Molecular Basis of Association of Receptor Activity-Modifying Protein 3 with the Family B G Protein-Coupled Secretin Receptor[†]

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ABSTRACT: The three receptor activity-modifying proteins (RAMPs) have been recognized as being important for the trafficking and function of a subset of family B G protein-coupled receptors, although the structural basis for this has not been well established. In the current work, we use morphological fluorescence techniques, bioluminescence resonance energy transfer, and bimolecular fluorescence complementation to demonstrate that the secretin receptor associates specifically with RAMP3, but not with RAMP1 or RAMP2. We use truncation constructs, peptide competition experiments, and chimeric secretin−GLP1 receptor constructs to establish that this association is structurally specific, dependent on the intramembranous region of the RAMP and TM6 and TM7 of this receptor. There were no observed changes in secretin-stimulated cAMP, intracellular calcium, ERK1/2 phosphorylation, or receptor internalization in receptor-bearing COS or CHO-K1 cells in the presence or absence of exogenous RAMP transfection, although the secretin receptor traffics normally to the cell surface in these cells in a RAMP-independent manner, resulting in both free and RAMP-associated receptor on the cell surface. RAMP3 association with this receptor was shown to be capable of rescuing a receptor mutant (G241C) that is normally trapped intracellularly in the biosynthetic machinery. Similarly, secretin receptor expression had functional effects on adrenomedullin activity, with increasing secretin receptor expression competing for RAMP3 association with the calcitonin receptor-like receptor to yield a functional adrenomedullin receptor. These data provide important new insights into the structural basis for RAMP3 interaction with a family B G protein-coupled receptor, potentially providing a highly selective target for drug action. This may be representative of similar interactions between other members of this receptor family and RAMP proteins.

Receptor activity-modifying proteins (RAMPs)[1] are type I single transmembrane proteins that associate with a subset of G protein-coupled receptors (GPCRs), thereby having the potential to affect their ligand binding specificity and affinity, signaling, and trafficking (*1*). In addition to these potential functional effects, RAMP−GPCR association has been proposed to represent an opportunity for the development of highly selective ligands that target this interface, providing an opportunity to achieve a greater degree of selectivity than those drugs that target only the particular GPCR molecule (*2*). However, little is currently understood regarding the molecular basis of RAMP association with GPCRs or the structural characteristics of the shared interface between these molecules.

The RAMPs are a family of three proteins, RAMP1, RAMP2, and RAMP3, having approximately 30% amino acid conservation. Each has a relatively large extracellular amino-terminal region, a single transmembrane segment, and a small intracellular carboxyl-terminal tail, with RAMP1 and RAMP3 approximately 148 amino acids in length, and RAMP2 having an additional 26 amino acids (*3*). RAMPs were first identified when they were found to be responsible for the translocation of the calcitonin receptor-like receptor (CLR) from the biosynthetic compartments (endoplasmic reticulum and golgi) of the cell to the plasma membrane (*4*). Of note, both this receptor and the RAMPS are expressed relatively poorly on the cell surface in the absence of an appropriate partner molecule. The ability of interacting GPCRs to enable translocation of RAMPs to the cell surface has become a key feature utilized in exploring their spectrum of association with other receptors. However, it is particularly noteworthy that there is currently only a small number of receptors recognized as interacting with RAMPs, yet RAMPs are expressed in many tissues in which these receptors are absent. Clearly, more information is needed regarding the basis of RAMP association with other molecules.

The specific RAMP association with CLR determines its pharmacology. RAMP1/CLR is phenotypically a calcitonin

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[1]Abbreviations: BRET, bioluminescence resonance energy transfer; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CTR, calcitonin receptor; DMEM, Dulbecco's modified Eagle's medium; GHRH, growth hormone releasing hormone; GPCR, G protein-coupled receptor; GLP, glucagon-like peptide; KRH, Krebs-Ringer-HEPES; NHERF1, sodium−hydrogen exchange regulatory factor; NSF, N-ethylmaleimide-sensitive factor; PBS, phosphate-buffered saline; PTH, parathyroid hormone; RAMPs, receptor activity-modifying proteins; Rlu, *Renilla* luciferase; SecR, human secretin receptor; VPAC, vasoactive intestinal polypeptide receptor; YFP, yellow fluorescent protein; YN, YFP(1−158); YC, YFP(159−238).

gene-related peptide (CGRP) receptor; RAMP2/CLR and RAMP3/CLR exhibit adrenomedullin receptor phenotypes (5). Similarly, RAMP association with the calcitonin receptor is necessary to express its amylin receptor phenotype (6). Other family B GPCRs, such as PTH1, PTH2, VPAC1, and glucagon receptors may associate with RAMPs, but to date no effects on their pharmacological profiles have been reported (7). Of interest, the VPAC1 receptor can associate with all three RAMPs, while the PTH1 and glucagon receptors associate only with RAMP2 and the PTH2 receptor associates only with RAMP3 (7, 8). Other family B GPCRs, like the VPAC2, GHRH, GLP1, and GLP2 receptors have not been found to associate with RAMPs (8). A recent study shows that RAMP1 and RAMP3 can also effectively interact with the family C calcium-sensing receptor, where they facilitate receptor glycosylation and its efficient delivery to the cell surface (9).

In the current work, we identified a new, previously unrecognized RAMP3-specific interaction with another family B GPCR, the secretin receptor, and have explored the molecular basis for this association. This receptor was the first family B GPCR to be isolated and has been extensively studied as a prototypical member of this receptor family (10). It is physiologically important as a mediator of pancreatic and biliary alkaline secretion that is critical for normal digestion (11). Our experience in working with this receptor, providing extensive insights into its structure (12, 13) and into the molecular basis of its constitutive dimerization (14−18), provide substantial insights into its function and key resources to explore the basis of its RAMP association. In the current project, we have utilized fluorescence, resonance energy transfer, and morphological techniques to establish the presence of a structurally specific association between RAMP3 and the secretin receptor, and have localized the basis of this interaction to an intramembranous region of the RAMP and specifically to TM6 and TM7 of this receptor.

EXPERIMENTAL PROCEDURES

Receptor Constructs. RAMP constructs fused in-frame at their carboxyl terminus with *Renilla* luciferase (Rlu) or yellow fluorescent protein (YFP) were prepared using GATEWAY technology from Invitrogen (Carlsbad, CA). The cDNAs encoding each human RAMP (RAMP1, RAMP2, and RAMP3) were amplified with Expand High Fidelity Enzyme blend (Roche) using a forward primer that introduced four nucleotides (CACC) immediately before the ATG initiation codon and a reverse primer that removed the receptor's natural stop codon. The PCR products were subcloned into the pENTR/D-TOPO vector using the pENTR directional TOPO Cloning Kit (Invitrogen), following the manufacturer's recommendations. The destination vectors were generated using the GATEWAY Vector conversion system, as previously described (15). The recombination reactions between pENTR-specific receptor cDNA and the destination vectors (pCR3.1-Rlu-dest and pEYFP-N1-dest) were performed using the LR recombinase kit. Additional RAMP constructs were prepared by inserting the amino- or carboxyl-terminal YFP fragments, YFP(1−158) (YN) or YFP(159−238) (YC), respectively, into the *Bgl*II/*Hin*dIII restriction sites before the TGA stop codon. The Δ(10−100) RAMP3 construct was created by inserting two XbaI sites after the relevant codons using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The nucleotide sequence between these sites was excised to yield RAMP3 sequences lacking 90 amino acids.

Amino- and carboxyl-terminal-truncated secretin receptor constructs, Rlu- and YFP-tagged human secretin receptor (SecR) constructs, the CLR receptor construct, and an Rlu-tagged CTR construct have been prepared previously (16, 18). Cmyc-tagged secretin receptor was prepared by primer extension PCR. cDNAs for N-ethylmaleimide-sensitive factor (pCMV6-NSF) and sodium−hydrogen exchange regulatory factor (pCMV6-NHERF1) were purchased from Origene (Rockville, MD). The secretin receptor mutant in which Gly^{241} within TM4 was replaced with Cys (G241C) was prepared by oligonucleotide-directed mutagenesis. All sequences were confirmed by direct DNA sequencing.

Chimeric secretin-GLP1 receptor constructs were prepared by fusion of complementary fragments of the two receptors, replacing segments of the secretin receptor with corresponding segments of the GLP1 receptor, again verifying the resulting sequences. Constructs consisted of the following: $(SecR_{1-124})$-$(GLP1R_{121-230})(SecR_{223-419})$ substituting the TM1-TM3 region of the secretin receptor with the corresponding region of the GLP1 receptor, denoted $Sec(GLP1_{TM1-3})R$; $(SecR_{1-230})$-$(GLP1R_{232-311})(SecR_{302-419})$ substituting the TM4-TM5 region of the secretin receptor with the corresponding region of the GLP1 receptor, denoted $Sec(GLP1_{TM4-5})R$; and $(SecR_{1-309})$-$(GLP1R_{319-388})(SecR_{377-419})$ substituting the TM6-TM7 region of the secretin receptor with the corresponding region of the GLP1 receptor, denoted $Sec(GLP1_{TM6-7})R$. Each of these constructs expressed in intact cells was shown to bind secretin and to signal normally, establishing their abilities to be synthesized and to traffick normally to the cell surface where their architecture was intact and able to couple with G_s (see data below).

Peptides. Synthetic peptides corresponding to each of the predicted transmembrane segments of the human secretin receptor were synthesized as described previously (17). This was achieved by manual solid-phase techniques using Pal resin (Advanced Chem Tech) and FMOC-protected amino acids. The peptides were purified to homogeneity using reversed-phase HPLC with an octadecylsilane reversed-phase column. Resulting peptides had their identities verified by mass spectrometry. For use, they were solubilized in dimethyl sulfoxide (DMSO) prior to dilution with KRH medium to yield a final concentration of 0.1% DMSO, with this concentration of DMSO shown to have no effect in the relevant assays.

Cell Culture and Transfection. African green monkey kidney (COS) cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA) were propagated in tissue culture plasticware in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% Fetal Clone II (Hyclone laboratories, Logan, UT) in a humidified environment including 5% CO_2. They were passaged approximately twice per week. For interaction studies, cells were transfected using the modified diethylaminoethyl (DEAE)-dextran method (17) and were studied approximately 72 h after transfection. For functional studies, COS cells were seeded at a density of 30 000 per well in a 96-well plate using DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) 24 h prior to transfection. Transfection of cmyc-tagged secretin receptor, RAMPs, NSF, and NHERF1 plasmids was performed according to cell type-specific recommendations in the Metafectine transfection reagent manual (Biontex Laboratories, GmbH). Chinese hamster ovary (CHO) K1 cells (American Type Culture Collection) were cultured in complete media (5% FBS, DMEM)

and seeded into 75 cm^2 culture flasks to achieve approximate 90% cellular confluency the next day. These cells were then transfected with cmyc-secretin receptor and human RAMP1, RAMP2, or RAMP3 DNA. DNA lipid complexes were formed in 1 mL of serum-free OPTIMEM1 (Invitrogen, Carlsbad, CA) using 1.6 μg of human secretin receptor and 11.4 μg of human RAMPs or pcDNA3.1 as mock control, along with 60 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 45 min, DNA−lipid complexes were added to flasks of cells already containing 4 mL of serum-free OPTI-MEM1. The cells were left to be transfected overnight or for 15 h. Cells were then recovered for 8 h in complete medium and subsequently passaged and seeded into 96-well plates and 25 cm^2 culture flasks to achieve 60−90% confluency for use the following day in functional assays.

Confocal Fluorescence Microscopy. Possible RAMP association with GPCRs was studied in a morphologic translocation assay in which fluorescent RAMP was evaluated for movement from the endoplasmic reticulum to the plasma membrane. In this assay, COS cells were transiently transfected with both the RAMP and GPCR constructs. After 24 h, the cells were seeded onto UV-sterilized 25-mm coverslips, washed with phosphate-buffered saline (PBS), and fixed in 2% (w/v) paraformaldehyde (in PBS) for 30 min at room temperature. Cells were then washed with PBS before being mounted on a microscope slide using Vectashield. YFP fluorescence was observed using a Zeiss LSM 510 confocal microscope (Thornwood, NY) with excitation, 488 nm argon laser; emission, LP505 filter; pinhole diameter 2.6 airy units, Plan-Apochromat 63X/1.4NA oil. Background-subtracted images were collected and assembled using Adobe Photoshop 7.0 (Mountain View, CA). Fluorescent labeling of secretin receptors on receptor-expressing cells were carried out by incubating the cells with 50 nM secretin(1−27)-Gly-Cys-Alexa^{488} (Alexa-secretin) (*19*) in PBS containing 0.08 mM CaCl$_2$ and 0.1 mM MgCl$_2$ at 4 °C for 90 min. After the incubation, the cells were washed twice with ice-cold PBS and fixed with 2% paraformaldehyde priior to being mounted on a slide using Vectashield. The Alexa fluorescence was observed using an epifluorescence Zeiss microscope with an FITC filter set (excitation, 480/20 nm; dichroic mirror, Q515 lp; and emission, 535/30 nm).

BRET Studies. Molecular associations were further evaluated using a bioluminescence resonance energy transfer (BRET) assay. In this, measurements were performed with a suspension of approximately 25 000 COS cells expressing relevant fluorescently tagged constructs in 96-well white Optiplates, as described previously (*17*). The measurements were initiated by adding the cell-permeant *Renilla* luciferase-specific substrate, coelenterazine *h* (Biotium, Hayward, CA), to achieve a final concentration of 5 μM. The resultant BRET signals were collected using the 2103 Envision fluorescence plate reader (PerkinElmer, Wellesley, MA) set up with the < 700 nm mirror and with emission filter sets for luminescence (460 nm, bandwidth 25 nm) and fluorescence (535 nm, bandwidth 25 nm). The BRET ratios were calculated based on the ratio of emissions, as described previously (*17*).

Saturation BRET experiments were performed for validation of the BRET signals, as described previously (*17*). Here, COS cells were transfected with a constant amount of donor construct (Rlu-tagged construct at 1.0 μg DNA/dish) and with increasing amounts of acceptor construct (YFP-tagged construct, ranging from 0.3 μg to 6 μg DNA/dish). BRET assays were performed 48 h after transfection. The BRET data were analyzed and were evaluated for quality-of-fit based on R^2 values using Prism 3.0 (GraphPad Software, San Diego, CA).

Bimolecular Fluorescence Complementation. Bimolecular fluorescence complementation assays were carried out in COS cells transiently expressing YN- and YC-tagged constructs. The COS cells were transfected with equimolar concentrations of YN-tagged secretin receptor and YC-tagged RAMP3 constructs. Twenty-four hours after transfection, the cells were lifted using 0.05% trypsin and were plated onto UV-sterilized coverslips and allowed to grow for 48 h before being fixed and mounted on a microscopic slide for fluorescence microscopy using standard YFP settings, as described above.

Functional Assays. Cells were harvested 16 h after transfection and seeded for use in cAMP, ERK1/2 phosphorylation, and calcium mobilization assays, or for antibody-binding experiments. Cells were allowed to adhere for 16 h, followed by serum-starvation for an additional 24 h prior to use in the functional assays.

Measurement of cAMP. Intracellular cAMP levels were determined using either AlphaScreen methodology with Fusion plate reader (PerkinElmer, Wellesley, MA) (*20*) or LANCE methods with Envision plate reader (PerkinElmer) (*17*), as have been fully described and validated in the respective laboratories previously. Each data point was assayed in duplicate, and the quantity of cAMP generated was calculated from the raw data using a cAMP standard curve. Results are expressed as the means ± SEM of data from three independent experiments.

Calcium Mobilization Assay. Transfected cells were seeded in poly-L-lysine-coated 96-well plates at a density of 50 000 cells/ well, incubated overnight, and serum-starved for an additional 24 h. Cells were washed three times with a modified Hanks buffered-saline solution (HBSS) (containing (in mM): NaCl, 150; KCl, 2.6; MgCl$_2$, 1.18; D-glucose, 10; HEPES, 10; CaCl$_2$, 2.2; probenecid, 2 and 0.5% (w/v) BSA). In light-diminished conditions, 100 μL of wash solution was added containing the cell-permeant calcium fluorophore, Fluo-4/AM (10 μM) and incubated for 1 h at 37 °C. The fluorophore solution was aspirated from the wells, and cells were washed twice then incubated for 30 min in modified HBSS at 37 °C. The assay plate was transferred to a FlexStation (Molecular Devices, Palo Alto, CA), which performed the robotic addition of ligands (10× stocks in modified HBSS). Receptor-mediated changes in intracellular calcium concentration were immediately recorded by the Flex-Station using an excitation wavelength of 485 nm and emission wavelength of 520 nm. Data were collected for each well every 1.52 s for a total of 135 s.

ERK1/2 Phosphorylation Assay. Transfected cells were seeded in 96-well plates at a density of 50 000 cells/well, and incubated for 16 h prior to being serum-starved overnight. On the day of assay, cells were pretreated with buffer or inhibitors (at the concentrations specified) and then stimulated with agonist at 37 °C. Time-course results demonstrated a peak response at 10 min for all receptor complexes following agonist stimulation; this time point was subsequently used in concentration−response studies. ERK1/2 phosphorylation was measured using the AlphaScreen-based ERK1/2 SureFire assay kit as previously described (*21*). Data are expressed as the percent response relative to stimulation with 10% FBS.

Determination of Cell-Surface Expression of Receptors Using Enzyme-Linked Immunosorbent Assay. COS and CHO-K1 cells were transfected as described above. All receptor constructs incorporated a cmyc epitope tag at the amino terminus,

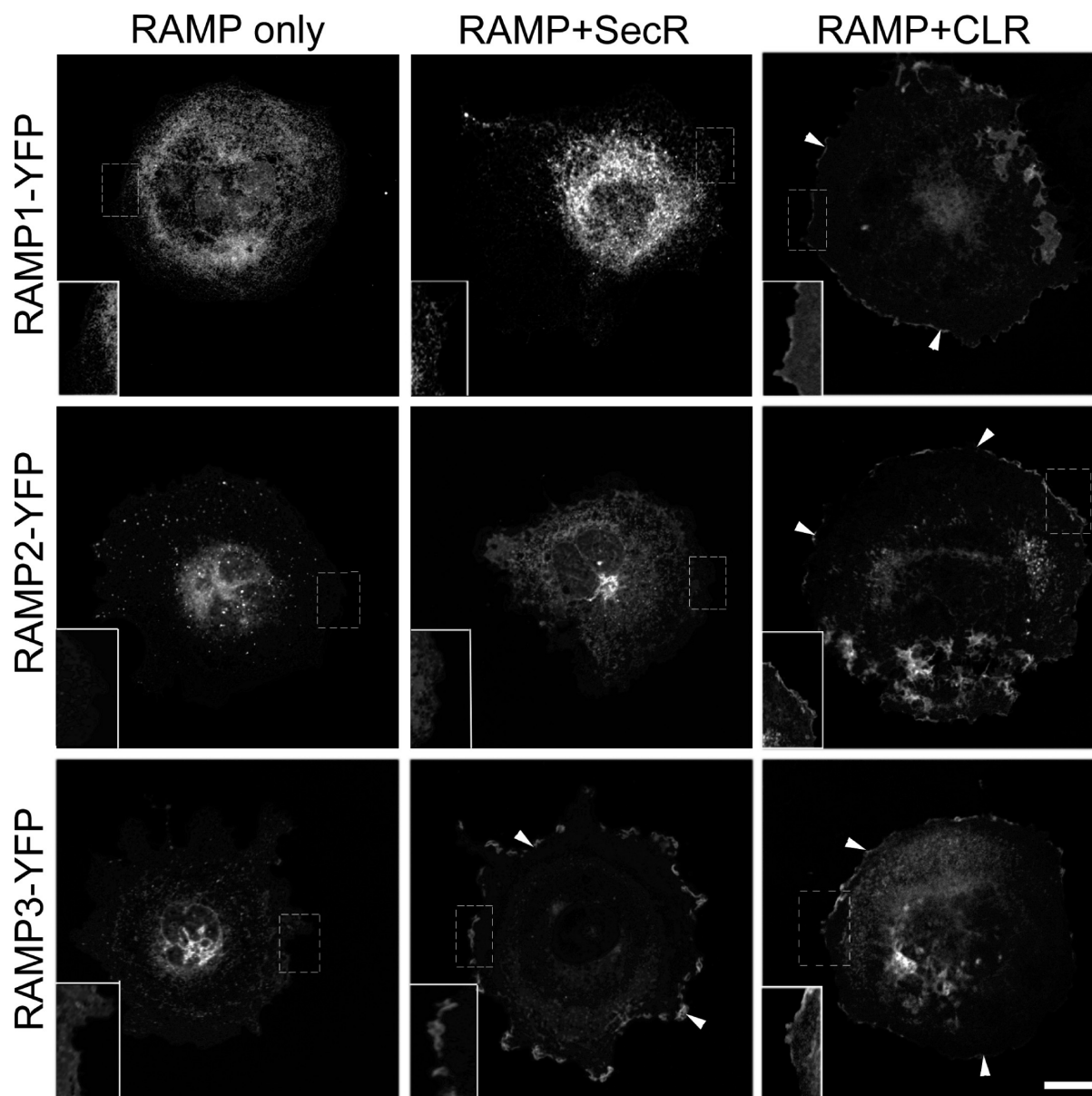## RAMP only   RAMP+SecR   RAMP+CLR

FIGURE 1: Morphologic assays for RAMP translocation to the plasma membrane. Shown are fluorescence images of the expression of each type of RAMP in COS cells, all localized to biosynthetic compartments, particularly representing the endoplasmic reticulum (left column). The right column shows the changes in distribution of the fluorescence when the calcitonin receptor-like receptor was coexpressed with each YFP-tagged RAMP. In all three conditions, there was significant translocation to the plasma membrane (highlighted with arrowheads). The middle column shows the changes in distribution of the fluorescence when the secretin receptor was coexpressed with each RAMP. Here, only RAMP3 distribution was changed, with significant translocation to the plasma membrane (highlighted with arrowheads). Insets show the boxed regions at higher magnification. The images shown are representative of four similar experiments. Bar 25 μm.

which enabled cell-surface expression to be determined by enzyme-linked immunosorbent assay as described previously (*22*). Results were normalized against data from nontransfected cells. All experiments were performed in quadruplicate.

Internalization Assay. Cells transfected with cmyc-secretin receptor, RAMPs, NSF or NHERF1 were used for an enzyme-linked immunosorbent assay-based internalization assay in which the surface expression of the secretin receptor was assessed at 1, 2, 3, 4, 6, 8, 10 s and then at 5 min intervals for 60 min after exposure to 100 nM secretin. Internalization profiles of cells transfected with cmyc-secretin and RAMPs were compared in the presense or absence of either NSF or NHERF1.

Data Analysis. cAMP, ERK1/2 phosphorylation, and calcium mobilization concentration−response data were analyzed via nonlinear regression using PRISM version 5 (GraphPad

Software, San Diego, CA). In all instances, data points are shown as the mean $\pm$ SEM.

RESULTS

Cellular Translocation Assay for Possible Interactions between RAMPs and the Secretin Receptor. The classical assay for RAMP association with a GPCR involves the demonstration of the ability of the receptor to translocate the fluorescent RAMP from the biosynthetic compartment to the plasma membrane (*4*, *7*). We performed this assay with COS cells transiently expressing fluorescent RAMPS and the secretin receptor, using CLR (known to associate with all three RAMPs) as a positive control. Shown in Figure 1 are COS cells transfected with YFP-tagged RAMP constructs in the absence or presence of GPCR constructs. In the absence of receptor cotransfection (left

column), the fluorescent RAMPs all remained in the biosynthetic compartments, particularly the endoplasmic reticulum. Following, cotransfection with CLR (right column), the fluorescent RAMP signals were clearly observed at the plasma membrane, demonstrating the known effect of association between the RAMPs and that receptor. Analogous experiments with the secretin receptor (middle column) generated a similar fluorescence signal at the plasma membrane for RAMP3, but none for RAMP1 or RAMP2. This suggests that the secretin receptor can interact in a structurally specific manner with RAMP3. This had not previously been observed or reported.

BRET Studies of Possible RAMP Interactions with the Secretin Receptor. We also utilized resonance energy transfer studies to examine the possible interactions between RAMPs and the secretin receptor (Figure 2A). In these studies, Rlu-tagged secretin receptor was used as donor and YFP-tagged RAMP constructs were used as possible acceptors. The well-established interactions between the calcitonin receptor and all three RAMPs (*3*) were used as positive controls in this series of studies. Indeed, in this assay, the calcitonin receptor expressed with RAMP1, RAMP2, or RAMP3 each generated a strong BRET signal above background; the latter established by the coexpression of RAMP-Rlu with cytosolic YFP. Of note, a similar positive BRET signal was generated by the coexpression of the secretin receptor with RAMP3. In contrast, the secretin receptor coexpressed with RAMP1 or RAMP2 did not produce a significant BRET signal above background. These data confirm the apparent structurally significant interaction between the family B G protein-coupled secretin receptor and RAMP3 that was suggested by the cellular translocation assay data.

The static BRET assay results were further validated using saturation BRET studies. Saturation BRET studies were performed to distinguish a specific and saturable molecular interaction between the secretin receptor and RAMP3, in contrast to a random molecular association between the donor and acceptor in a crowded cellular compartment. Figure 2B shows the BRET signals typical of a specific interaction in COS cells expressing a fixed concentration of donor construct (SecR-Rlu) with increasing amounts of acceptor construct (RAMP3-YFP), with the BRET signal increasing and then reaching a plateau. This was similar to the positive control that was generated when the calcitonin receptor was coexpressed with RAMP1, RAMP2, or RAMP3. In contrast, the BRET signals reflecting possible secretin receptor interaction with RAMP1 were not different from a linear fit through the origin, as is typical of a nonspecific interaction.

The agonist-dependency of the interaction between the secretin receptor and RAMP3 was also studied using an analogous BRET assay (Figure 2C). There was no significant effect of ligand occupation on the signal in this assay using stimulation with secretin concentrations as high as 1 μM.

Bimolecular Fluorescence Complementation to Demonstrate Secretin Receptor–RAMP3 Interactions. The bimolecular fluorescence complementation approach was used to further demonstrate the specific interaction between the secretin receptor and RAMP3 (Figure 3). RAMP3-YFP expressed in the absence of receptor transfection was observed to reside in cellular biosynthetic compartments, particularly the endoplasmic reticulum. When RAMP3 was tagged with YN or YC, no fluorescence was observed. However, when RAMP-YN and RAMP-YC were coexpressed, the fluorescence pattern was similar to that of RAMP3-YFP, consistent with the ability of RAMP molecules
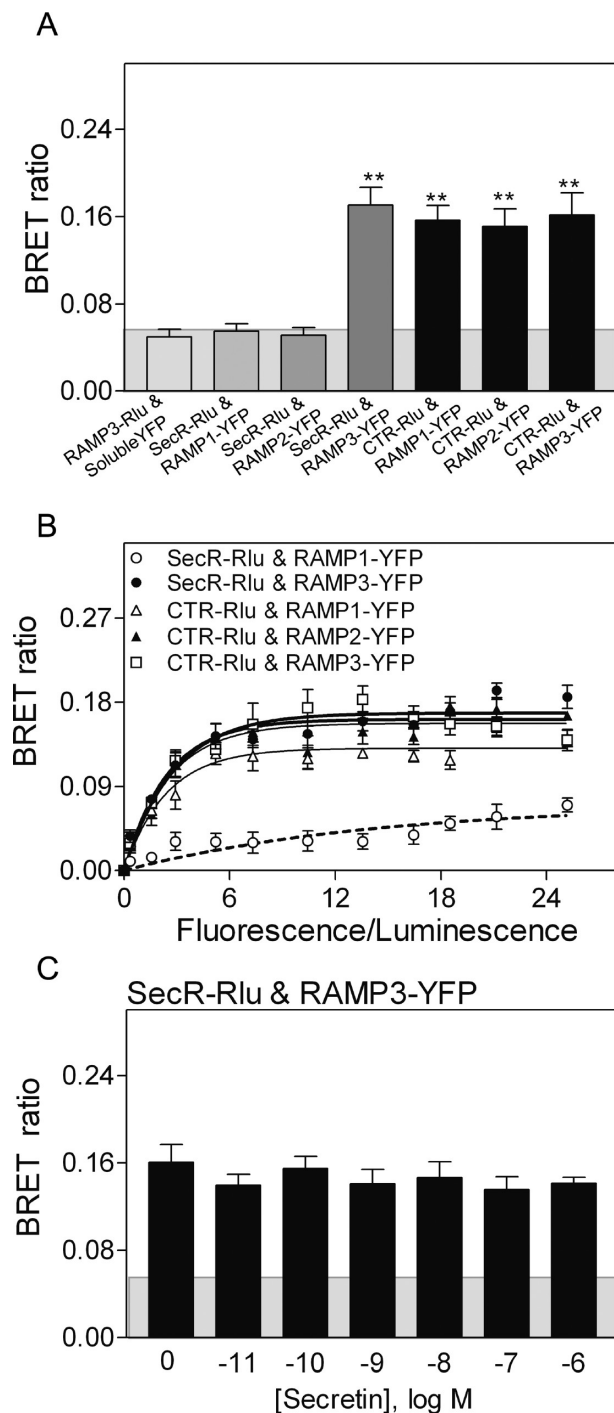
FIGURE 2: BRET analysis of association between RAMPs and the secretin receptor. Shown are BRET signals in static and saturation assays evaluating the potential interaction of the secretin receptor and RAMPs. In the static assays shown in panel A, background BRET signals were determined by expression of the Rlu-tagged RAMPs and soluble YFP. The coexpression of YFP-tagged RAMP1 and RAMP2 produced only background BRET. In contrast, coexpression of RAMP3 produced a significant BRET signal. This was similar to that produced by the positive control, the coexpression with the Rlu-tagged calcitonin receptor. Panel B shows the saturation BRET data, confirming the significance of the signal generated by coexpression of RAMP3 and the secretin receptor. In panel C, the effects of increasing concentrations of secretin on the BRET signal were studied. The cells were incubated with specified concentrations of secretin at 37 °C for 5 min before adding the coelenterazine h (5 μM) to initiate the BRET signal. There was no significant effect of agonist on secretin receptor-RAMP3 interaction. The values plotted represent means ± SEM of data from five independent experiments. ** $p < 0.001$ significantly above background BRET signals.
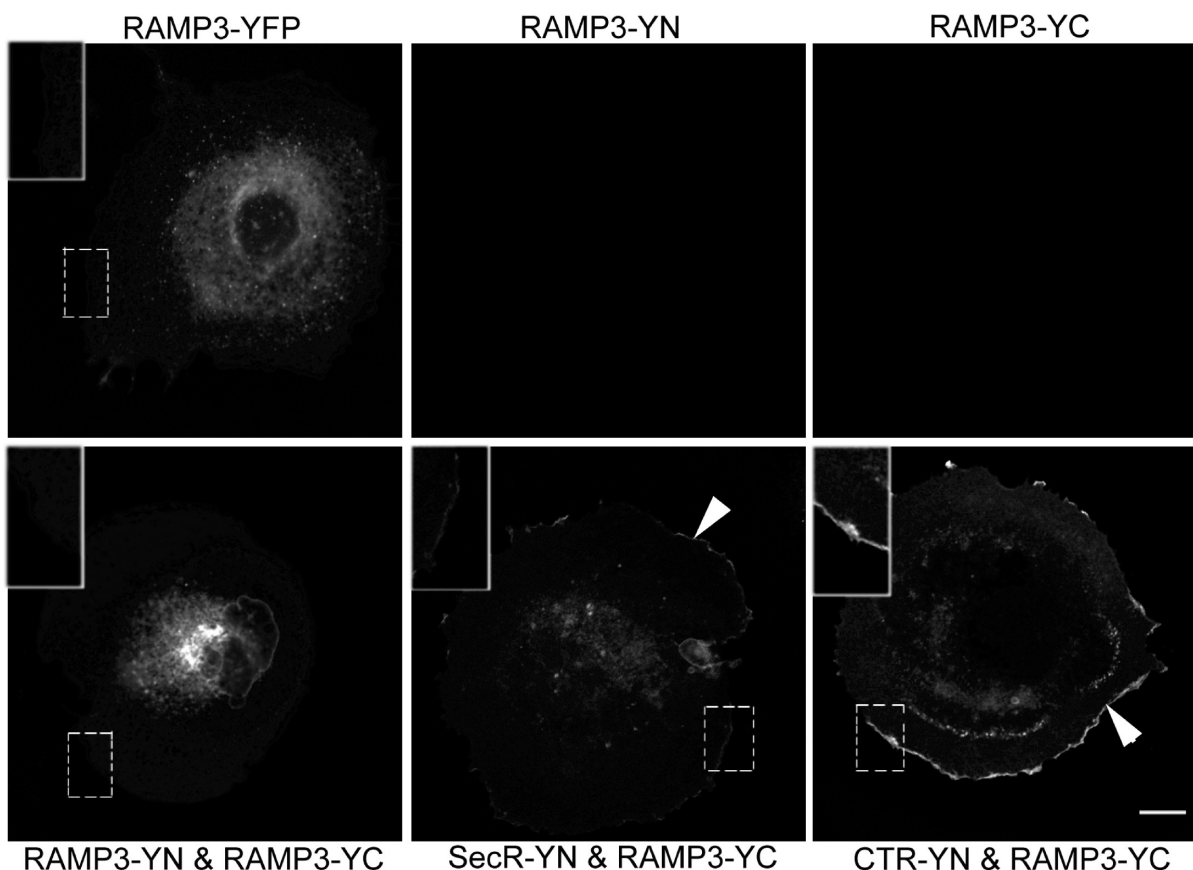
FIGURE 3: Bimolecular fluorescence complementation assays. Shown are typical fluorescence images of COS cells expressing the YFP-, YN-, and YC-tagged constructs, as noted. Significant fluorescence was noted at the cell surface (highlighted with arrowheads) when YC-tagged RAMP3 was coexpressed with the calcitonin receptor tagged with YN or with the secretin receptor tagged with YN. RAMP dimerization in the intracellular biosynthetic compartments was observed when RAMP-YN and RAMP-YC were coexpressed. Insets show the boxed regions at higher magnification. Bar 25 μm.
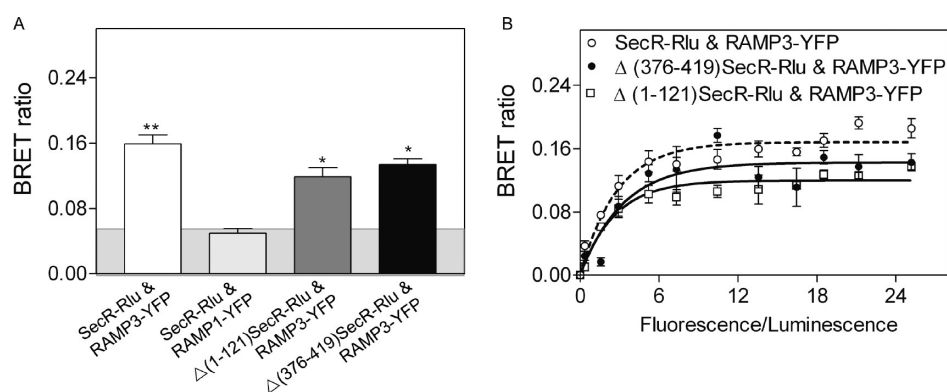

FIGURE 4: BRET analysis of secretin receptor−RAMP3 interaction using truncated receptor constructs. Shown are the static (A) and saturation (B) BRET signals obtained from COS cells coexpressing Rlu-tagged truncated secretin receptor with YFP-tagged RAMP3. Significant BRET signals above nonspecific (shown in the shaded area) are marked *$p < 0.01$, **$p < 0.001$. The values plotted represent means ± SEM of data from five independent experiments.

to dimerize, as has been reported (*3*), yet this signal was limited to the intracellular biosynthetic compartments. When the nonfluorescent SecR-YN construct was coexpressed with the RAMP3-YC construct, a fluorescent signal was observed at the level of the plasma membrane. This pattern of response was similar to that observed when analogous studies were performed with the known RAMP3 partner, the calcitonin receptor, representing a positive control for this assay.

Exploring the Region of the Secretin Receptor That Contributes to Its Interaction with RAMP3. To explore

the molecular basis for the interaction between the secretin receptor and RAMP3, BRET and cellular translocation studies were performed with truncated receptor constructs. In the static and saturation BRET studies, secretin receptor constructs with truncation of the amino-terminal region (residues 1−121) or truncation of the carboxyl-terminal region (residues 376−419) displayed similar levels of energy transfer to those observed with the intact wild type secretin receptor, suggesting that the terminal regions were not critical for RAMP association with this receptor (Figure 4A,B). Morphological translocation studies of
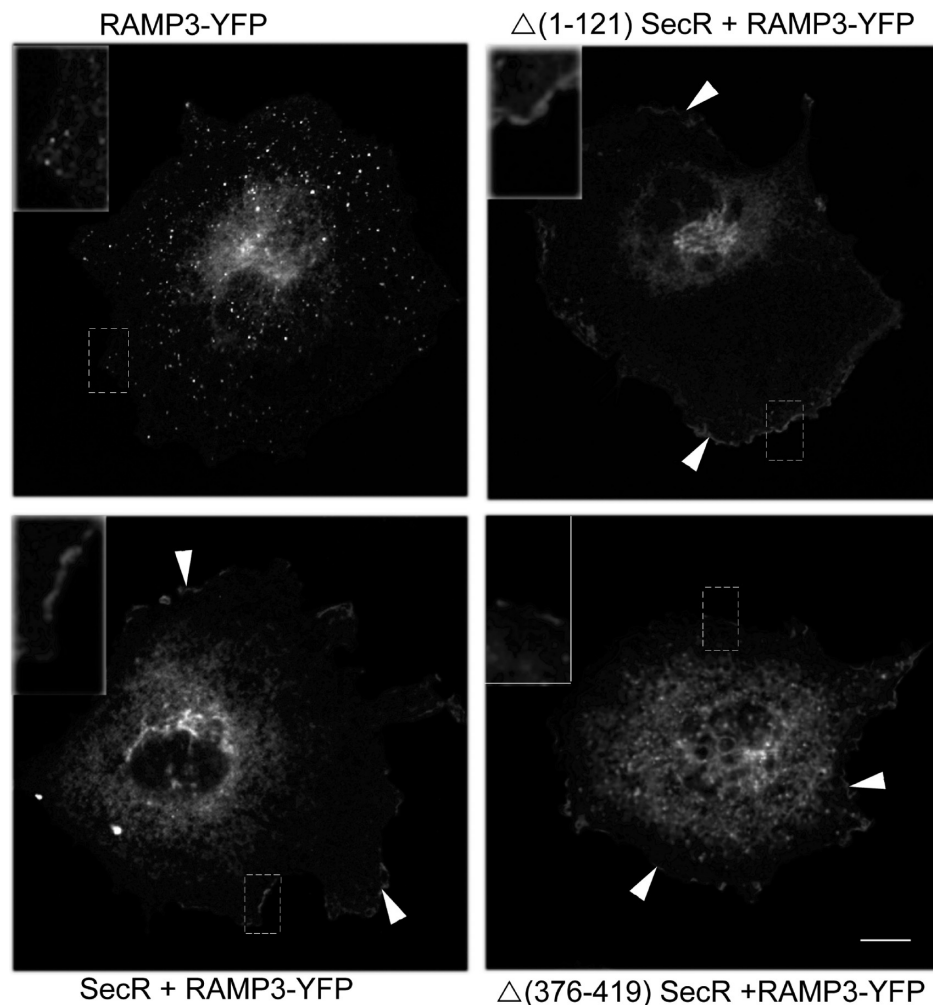
FIGURE 5: Morphological RAMP translocation assay. Shown are representative confocal microscopic images of COS cells expressing either YFP-tagged RAMPs alone or coexpressed with wild type or amino-terminal region ($\triangle 1-121$) truncated or carboxyl-terminal region ($\triangle 376-419$) truncated secretin receptor constructs. Each of the truncated secretin receptor constructs translocated the YFP-tagged RAMP3 to the cell surface (arrowheads) similar to wild type secretin receptor. These images shown are representative of four similar experiments. Insets show the boxed regions at higher magnification. Bar 25 μm.

fluorescently tagged RAMP3 were also consistent with the BRET data (Figure 5).

The possible contribution of the transmembrane (TM) helical bundle region of the secretin receptor to its interaction with RAMP3 was explored using a different experimental strategy. In this, peptides representing each of the TM segments were used to compete for a critical epitope in the bimolecular fluorescence complementation assay with YN-tagged RAMP3 and YC-tagged secretin receptor that normally yields fluorescence at the level of the plasma membrane. When a critical epitope for interaction between the secretin receptor and RAMP3 would be delivered to this system, the plasma membrane fluorescence would be expected to be reduced or eliminated. Indeed, as observed in Figure 6, this was the case for the peptides encompassing TM6 and TM7. Each of the other secretin receptor TM segment peptides elicited no competitive disruption of this signal.

To further confirm these observations, receptor chimeras were created between the GLP1 receptor that is known to not associate with any RAMP (7), and the secretin receptor. These chimeric receptor constructs trafficked normally and were structurally and functionally intact, as reflected by their abilities to bind and signal in response to secretin. The following concentrations of secretin (nM) were able to stimulate half-maximal cAMP responses in

COS cells expressing the respective constructs, as determined by the LANCE methodology (17): SecR, 0.4 ± 0.15; Sec-$(GLP1_{TM1-3})$R, 4.4 ± 0.12; Sec$(GLP1_{TM4-5})$R, 5.9 ± 1.84; and Sec$(GLP1_{TM6-7})$R, 6.8 ± 1.1. Basal levels of cAMP in these cells were 2.1 ± 0.5 picomol per million cells, while maximal levels achieved (E_{max} values) represented the following for each construct were SecR, 178 ± 24; Sec$(GLP1_{TM1-3})$R, 153 ± 14; Sec$(GLP1_{TM4-5})$R, 149 ± 15; and Sec$(GLP1_{TM6-7})$R, 165 ± 15 picomol per million cells. As predicted by the competitive TM peptide approach, only the chimeric constructs incorporating the secretin receptor TM6 and TM7 regions were able to translocate the fluorescent RAMP3 construct (Figure 7). The chimeric secretin receptors incorporating TM1-TM3 and TM4-TM5 regions of the GLP1 receptor behaved like the wild type secretin receptor, transporting the fluorescent RAMP3 construct to the cell surface. However, when the TM6-TM7 regions of the GLP1 receptor were included in the chimeric construct, this RAMP remained intracellularly in the biosynthetic compartment.

Exploring the Region of RAMP3 That Contributes to Its Interaction with the Secretin Receptor. RAMP3 is a type I single transmembrane protein that has a large extracellular amino-terminal region and a very small intracellular carboxyl-terminal region. In this series of studies, most of the amino-terminal
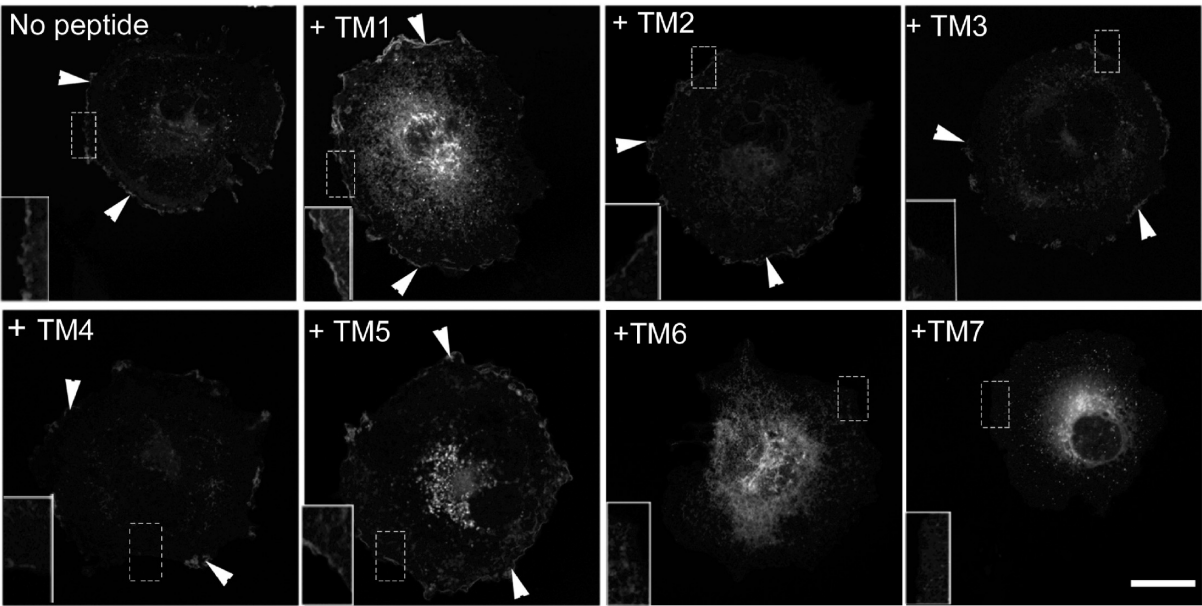
RAMP3-YN + SecR-YC

FIGURE 6: Morphological bimolecular fluorescence complementation assay of RAMP3 interaction with the secretin receptor, determining the ability of transmembrane peptides to competitively disrupt the complex. Shown are the representative confocal images of COS cells coexpressing YN-tagged RAMP3 and YC-tagged secretin receptor treated with or without the specified secretin receptor transmembrane peptides at a concentration of 40 μg/mL for 2 h prior to fixation of the cells. Cell surface fluorescence (highlighted with arrowheads) was competitively disrupted only by TM6 and TM7 peptides. Images shown are representative of images from three similar experiments. Insets show the boxed regions at higher magnification. Bar 25 μm.

Sec(GLP1$_{TM1-3}$)R   Sec(GLP1$_{TM4-5}$)R   Sec(GLP1$_{TM6-7}$)R
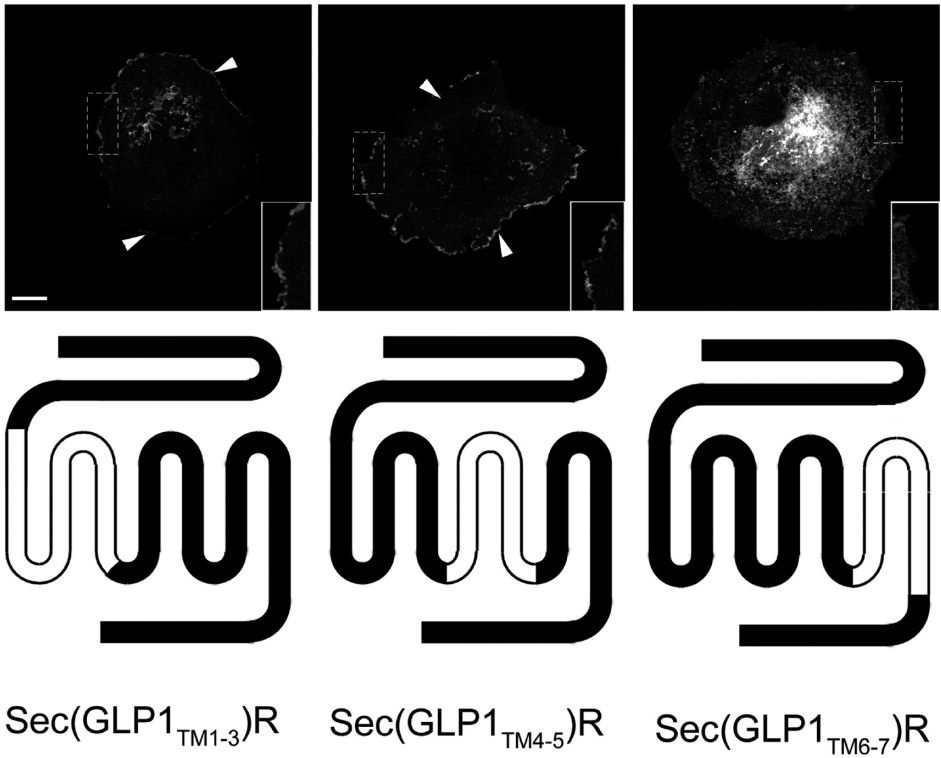
FIGURE 7: Morphologic fluorescence analysis of chimeric receptor association with RAMP3. Three chimeric secretin−GLP1 receptor constructs were studied for their abilities to translocate the fluorescent RAMP3 construct to the transfected COS cell surface. Sec(GLP1$_{TM1-3}$)R and Sec(GLP1$_{TM4-5}$)R behaved like wild type secretin receptor, associating with this RAMP and translocating it to the COS cell surface. However, Sec(GLP1$_{TM6-7}$)R did not have this effect, behaving instead like the wild type GLP-1 receptor. This supports the interpretation that the TM6 and TM7 regions of the secretin receptor are responsible for its association with RAMP3. Insets show the boxed regions at higher magnification. Bar 25 μm.

tail of RAMP3 was deleted (residues 10−100). This was studied in BRET and morphological translocation assays using YFP-tagged ($\triangle$10−100)RAMP3 and Rlu-tagged secretin receptor in

COS cells. Figure 8A,B shows that this RAMP3 deletion construct behaved like wild type RAMP3, suggesting that the amino terminus does not contribute significantly to the molecular
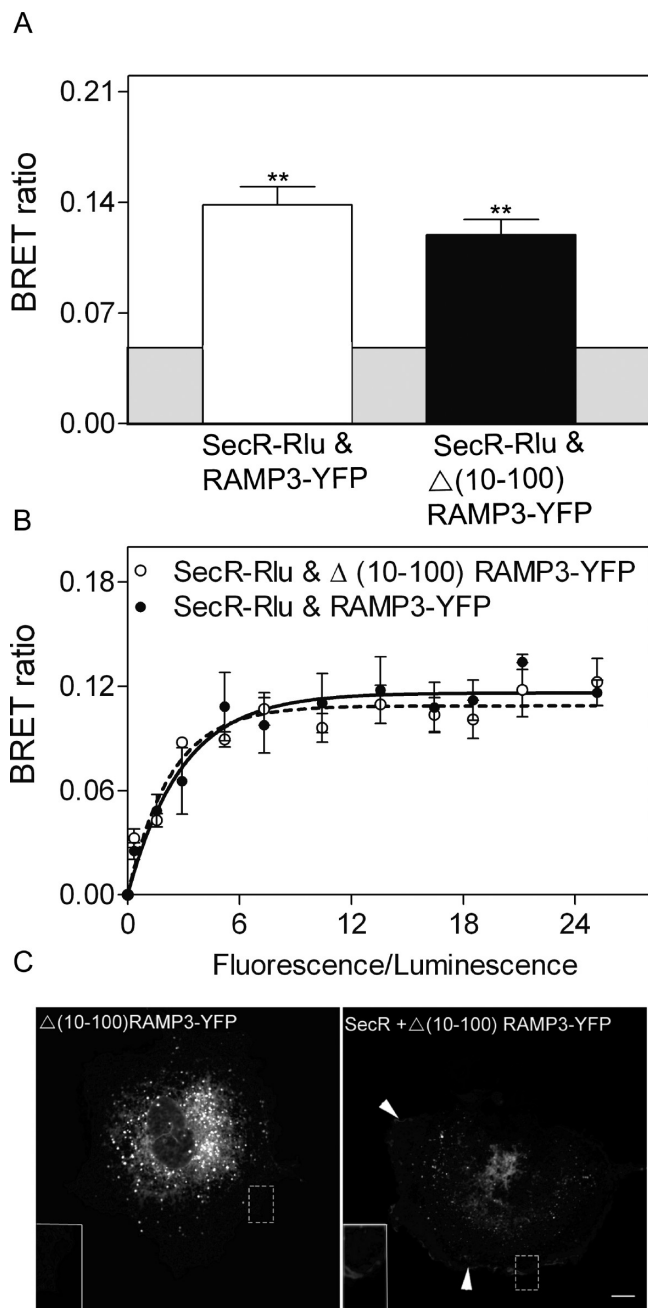
A

B

C

FIGURE 8: BRET analysis of secretin receptor−RAMP3 interaction using the truncated RAMP construct. Shown are the static (A) and saturation (B) BRET signals obtained from COS cells coexpressing Rlu-tagged secretin receptor with YFP-tagged amino-terminally truncated RAMP3. Significant BRET signals above nonspecific (shown in the shaded area) are marked **$p < 0.001$. The values plotted represent means ± SEM of data from five independent experiments. Also shown are representative images from morphological translocation assay (C). Confocal microscopic images were collected from COS cells coexpressing YFP-tagged truncated RAMP3 with untagged secretin receptor. The truncated RAMP3 was able to translocate the secretin receptor to the cell surface (arrowheads). The data marked **$p < 0.001$ level in figure A represent a significant BRET signal above the nonspecific signal shown in the shaded area. Insets show the boxed regions at higher magnification. The values plotted represent means ± SEM of data from five independent experiments. Bar 25 μm.

association. This was further supported by the morphologic translocation assay where ($\triangle10-100$)RAMP3 retained the ability to translocate to the cell surface when coexpressed with the secretin receptor (Figure 8C).

Functional Assays. RAMP-receptor interaction, in a receptor-specific and RAMP-specific manner, has been proposed to alter ligand recognition (*4, 6*), signal strength and preference (*7, 23*), and receptor internalization and recycling (*24, 25*). We therefore sought to determine if interaction of RAMP3 with the secretin receptor could alter gross receptor function in signaling and internalization assays, in two cellular backgrounds (COS and CHO-K1 cells). It must be remembered, however, that the secretin receptor is capable of trafficking normally to the plasma membrane even in the absence of RAMPs. Thus, unlike the early functional assays using CLR where only RAMP-associated receptor reaches the plasma membrane, here RAMP-free and RAMP3-associated secretin receptor are present on the cell surface. In COS cells, RAMP3 coexpression did not alter secretin-induced increases in cAMP production, intracellular calcium mobilization, or phosphorylation of ERK1/2 (Table 1). Similarly, transient coexpression of the secretin receptor with each individual RAMP failed to elicit any specific change in these parameters, except for RAMP1 where a decrease in secretin potency in the COS cell cAMP assay was observed (Table 1) (*20*). No difference in intracellular calcium mobilization or cAMP response was observed in CHO-K1 cells upon cotransfection with any of the RAMPs (Table 1). The lack of a consistent effect by RAMP1 on cAMP response across the two cellular backgrounds suggests that the effect in COS cells is a nonspecific response of these cells, potentially via altered $G_{\alpha s}$ levels. We further monitored internalization of cmyc-tagged secretin receptor in response to 100 nM secretin peptide. In CHO-K1 cells expressing the secretin receptor alone, secretin induced a rapid internalization of the receptor, with a $t_{1/2}$ of ~1.5 min. Coexpression of individual RAMPs did not alter the rate or extent of receptor internalization (Figure 9A). Furthermore, additional coexpression of either NSF, or NHERF1, which modify the internalization or recycling of the RAMP3-based adrenomedullin 2 receptor (*24, 25*), failed to affect secretin receptor internalization, either alone or in the presence of RAMP3 (Figure 9B, C). Cell surface expression of the cmyc-tagged secretin receptor, as determined by enzyme-linked immunosorbent assay, was not significantly different between receptor expressed alone or receptor expressed in the presence of RAMPs (data not shown).

Additional studies were performed to further explore potential function of the RAMP3−secretin receptor association. In the first series of these studies, a secretin receptor mutant in which Gly[241] in TM4 was replaced with Cys (G241C), resulting in a receptor mutant that did not traffick normally to the plasma membrane. This construct was trapped in the biosynthetic machinery, likely the endoplasmic reticulum (*26*). In the presence of RAMP3, this defect was partially corrected, resulting in more receptor trafficking to the plasma membrane and the emergence of demonstrable biological activity in response to secretin stimulation in intact cells (Figure 10). Furthermore, the transfection of increasing amounts of the secretin receptor along with constant amounts of CLR and RAMP3 resulted in attenuation of the adrenomedullin response (Figure 11), with a parallel loss of CLR at the cell surface as monitored by ELISA (data not shown). This suggests that the secretin receptor was able to compete with CLR for RAMP3, resulting in less CLR-RAMP3 adrenomedullin receptor complex at the cell surface.

DISCUSSION

RAMPs were first discovered based on their function in an expression cloning strategy, seeking a calcitonin gene-related peptide (CGRP) receptor (*4*). It ultimately became clear that an

Table 1: Impact of RAMP Expression on Secretin-Stimulated Biological Responses at the Secretin Receptor[a]

	EC_{50} (nM)				E_{max}[b] (cAMP, nM; Ca^{2+}, RFU; ERK1/2, % FBS 10%)			
	SecR				SecR			
	SecR	+ RAMP1	+ RAMP2	+ RAMP3	SecR	+ RAMP1	+ RAMP2	+ RAMP3
COS Cells								
cAMP	0.3 ± 0.1	3.7 ± 1.0*	0.3 ± 0.1	0.2 ± 0.2	3392 ± 154	4477 ± 162	4466 ± 55	4026 ± 283
Ca^{2+}	2.4 ± 1.0	2.4 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	33404 ± 151	35598 ± 166	36261 ± 133	36082 ± 134
ERK1/2	3.6 ± 1.0	2.0 ± 0.1	2.4 ± 1.0	2.3 ± 1.0	40 ± 0.2	44 ± 0.2	44 ± 0.2	47 ± 0.2
CHO-K1 Cells								
cAMP	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	81 ± 6	78 ± 8	73 ± 5	74 ± 6
Ca^{2+}	0.9 ± 0.3	0.9 ± 0.2	0.7 ± 0.1	0.8 ± 0.2	10104 ± 1516	12365 ± 2264	10768 ± 1958	12378 ± 1551

[a]Shown are the responses to secretin in COS cells and CHO-K1 cells transiently expressing the cmyc-tagged secretin receptor either alone, or in the presence of individual RAMPs. Shown are cAMP (20), intracellular calcium, and phospho-ERK1/2 responses to secretin stimulation for 10 min. Values represent means $\pm$ SEM of data from three to five independent experiments. No significant RAMP3-induced changes were observed for any of the responses studied. [b]Basal levels: In COS cells, cAMP: SecR 354 ± 97, +RAMP1 932 ± 53, +RAMP2 1182 ± 34, +RAMP3 824 ± 183; Ca^{2+}: SecR 343 ± 158, +RAMP1 369 ± 173, +RAMP2 487 ± 163, +RAMP3 433 ± 159; ERK1/2: SecR 0.43 ± 0.19, +RAMP1 0.44 ± 0.20, +RAMP2 0.44 ± 0.20, +RAMP3 0.48 ± 0.22. In CHO-K1 cells, cAMP: SecR 48 ± 3, +RAMP1 47 ± 2, +RAMP2 46 ± 1, +RAMP3 48 ± 5; Ca^{2+}: SecR 2976 ± 1100, +RAMP1 1307 ± 270, +RAMP2 1025 ± 113, +RAMP3 1621 ± 206.

accessory protein was necessary to be combined with the calcitonin receptor-like receptor (CLR) to facilitate its glycosylation and trafficking to the cell surface where it could bind and be activated by CGRP. This accessory protein was the first RAMP. This same type of survey was subsequently successful in identifying RAMP partners for the calcitonin receptor (CTR) and for the CLR, to provide molecular complexes that respond to amylin or adrenomedullin as well.

With both of these early recognized RAMP partners identified as family B GPCRs, many other members of the family have been screened for RAMP association. This was largely dependent on a fluorescent RAMP translocation assay in which receptor interaction with the RAMP, in transit through the biosynthetic compartments, leads to translocation of the RAMP to the plasma membrane. Indeed, this approach has been successful in demonstrating RAMP- and receptor-specific interactions where the PTH1, PTH2, VPAC1, and glucagon receptors all associate with one or more RAMPs, while the VPAC2, GHRH, GLP1, and GLP2 receptors do not appear to associate with any RAMPs (7, 8). More recently, association of RAMP1 and RAMP3 with the calcium-sensing receptor has been reported (8). Despite this growing list of RAMP−receptor interactions, relatively little is known regarding the molecular mechanism(s) underlying the interaction. In the current study, we have identified the secretin receptor as a novel, specific partner for RAMP3 and have elucidated the interaction interface as TM6 and TM7 of the receptor.

To date, the best studied RAMP−GPCR interaction is that of CLR and RAMP1. For this receptor, a major component of the interaction interface is believed to be the amino-terminal domain of the receptor. Both the isolated amino-terminal domain of RAMP1 and the amino-terminal domain of RAMP1 linked to platelet-derived growth factor (PDGF) receptor transmembrane region are capable of eliciting cell surface expression of CLR and to engender a CGRP receptor phenotype (27). This is further supported by loss of cell surface expression of CLR following mutagenesis of specific aromatic amino acids in helix 3 of the RAMP1 amino terminus (1, 2). A similar interaction between CLR and RAMP2 and RAMP3 is also likely as mutation of conserved amino-terminal histidine residues in these proteins also disrupts the ability of RAMPs and CLR to translocate to the cell surface, although the specific role of individual histidines can differ between RAMPs (28). Nonetheless, the RAMP transmembrane region also played a contributory role in the interaction with CLR with both the isolated amino-terminal domain and the RAMP1−PDGF receptor chimera unable to fully reconstitute RAMP1 function (27).

The critical role of the amino-terminal domains of CLR and the RAMPs contrasts with the lack of importance of these domains for interaction between the secretin receptor and RAMP3 observed in the current study. Deletion of either the secretin receptor amino terminus or the RAMP3 amino terminus had little effect on trafficking of RAMP3 to the cell surface. Use of isolated receptor transmembrane peptides and chimeras between the secretin receptor and the GLP1 receptor refined the key site of interaction to TM6 and TM7. A key role for the transmembrane region of RAMPs in receptor interaction has also been observed for the CTR where chimeras of RAMP1 and RAMP2 implicated the transmembrane region as the key determinant of the difference in strength of induction of amylin receptor phenotype (29, 30), however, the site of this interaction within the CTR has not been determined.

Phenotypically, all GPCR−RAMP interactions identified to date enable cell surface translocation of intracellularly retained RAMPs, which is not surprising as this is the underlying tenet for most assays for interaction. However, despite their initial description as modulators of peptide-binding specificity in association with CLR (4), the alteration of binding profiles has only been observed for CLR and the closely related CTR (1, 2). For other interacting receptors, including the VPAC1 and PTH1 receptor, no effect on peptide affinity has been observed (7). A similar lack of effect by RAMP3 on secretin interaction with its receptor was found in the current study, with no apparent change in secretin potency across a broad range of assays including cAMP accumulation, intracellular calcium mobilization and phosphorylation of ERK1/2.
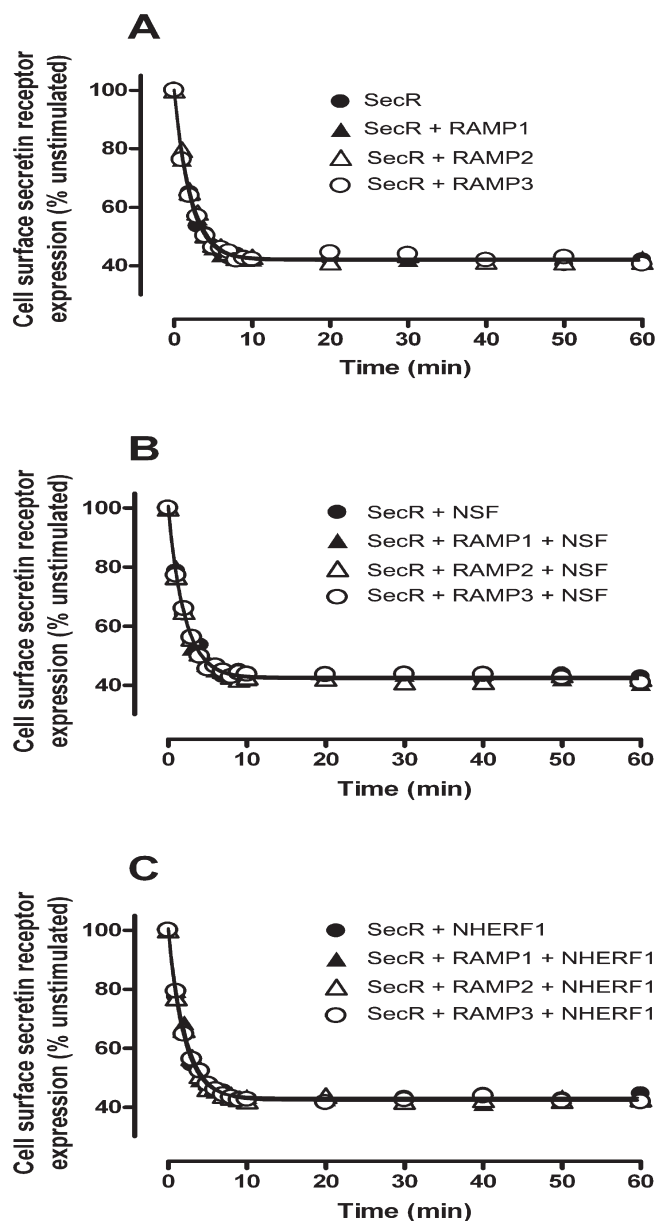
FIGURE 9: Internalization of cmyc-tagged secretin receptor following stimulation with secretin peptide (100 nM). Shown is internalization of the secretin receptor in transiently transfected CHO-K1 cells measured by loss of cell surface receptor expression in an anticmyc enzyme-linked immunosorbent assay. (A) secretin receptor internalization in the presence or absence of individual RAMPs, (B) secretin receptor internalization in the presence of NSF, in the presence or absence of individual RAMPs, and (C) secretin receptor internalization, in the presence of NHERF1, in the presence or absence of individual RAMPs. The data plotted represent means ± SEM of data from three independent experiments. Where error bars are not visible, these are contained within the plotted symbols. No RAMP3-specific alteration to responses was observed.

As with most receptors, the secretin receptor does not require RAMP coexpression for cell surface expression. Therefore, we cannot rule out the possibility that the lack of a specific demonstrable signaling phenotype in these assays is due to the expression of "uncomplexed" secretin receptor at the cell surface that overwhelms the response of RAMP3−secretin receptor complexes, since the percentage of complexed and uncomplexed receptor is unknown. However, under the varying conditions of assay for BRET and bimolecular fluorescence complementation, the level of interaction between RAMP3 and the secretin receptor

was similar to that seen for CLR or CTR interaction with RAMP3, the classic RAMP-associating receptors. For the CTR, this level of interaction clearly enables resolution of signaling from uncomplexed and complexed receptor despite a high level of background uncomplexed CTR phenotype (*6, 23, 29, 31*).

Functional consequence of the secretin receptor−RAMP3 interaction was demonstrated in two additional assays. In the first series of studies, a trafficking-defective secretin receptor mutant was rescued by RAMP3 coexpression. This suggests that the RAMP can play a chaperone-like role for an interacting receptor, even when this is not required for routine cell surface receptor expression. In the second series of studies, secretin receptor competed for the RAMP3 interaction with CLR, an interaction that is critical for the establishment of a functional adrenomedullin receptor. Thus, we also need to be cognizant of how interacting molecules might saturate and compete for other functionally important molecular associations.

The lack of effect in modulation of apparent secretin affinity is consistent with the lack of interaction between the amino-terminal domains of the secretin receptor and RAMP3. Indeed, it implies that lack of significant association between the amino-terminal domains may be the norm rather than the exception for most family B GPCR−RAMP interactions. The identification of TM6 and TM7 as the secretin−RAMP3 interface is therefore the first mechanistic guide to how most RAMP−family B receptor associations may occur.

Of note, the RAMP−secretin receptor interface is distinct from the homodimerization interface for secretin receptors that we recently identified as TM4 of this receptor (*17*). Homodimerization of the secretin receptor appears to be important for efficient signaling of the receptor (*17*), and our current data are consistent with an ability of RAMP3 to interact with an intact secretin receptor homodimer. For the CLR, elegant work has revealed that it is also a homodimer of this receptor that interacts with RAMPs, with the RAMP present as a monomer (*32*), although the final stoichiometry of the complex is not clear. Assuming that there is conservation of function across family B GPCRs, these data provide support for the potential extrapolation of our current findings as a model for RAMP-family B GPCR transmembrane region interactions, however, an alternate interface involving TM1 and TM2 of CLR cannot be ruled out.

The other key RAMP-dependent phenotype that has been observed for CLR−RAMP3 interactions is an alteration to adrenomedullin-induced internalization and recycling of the AM_2 receptor (*24, 25*). The AM_2 receptor, but not the RAMP2-associated AM_1 receptor, may be alternatively targeted for lysosomal degradation, rapidly recycled or not internalized at all depending upon interaction of the carboxyl-terminal RAMP3 PDZ domain with either NSF or NHERF1. Consequently, we studied internalization of the secretin receptor in the presence and absence of RAMPs and also in the context of overexpression of either NSF or NHERF1. However, internalization of the secretin receptor was not altered by RAMP3, either alone or in the presence of NSF or NHERF1, suggesting that the protein−protein interactions engendered by RAMP3 are contextual on the presentation of the PDZ epitope by its receptor partner.

The resolution of the site of transmembrane interaction between RAMP3 and the secretin receptor provides impetus toward understanding the molecular basis for the specificity of RAMP−GPCR interactions. If this interaction interface is
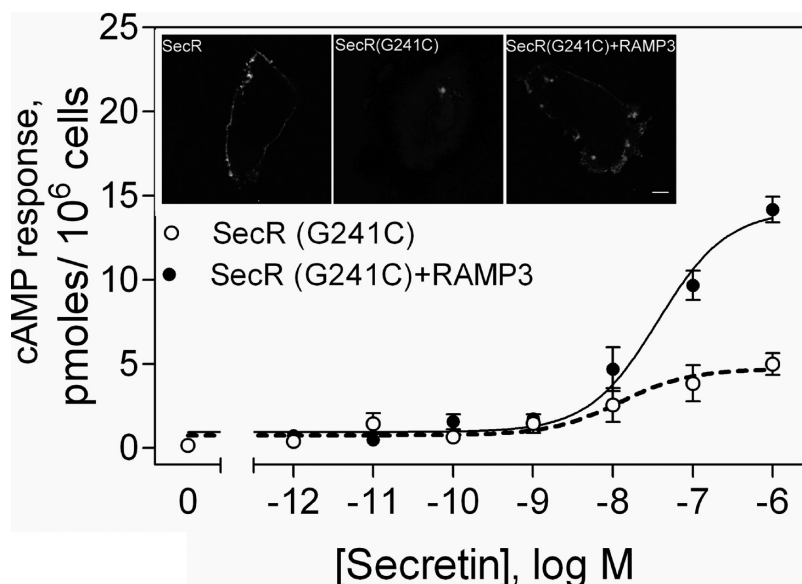
FIGURE 10: Chaperone function of RAMP3 at a trafficking-defective secretin receptor mutant. Shown are cAMP concentration−response curves to secretin stimulation of intact COS cells expressing G241C secretin receptor mutant with or without RAMP3 (*17*). Shown in the inset are the microscopic images of Alexa-secretin-labeled cells showing the increased surface expression of the mutant receptor in the presence of RAMP3. The values plotted represent means ± SEM of data from three independent experiments. Bar 25 μm.
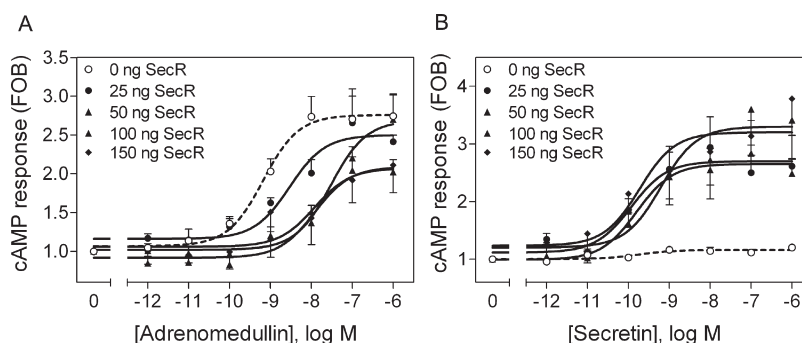

FIGURE 11: Competitive inhibition of adrenomedullin action on the CLR−RAMP3 complex by secretin receptor expression. Shown are cAMP responses to adrenomedullin (A) and secretin (B) in COS cells transfected with a fixed amount of cmyc-CLR (50 ng) and RAMP3 (100 ng), and increasing amounts of secretin receptor construct (noted for each curve). The values plotted represent means ± SEM of data from three or four independent experiments.

confirmed for other GPCRs, it will enable a bioinformatic approach to prediction of RAMP−GPCR interactions. For example, the secretin receptor and the PTH2 receptor specifically interact with RAMP3, while the PTH1 and glucagon receptors specifically interact with RAMP2. There has been substantial work to explore the molecular basis of helix−helix interactions within the lipid bilayer (*33−35*). Within the helical domain of the three RAMPs, there is approximately 30% identity of the amino acid residues and 60% homology. The differences between the RAMPs should be adequate to explain the specificity of interaction between a given GPCR and a given RAMP. Conservation of the site of transmembrane interaction would therefore allow rules to be developed for predicting such interactions.

Our data also provides further evidence for complexity in the functional consequence of RAMP−GPCR interaction with existing phenotypes varying greatly across interacting receptors and also many examples of lack of understanding of the functional significance of RAMP−GPCR interaction, as currently is the case for the secretin receptor−RAMP3 interaction. The broad distribution of RAMPs suggests that substantial work is still required to fully understand their role in physiology and disease.

SUPPORTING INFORMATION AVAILABLE

Supplemental data are available that show the alignment of the sequences of the human secretin and GLP1 receptors that was utilized to determine the sequences of the chimeric receptor constructs used in the current report. This material is available free of charge via the Internet at http://pubs.acs.org

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