiments.

Analysis of the Glycosylation Status of the Receptor—To allow for immunodetection of CRLR, we FLAG-tagged the protein by subcloning the gene into *Nco*I/*Xba*I sites of CP3146 (*2μ ori AmpR LEU2 REP3 PGK*-promoter-FLAG). Membrane extracts from cells expressing CRLR-FLAG alone or in combination with RAMPs were prepared by standard procedures. Briefly, cells were grown to mid-logarithmic phase, washed with sterile cold water, and resuspended in lysis buffer (20 mM Hepes, pH 7.3, 50 mM NaCl, 2 mM EDTA, plus protease inhibitors). Yeast cells were glass bead-disrupted, and extracts were cleared by centrifugation at 2200 rpm for 5 min in a Sorvall RT6000D. Enriched membrane fractions were obtained by centrifugation of the resulting extracts at 48,000 rpm for 30 min in Optima TL Ultracentrifuge (Beckman, rotor TLA 100.3) and resuspension of the pellet in lysis buffer. Protein concentration was determined with the Dc protein assay (Bio-Rad) and endoglycosidase H digestions were performed following the manufacturer's instructions (New England Biolabs). Western blot analysis was performed by standard procedures, and CRLR-FLAG was detected by enhanced chemiluminescence using an Anti-FLAG M2 monoclonal antibody-peroxidase conjugate (Sigma A8592).

RESULTS

Co-expression of CRLR and RAMP1 in Yeast Is Sufficient for a CGRP Response—Haploid *S. cerevisiae* cells contain a cell type-specific seven-transmembrane receptor (Ste2 in MATa cells or Ste3 in MATα cells) coupled to a heterotrimeric G protein that consists of a Gα subunit (*GPA1*), a Gβ subunit (*STE4*), and a Gγ subunit (*STE18*). Stimulation of the receptor by mating factor leads to activation of a mitogen-activated protein kinase pathway and the induction of several genes, including *FUS1*, a gene involved in fusion of mating partners. By placing the *lacZ* reporter gene under the control of the *FUS1* promoter, pathway activity in these cells can be conveniently monitored by measuring β-galactosidase activity (22). Functional expression of mammalian GPCRs is typically achieved in cells lacking the endogenous mating factor receptor and containing a yeast/mammalian chimeric G protein. Because CGRP responsiveness in mammalian cells requires co-expression of RAMP1 and CRLR, we created yeast vectors that allow for co-expression of receptor and accessory factor under the control of strong yeast promoters. We used *LEU2*-marked

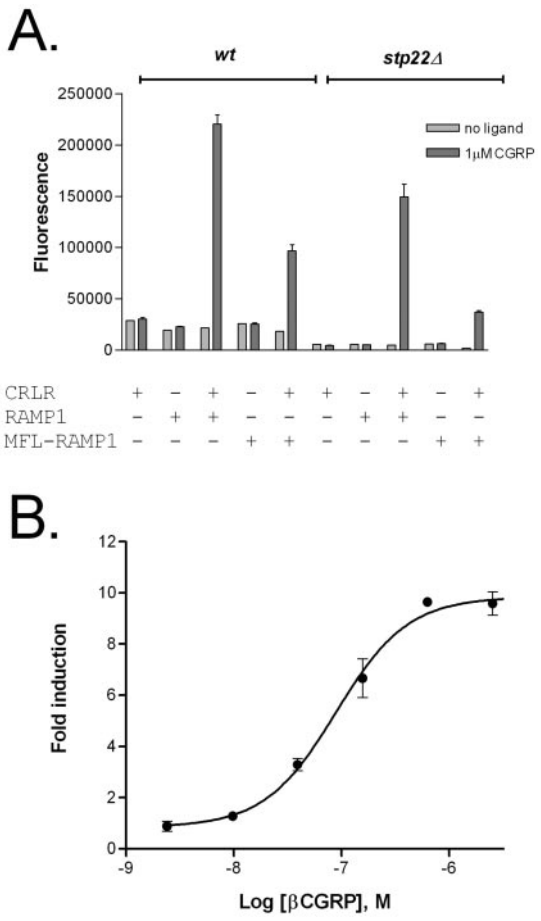

FIG. 1. **βCGRP stimulates the pheromone signaling pathway in yeast cells co-expressing RAMP1 and CRLR.** *A*, wild type (CY16463) and *stp22*Δ (CY19888) yeast strains were transformed with the indicated combinations of CRLR and RAMP expression constructs. The strains were grown overnight to an A_{600} of 0.3–0.8 in selective media at pH 7.2. The culture was diluted to an A_{600} of 0.2 and incubated in the same media plus 0.1% BSA, 0.1% Bactopeptone with 1 μM βCGRP for 4 h at 30 °C. β-Galactosidase activity was then determined as described in the text. *B*, strain CY16463 (*wt*) transformed with pRAMP1 and pCRLR was used to determine the βCGRP dose response. The yeast culture was grown as described in *A* and incubated with various concentrations of βCGRP for 4 h at 30 °C. The induction of *FUS1p*::*lacZ* was determined by measuring the levels of β-galactosidase activity.

2-μm vectors for expression of CRLR from the *PGK* promoter and *URA3*-marked 2-μm vectors for expression of RAMP1 from the *ADH* promoter. Because many mammalian proteins show improved plasma membrane localization in yeast when they are fused to the α-factor leader sequence,[2] we made expression constructs for CRLR and RAMPs with and without this leader sequence (the resulting vectors are indicated as pCRLR, pMFL-CRLR, pRAMP1 and pMFL-RAMP1). Specific proteases in the Golgi apparatus cleave the α-leader sequence, allowing expression of unmodified proteins at the plasma membrane.

The CGRP and ADM receptors couple to $G\alpha_s$ in mammalian cells. We therefore expressed various combinations of CRLR and RAMP1 constructs in a yeast strain that contains a rat $G\alpha_s$ (CY16463) and treated cells with βCGRP for 4 h before measuring β-galactosidase activity. Fig. 1*A* shows that expression of CRLR or RAMP1 alone did not result in a functional response. However, up to a 10-fold induction of β-galactosidase was observed when CRLR and RAMP1 were co-expressed. The largest induction was found when both CRLR and RAMP1 were expressed without leader sequence (Fig. 1*A*). We hypothesized that the number of receptors at the plasma membrane might be affected by yeast systems that recognize damaged, misfolded, or heterologous receptors and that target proteins to the vacuole for degradation (27). We have found that functional expression of several mammalian GPCRs in yeast is aided by deletion of *STP22*, a gene involved in this type of "quality control" (27).[3] Therefore, we also tested CRLR and RAMP expression in an *stp22*-deficient strain (CY19888). Reporter gene expression in the absence of ligand was found to be lower in *stp22*-deficient cells, whereas robust induction of the signal by ligands was still observed (Fig. 1*A*). As a consequence, the βCGRP-responsiveness was increased to 30-fold for the CRLR/RAMP1 and 20-fold for the CRLR/MFL-RAMP1 combinations (Fig. 1*A*). This level of induction appears to be stronger than for the UDP-glucose receptor KIAA0001 (10-fold induction) or the LPA receptor Edg-2 (5-fold induction) coupled in yeast (24, 28), but not as strong as for some other mammalian GPCRs or for the mating factor receptor coupled to the endogenous *GPA1* gene (which can be in excess of 100-fold induction).[2] MFL-CRLR did not show any functional response in any of the combinations tested (data not shown).

We then tested whether the reconstituted receptors responded to βCGRP in a dose-dependent manner. Fig. 1*B* shows a classic sigmoidal dose-response curve with an EC_{50} of 89 ± 30 nM for strain CY16463 carrying pCRLR/pRAMP1. We obtained similar results with the *stp22*-deficient strain containing either pCRLR/pRAMP1 or pCRLR/pMFL-RAMP1 (data not shown). In every case, the sensitivity of the response was affected by the pH of the incubation media (pH 7.2 was optimal) and the presence of BSA and peptone (data not shown). The addition of BSA and peptone improved the potency of βCGRP somewhat, possibly by preventing nonspecific ligand interactions with yeast cell wall proteins or with the plastic of the reaction wells. Taken together, these data indicate that co-expression of CRLR and RAMP1 is necessary and sufficient for βCGRP responsiveness in yeast.

ADM Responsiveness in Yeast Requires Co-expression of CRLR and RAMP2/3—In contrast to RAMP1, co-expression of CRLR with RAMP2 or RAMP3 in mammalian cells results in cAMP production through an ADM receptor. This is the only ADM receptor described to date for which the molecular nature appears to be reasonably clear (13, 29). To test whether RAMP2 and RAMP3 are sufficient to confer to CRLR the properties of an ADM receptor, we co-expressed various combinations of RAMP2, RAMP3, and CRLR in wild type (CY16463) and *stp22*-deficient yeast (CY19888), as described above for RAMP1 and CRLR. As observed with the response to CGRP, expression of either RAMP2 or CRLR alone gave no response to ADM, but co-expression of RAMP2 and CRLR resulted in a ligand-dependent signal (Fig. 2*A*). The optimal responsiveness to ADM (~10-fold induction in β-galactosidase activity) was observed when CRLR and pMFL-RAMP2 were expressed in an *stp22*-deficient strain (Fig. 2*A*). This combination also responded to agonist in a dose-dependent fashion, displaying a sigmoidal curve with an EC_{50} of 274 ± 50 nM (Fig. 2*B*). The assay had similar pH, BSA, and peptone requirements as the RAMP1/CRLR receptor (data not shown).

We performed comparable experiments using CRLR in combination with RAMP3 and observed a 5–10-fold induction of β-galactosidase activity in wild type CY16463 and a 10–15-fold induction in the *stp22*-deficient CY19888 (Fig. 3*A*) with an EC_{50} of 115 ± 50 nM (Fig. 3*B*). Just as observed in mammalian

[2] J. J. Miret, L. Rakhilina, L. Silverman, and B. Oehlen, unpublished observations.

[3] K. Ostanin, unpublished observations.

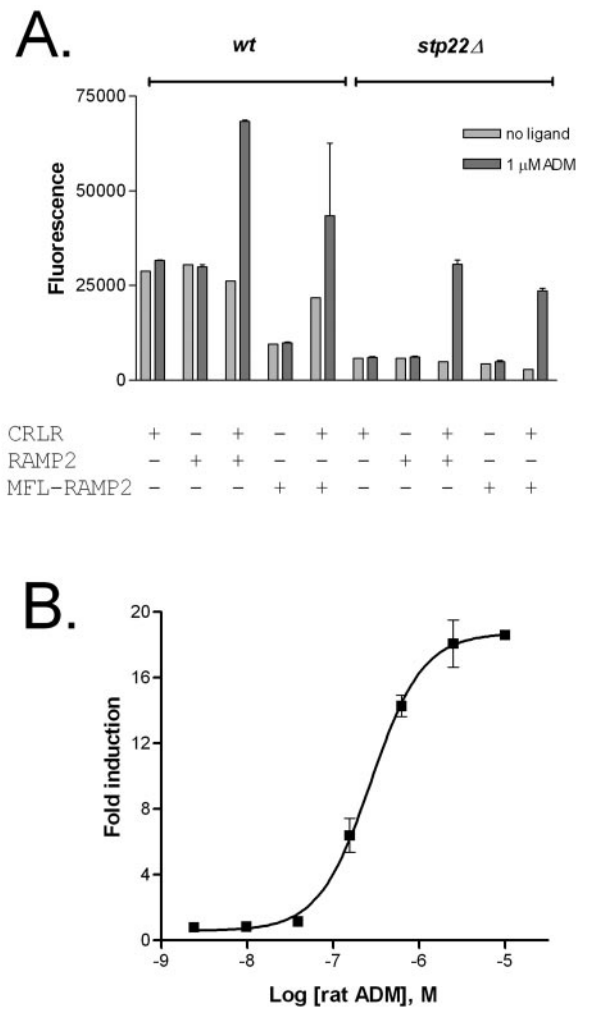

FIG. 2. ***FUS1p*::*lacZ* is induced by ADM in a dose-dependent manner in yeast cells expressing CRLR and RAMP2.** *A*, yeast strains CY16463 (*wt*) and CY19888 (*stp22*Δ) transformed with the indicated CRLR and RAMP2 plasmids were incubated with 1 μM rat ADM for 4 h at 30 °C, and β-galactosidase activity was determined as described in the text. *B*, CY19888 (*stp22*Δ) transformed with pMFL-RAMP2 and pCRLR was used to determine dose dependence of the response to rat ADM. The yeast culture was prepared as described in the text and incubated with increasing amounts of rat ADM for 4 h at 30 °C. The induction of *FUS1p*::*lacZ* was determined by measuring the levels of β-galactosidase activity.

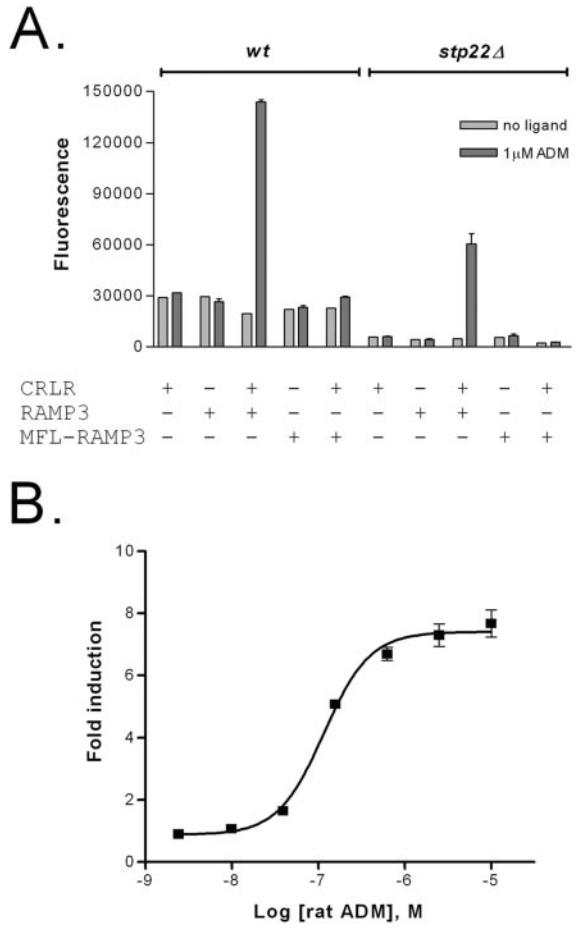

FIG. 3. **Co-expression of RAMP3 and CRLR confers to yeast a dose-dependent ADM response.** *A*, yeast strains CY16463 (*wt*) and CY19888 (*stp22*Δ) were transformed with the indicated CRLR and RAMP plasmids. Strains were incubated with 1 μM rat ADM for 4 h at 30 °C, and β-galactosidase activity was determined as described in the text. *B*, CY16463 (*wt*) transformed with pRAMP3 and pCRLR was used to determine a ADM dose-response curve. The yeast culture was prepared as described in the text and incubated with increasing amounts of rat ADM for 4 h at 30 °C. The induction of *FUS1p*::*lacZ* was determined by measuring the levels of β-galactosidase activity.

cells, co-expression of CRLR with either RAMP2 or RAMP3 in yeast resulted in ADM responsiveness.

RAMP1 Is Sufficient to Impart CRLR with CGRP Receptor Pharmacology—The agonist rank order of potency for different members of the calcitonin family of peptides defines the CGRP receptor. βCGRP and αCGRP are the most potent agonists for this receptor, followed by adrenomedullin and, with significantly lower potency, calcitonin and amylin (29). We determined the dose-response relationship for these peptides in yeast cells expressing CRLR and RAMP1 (Fig. 4*A*). βCGRP was found to be the most potent agonist followed by αCGRP and ADM, whereas calcitonin and amylin did not have any effect. No receptor stimulation was observed with the linear CGRP-related peptide (human $Cys(Acm)^{2,7}$ CGRP), which has been described as a CGRP2-specific agonist (30). The rank order of agonist activity of these peptides is consistent with reconstitution of CGRP receptor pharmacology in yeast. To characterize further the pharmacological properties of the receptor in yeast, we studied the effect of the short CGRP peptide CGRP-(8–37), a well characterized CGRP receptor antagonist (29, 31–33). As observed in mammalian systems, this peptide acted as a competitive antagonist; it inhibited the response to βCGRP (100 nM) in yeast with an IC_{50} of 4 μM (Fig. 4*B*), and addition of increasing concentrations produced an incremental shift of the βCGRP dose-response curves to the right (data not shown). CGRP-(8–37) has been reported to act also as an antagonist when ADM is the ligand for the CGRP receptor. This is a key differentiator between the response of the CGRP receptor to ADM and *bona fide* ADM receptors, which are insensitive to this antagonist (29, 34). Fig. 4*C* illustrates that CGRP-(8–37) inhibits the ADM response (at 2 μM rat ADM) with an IC_{50} of ~1 μM, whereas the specific ADM antagonist, ADM-(22–52), did not antagonize the effects of ADM on this receptor. These data combined show that the presence of RAMP1 is sufficient to confer to CRLR pharmacological properties of a CGRP receptor.

RAMP2 Confers to CRLR an ADM Receptor Pharmacology—The best characterized ADM receptors so far are CRLR/RAMP2 and CRLR/RAMP3, which have very similar pharmacological properties in mammalian cells (19, 35). The agonist rank order for these receptors is ADM > CGRP ≫ amylin and calcitonin. We tested the effect of various agonists on *stp22*-deficient yeast cells expressing CRLR and MFL-RAMP2, the combination that was found to give optimal response to ADM (Fig. 2*A*). As shown in Fig. 5*A*, the CRLR/RAMP2 receptor reconstituted in yeast

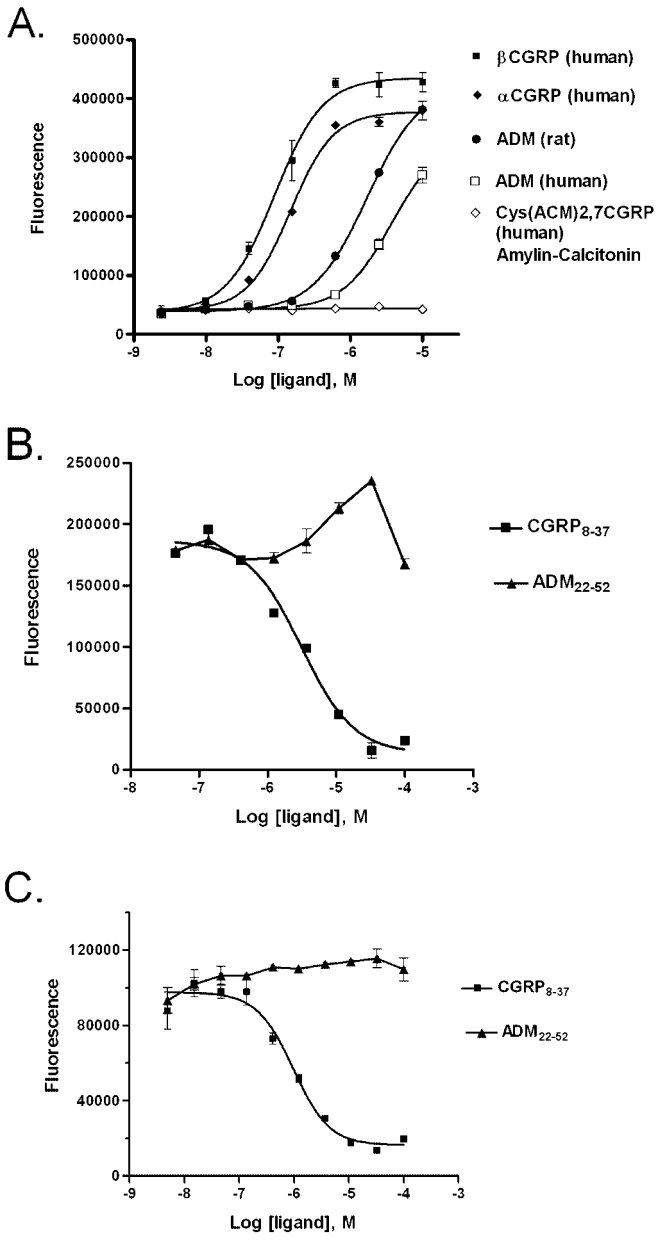

FIG. 4. **RAMP1 confers to CRLR a CGRP receptor pharmacological profile.** *A*, CY16463 (*wt*) cells containing pRAMP1/pCRLR were incubated for 4 h at 30 °C with various concentrations of βCGRP (human), αCGRP (human), ADM (rat), ADM (human), Cys(ACM)^{2,7} CGRP (human), amylin (human), and calcitonin (human). The induction of *FUS1p*::*lacZ* was determined by measuring the levels of β-galactosidase activity. *B*, CY19888 (*stp22*Δ) containing pRAMP1 and pCRLR was incubated with 100 nM of the agonist βCGRP and increasing concentrations of CGRP-(8–37) or ADM-(22–52). The assay conditions were as described in the text, and β-galactosidase activity was determined after treatment for 4 h at 30 °C. *C*, the same cells as in *B* were incubated with 2 μM of rat ADM and increasing concentrations of CGRP-(8–37) or ADM-(22–52). The assay conditions were as described in the text, and β-galactosidase activity was determined after treatment for 4 h at 30 °C.

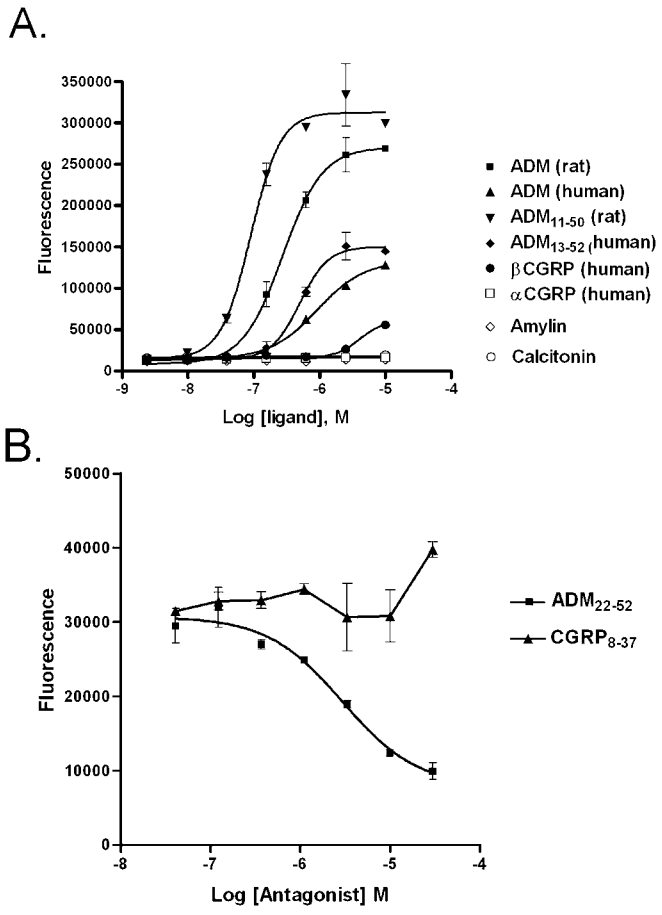

FIG. 5. **Cells co-expressing CRLR and RAMP2 display the pharmacological profile of an ADM receptor.** *A*, the effect of different agonists on yeast cells expressing RAMP2 and CRLR. CY19888 (*stp22*Δ) cells containing pMFL-RAMP2 and pCRLR were incubated for 4 h at 30 °C with various concentrations of rat ADM, human ADM, rat ADM-(11–50), human ADM-(13–52), human βCGRP, human αCGRP, human amylin, or human calcitonin. The induction of *FUS1p*::*lacZ* was determined by measuring the levels of β-galactosidase activity. *B*, the effect of ADM-(22–52) on the response to ADM. CY19888 (*stp22*Δ) containing pMFL-RAMP2 and pCRLR was incubated with 200 nM rat ADM and increasing concentrations of ADM-(22–52) or CGRP-(8–37). The assay conditions were as described in the text, and β-galactosidase activity was determined after treatment with ligands for 4 h at 30 °C.

presents the same rank order as observed previously in mammalian cells; adrenomedullins were more potent agonists than α- and βCGRP, which only moderately activated this receptor at high concentrations (Fig. 5*A*). The short versions of rat and human ADM, rat ADM-(11–50) and human ADM-(13–52), were more potent than full-length peptides in the yeast assay (Fig. 5*A*), even though their potency in mammalian systems was similar to full-length ADM (35, 36). We have observed several cases where shorter peptides are better agonists in yeast than their full-length counterparts.[2] This presumably reflects the fact that they can penetrate the yeast cell wall more efficiently and thus achieve a higher effective concentration at the plasma membrane (see also "Discussion").

The only antagonist reported for the ADM receptor is an ADM-derived peptide, ADM-(22–52), that acts as a competitive inhibitor to ADM (29). We tested the effect of ADM-(22–52) on yeast cells stimulated with 200 nM ADM and found antagonistic activity (Fig. 5*B*). CGRP-(8–37) had no effect on ADM-stimulated RAMP2/CRLR-expressing yeast cells (Fig. 5*B*). Similar agonist and antagonist experiments in CRLR/RAMP3-expressing cells yielded comparable results (data not shown). To gain information on the number of ligand-binding sites, we performed ligand-binding studies with radioactively labeled CGRP and ADM on yeast membranes prepared from strains expressing CRLR/RAMP1, CRLR/MFL-RAMP2. However, we were unable to detect any specific binding in these yeast membranes, even though control membranes prepared from mammalian cells expressing the ADM and CGRP receptors showed significant specific binding (data not shown). Nevertheless, taken together, our data in the reporter-based assays indicate that the presence of RAMP2 or RAMP3 in yeast is sufficient for

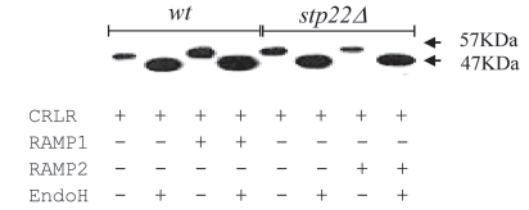

FIG. 6. **RAMPs do not alter CRLR glycosylation in yeast.** CY16463 (*wt*) cells containing pCRLR-FLAG or pRAMP1 + pCRLR-FLAG and CY19888 (*stp22*Δ) cells expressing pCRLR-FLAG or pMFL-RAMP2 + pCRLR-FLAG were grown to exponential phase. Membrane preparations were made as described under "Materials and Methods," and a portion of the samples was treated with endoglycosidase H (*EndoH*), whereas the other portion was mock-treated. Samples were analyzed by Western blot using an anti-FLAG antibody.

CRLR to display a pharmacological profile that is comparable with that of the ADM receptor in mammalian cells.

RAMPs Do Not Alter the Glycosylation Status of CRLR in Yeast—In mammalian cells CRLR is terminally glycosylated in the presence of RAMP1, whereas CRLR/RAMP2 and CRLR/RAMP3 display core glycosylation (13, 19). This observation led to the hypothesis that the nature of the oligosaccharides attached to CRLR might have a role in determining the pharmacological properties of the receptor. Studies using chimeras between RAMP1 and RAMP2 yielded results that are consistent with this idea; only constructs carrying the amino-terminal domain of RAMP1 were capable of altering CRLR glycosylation and presented a CGRP pharmacological profile (35). However, recent experiments in Schneider 2 insect cells cast doubt regarding the role of glycosylation in determining CRLR pharmacological properties; in these cells RAMPs did not affect CRLR glycosylation status, but they still defined its pharmacological properties (37). Unlike mammalian cells, *S. cerevisiae* lacks the ability to process the core mannose oligosaccharides that are covalently attached to proteins in the endoplasmic reticulum, and no terminal glycosylation is therefore observed in this organism. In a further test of the hypothesis that the glycosylation status of CRLR correlates with pharmacological properties, we evaluated the glycosylation status of CRLR in the presence of RAMPs in yeast. Yeast extracts from cells expressing CRLR-FLAG, CRLR-FLAG/RAMP1, or CRLR-FLAG/MFL-RAMP2 were analyzed by Western blotting. Digestion with endoglycosidase H, which is specific for core glycosylated proteins, was used to examine the glycosylation status of CRLR. In the absence of RAMPs, CRLR migrated as 57-kb band, which was reduced to an apparent mobility of 47 kb after endoglycosidase H digestion (Fig. 6), indicating that CRLR is core-glycosylated in yeast. Co-expression with either RAMP1 or RAMP2 did not alter the migration pattern of FLAG-tagged CRLR in SDS-PAGE gels, whether samples were treated or not with endoglycosidase H (Fig. 6). The lack of correlation between glycosylation status and pharmacological properties of different CRLR/RAMP combinations in yeast indicates that glycosylation is not the prime factor responsible for determining the pharmacological properties of these receptors and corroborates the results obtained in Schneider-2 cells.

DISCUSSION

In this study, CRLR and various RAMPs were co-expressed in the yeast *S. cerevisiae* to reconstitute CGRP and ADM receptors that functionally couple to a mitogen-activated protein kinase signaling cascade. Based on the rank order of agonists and the activity of specific antagonists, we found that co-expression of CRLR with RAMP1 results in the generation of a CGRP receptor, whereas expression with RAMP2 or RAMP3 imparted ADM receptor pharmacology. Because no additional mammalian factors are needed for these pharmacological responses in yeast, these data strongly support the notion that RAMPs alone determine the most important features of CRLR receptor pharmacology, *i.e.* ligand specificity. To our knowledge, the functional expression of the CGRP and ADM receptors in yeast provides the first example of reconstitution of a heteromeric seven-transmembrane receptor in this model system. Yeast offers several advantages for the study of heteromeric GPCRs. Unlike other expression systems, like various mammalian cell lines, *Xenopus* oocytes or *Drosophila* Schneider 2 cells, yeast provides a defined experimental system with virtually a null background for GPCRs and G proteins. The "null background" feature of the yeast system has allowed for the better definition of the pharmacological properties of the monomeric adenosine and lysophosphatidic acid receptor families, which have various subtypes with similar pharmacological properties and which are commonly expressed in mammalian expression systems (28, 38). Our findings with CGRP and ADM receptors in yeast extend the benefits of the null background feature to the study of heteromeric receptors.

In many respects, the pharmacological responses of CRLR/RAMP receptors in yeast are very similar to the pharmacological properties described in other expression systems. The rank order of agonists, the properties of antagonists, and the differences between RAMP1 and RAMP2/3 co-expression are all very similar to results described previously (29) that were obtained with mammalian cells. However, with respect to the absolute potency of various ligands, the response of the CRLR/RAMP receptors in the yeast-based assay is significantly reduced compared with the responses in mammalian cell lines. In the case of the CGRP receptor, our results show that βCGRP activates the mating factor pathway with an EC_{50} of ~90 nM, about 2 orders of magnitude higher than required for cAMP induction in HEK293 cells (18). There are many factors that could explain this discrepancy, including the use of hybrid mammalian-yeast G proteins and the size of the ligands. The ligands in this study, CGRP and ADM (M_r 3790 and 5729, respectively), are much larger than adenosine (M_r 267), for example, for which ligand potencies were found to be very similar in mammalian and yeast expression systems (38, 39). The size of the CGRP and ADM ligands and the efficiency with which they penetrate the yeast cell wall are likely an important contributor to the difference in potency in yeast and mammalian expression systems. The fact that both short versions of the rat and human ADM were more efficient agonists is consistent this notion. Also, we have observed similar differences in potency between the yeast and mammalian expression systems for other receptors with peptide ligands of similar size as CGRP and ADM.[2] The difference in absolute potency between yeast and mammalian expression systems might put some restrictions on the use of yeast for the pharmacological characterization of certain types of receptors.

Co-expression of RAMPs and CRLR in mammalian cells led to the idea that RAMPs are the main factors regulating ligand selectivity for these receptors. However, given the complex nature of these experimental systems, it was unknown whether RAMPs by themselves are sufficient to determine ligand specificity or whether additional cellular factors might be required. Yeast cells do not have endogenous RAMP or CRLR homologues, but they do have a gene (*YJL011C*) with some homology to the receptor component protein RCP (37% identity), a factor affecting the signaling properties of CRLR-RAMP complexes (14). The function of this gene in yeast is unknown; knockout of the gene in haploid cells is lethal (*Saccharomyces* Genome Data base at Stanford University), and there is no evidence that this protein serves as a true homologue of human RCP. High level expression of human RCP in yeast does not alter the pharma-

cological properties of either the CGRP or ADM receptors in this organism.[2] Therefore, our results strongly support the notion that RAMPs alone are sufficient to define the fundamental features of CRLR pharmacology. The initial observation that terminal glycosylation of CRLR is dependent on co-expression with RAMP1 and is not observed when the receptor is expressed alone or in combination with RAMP2 led to the hypothesis that the glycosylation state of the receptor correlates with certain aspects of its pharmacology (13). Experiments using RAMP chimeras showed that the amino-terminal portion of these accessory proteins determines both the glycosylation state of CRLR and its ligand specificity and confirmed a correlation between pharmacological properties of CRLR and its glycosylation state (35). However, subsequent work in an insect cell expression system failed to show RAMP-specific differences in glycosylation, whereas differences in pharmacology between the CRLR/RAMP1 and CRLR/RAMP2 receptors were as observed in mammalian cells (37). The *S. cerevisiae* glycosylation pathway is fundamentally different from mammalian cells, as yeast cells can only synthesize core or high mannose oligosaccharides and do not have the ability to synthesize mature or complex oligosaccharides. Our observations in yeast, along with the studies in insect cells, therefore provide evidence that glycosylation of CRLR is not a major determinant of CGRP or ADM pharmacology.

There is an increasing number of GPCRs that form heteromeric complexes with other GPCRs or that interact with unrelated proteins required for their function (1, 8). Because of the fact that GPCRs are the most abundant family of cell-surface receptors and these heteromeric interactions present a new level of complexity, the identification of the exact molecular nature of many receptors will continue to be a major scientific challenge. Our results with the CRLR/RAMP system suggest that yeast could be a useful model system to study certain aspects of heteromeric GPCRs. Besides the benefits of the null background of interfering endogenous signaling molecules, which allows for the precise molecular definition of signaling complexes, the yeast system also provides a versatile research tool for the genetic dissection of these heteromeric complexes. Several genetic strategies have been used in yeast to identify factors that affect GPCR signaling. The commonly used yeast two-hybrid system has been valuable in the identification of some of the novel GPCR-associated proteins (40–42). Other types of genetic screens in the yeast system have identified AGS1 as a novel mammalian protein involved in the regulation of G protein signaling (43), residues in the C5a receptor that are important for receptor activation (44), or surrogate ligands for orphan GPCRs (24, 45). Our demonstration that some GPCR-associated protein interactions can be functionally reproduced in yeast suggests the possibility of employing similar genetic selection strategies for the analysis of complex receptors in yeast.

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MECHANISMS OF SIGNAL TRANSDUCTION:

Functional Expression of Heteromeric Calcitonin Gene-related Peptide and Adrenomedullin Receptors in Yeast

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