Transmembrane Segment IV Contributes a Functionally Important Interface for Oligomerization of the Class II G Protein-coupled Secretin Receptor

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Oligomerization of the Class II G protein-coupled secretin receptor has been reported, but the molecular basis for this and its functional significance have not been determined. In the current work, we have examined the possible contribution of each of the transmembrane (TM) segments of this receptor to its homo-oligomerization, using the method of competitive disruption screening for inhibition of receptor bioluminescence resonance energy transfer signal. TM IV was the only segment that was found to disrupt receptor bioluminescence resonance energy transfer. Evaluation of predicted interhelical and lipid-exposed faces of this TM segment demonstrated that its lipid-exposed face represented the determinant for oligomerization. This was further confirmed by mutagenesis of the intact secretin receptor. Morphological FRET was utilized to demonstrate the lack of effect of the glycophorin receptor. Although disruption of the receptor oligomerization interface and that this oligomerization was disrupted by mutating Gly243 and Ile247, key residues within the lipid-exposed face of TM IV. Although disruption of the receptor oligomerization interface had no effect on secretin binding parameters, it reduced the ability of secretin to stimulate intracellular cAMP. This supports a clear functional effect of oligomerization of this receptor. Such an effect might be particularly relevant to clinical situations in which this receptor is overexpressed, such as in certain neoplasms.

Dimerization of plasma membrane receptors represents a timely theme of substantial interest and potential importance (1). For single transmembrane tyrosine kinase receptors, dimerization has been shown to provide a molecular mechanism for cross-molecular phosphorylation and receptor regulation (2). For some G protein-coupled receptors, dimerization or oligomerization has also been demonstrated, although the rules for establishment of such complexes, the effects of ligand binding to these complexes, and its functional implications have been quite varied (3–6). The role of such complexes in normal physiology is also unclear, with most demonstrations of G protein-coupled receptor oligomerization occurring in the setting of receptor overexpression systems (7, 8).

We previously demonstrated the oligomerization of the Class II G protein-coupled secretin receptor, based on a positive bioluminescence resonance energy transfer (BRET) signal from tagged receptors expressed in COS cells that was structurally specific, not shared with similar levels of overexpression of a Class I G protein-coupled receptor with the secretin receptor (9). Subsequently, using the same technique and complementing this with morphologic fluorescence resonance energy transfer (FRET) analysis, the secretin receptor oligomerization was shown to occur constitutively at the cell surface, without being disrupted by secretin agonist ligand binding (10). Further, the secretin receptor was shown to be capable of forming similar hetero-oligomers with structurally related VPAC1 and VPAC2 receptors (10). Of interest, expression of a secretin receptor splice variant missing a portion of its amino-terminal tail in pancreatic cancer cells along with wild type secretin receptor was shown to result in a hetero-oligmeric receptor complex, where the variant acted as a dominant-negative inhibitor of secretin signaling (9). In the present work, we have explored the molecular basis of secretin receptor oligomerization and its functional significance.

Oligomerization of membrane proteins may be influenced by extramembranous or intramembranous regions of these molecules. We previously studied truncated receptors to rule out contributions of the amino-terminal or carboxyl-terminal tail regions of the secretin receptor in its oligomerization, again utilizing receptor BRET and morphologic FRET analysis in COS cells (11). Also, alanine substitution mutagenesis was utilized to demonstrate the lack of effect of the glycoprotein GXXXG helix-helix interaction motif within transmembrane helix seven of the secretin receptor for its oligomerization (11). In the current work, we have systematically studied the contributions of the transmembrane (TM) segments of this receptor. We initially utilized an experimental strategy reported by Hebert et al. (12) to demonstrate the importance of TM segment VI in the dimerization of the Class I G protein-coupled β2-adrenergic receptor. In this strategy, TM segment peptides are mixed with the tagged intact receptor in an attempt to compete for interacting domains to thereby disrupt receptor dimerization. This was further supported by mutating Gly234 and Ile247, key residues within the lipid-exposed face of TM IV. Although disruption of the receptor oligomerization interface had no effect on secretin binding parameters, it reduced the ability of secretin to stimulate intracellular cAMP. This supports a clear functional effect of oligomerization of this receptor. Such an effect might be particularly relevant to clinical situations in which this receptor is overexpressed, such as in certain neoplasms.
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This strategy also provides a basis for studying the predicted face of the transmembrane helix, and even specific residues within that face, that are most critical to the process of receptor oligomerization.

Based on sequence analysis, the heptahelical bundle of Class II G protein-coupled receptors has been suggested to be structurally quite distinct from the more extensively studied Class I receptors (13, 14). Although oligomerization has been reported for several of the members of Class II G protein-coupled receptors (10, 15, 16), prior to this none have had their oligomerization interface mapped. Indeed, we have been able to identify a single TM segment of the Class II secretin receptor, as well as the face of this helix, that is critical for oligomerization. The peptide competition BRET strategy was confirmed and extended using intact mutated receptor BRET. This confirmed the face of the helical segment identified in the competition BRET studies and was able to further refine the critical residues to two distinct residues within that segment. Use of these complementary approaches helped to support the direct effect of the residues identified rather than their modification disrupting a normal structure to account for loss of BRET signal. Although disruption of the critical helix could explain the mutagenesis data, such structural disruption of the helical structure of the peptide would likely not effectively compete for the helical face normally involved in the dimerization interface.

This also provided an experimental strategy to study the functional importance of secretin receptor oligomerization. This was accomplished both using the competing TM peptides as well as the mutant receptors disrupting receptor oligomerization. Of note, whereas this disruption had no effect on the binding of secretin, it reduced secretin-stimulated intracellular cAMP accumulation. This effect was confirmed using a neuroblastoma/glioma cell line, NG108-15, expressing a low density of secretin receptors (17). Thus, the oligomerization of the Class II secretin receptor appears to be important for signaling, although the molecular basis for this is not yet clear.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology reagents were purchased from Invitrogen (Carlsbad, CA). A LANCE assay kit for cAMP measurement and white Optiplates were obtained from Perkin-Elmer Life Sciences. NG108-15 neuroblastoma/glioma cells were obtained from the American Type Culture Collection (Manassas, VA). Fetal Clone II in a 5% CO₂ environment. The cells were passed twice/week. Coelenterazine h was from Biotium (Hayward, CA). Other reagents were analytical grade.

Secretin Receptor Constructs—Human secretin receptor constructs tagged at the carboxyl terminus with either Renilla luciferase (Rlu) or yellow fluorescent protein (YFP) ligated into the pcDNA3 expression vector were used as donor and acceptor, respectively, for BRET studies. For donor in morphologic FRET studies, an analogous secretin receptor construct with a carboxyl-terminal CFP tag was prepared. Each of these tagged secretin receptor constructs bound secretin and signaled in response to secretin in manner similar to wild type receptor (see supplemental material) (10). For this study, we have also prepared a series of secretin receptor constructs with mutations in positions 243, 247, 254, and 257, predicted to reside within the lipid-exposed face of the fourth transmembrane (TM IV) segment (see Fig. 1B) (18). These constructs changed these four residues to alanine residues individually or in pairs (243 and 247; 254 and 257) using a QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide primers represented the following: 243,247 construct: sense primer, 5’-CCGATGGGCTTCTCCAGGCGCTTTGGTTGCTTTGTTG-3’, and antisense primer, 5’-CCAAAAGC-ACAAAGAGCGGCTGAGAACCCATCG-3’; 254,257 construct: sense primer, 5’-GCTTTGTGCGTGTCGA-CAGAGGCTTTGAGATGATGTTG-3’, and antisense primer, 5’-CCCAATCTTCCAGAAGGCTTGCGCACGCCCCAAAGG-3’. All of the receptor sequences were verified by direct DNA sequencing.

Secretin Receptor Transmembrane Segment Peptides—A series of peptides representing each of the TM segments predicted for the secretin receptor were synthesized (see Fig. 1A) (18). Two variant peptides of TM IV having predicted interhelical (Ala241,244,248,252) and lipid-exposed (Ala243,247,254,257) faces modified to replace the natural residues with alanines were also prepared (see Fig. 1B) (18). The transmembrane peptides were synthesized as carboxyl-terminal amides using manual solid phase techniques with Pal resin (Advanced Chem Tech) and Fmoc (N-(9-fluorenylmethoxycarbonyl)-protected amino acids (19). The unprotected peptides were dissolved in acetonitrile/water (50% v/v) and purified using semi-preparative reversed phase HPLC. Immediately before use, the peptides were solubilized in Me₂SO and diluted in Krebs-Ringers-HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄) to yield a final concentration of 1% Me₂SO.

Cell Preparations—Chinese Hamster Ovary cells engineered to stably express the human secretin receptor (CHO-HSecR cells) were used as a source of wild type receptor for evaluation of the effects of TM segment peptides on receptor binding and biological activity. These cells were prepared using the secretin receptor cDNA isolated by Ulrich et al. (20) inserted into the pcDNA1/Neo expression vector and transfecting CHO-K1 cells that do not naturally express secretin-responsive receptors. These were selected based on G418 insensitivity and were then cloned by successive cycles of limiting dilution, screening for secretin binding and biological responsiveness. Binding was performed using a particulate fraction enriched in plasma membranes (2 pmol receptor/mg of protein), although intact CHO-HSecR cells (39,000 receptor binding sites/cell) were used for monitoring agonist-dependent cAMP responses. The cells were grown in Ham’s F-12 medium supplemented with 5% Fetal Clone II in a 5% CO₂ environment. The cells were passed twice/week.

COS cells were used for the transient expression of Rlr-, YFP-, and CFP-tagged wild type or mutant secretin receptor constructs for BRET and morphologic FRET studies and for the functional characterization of mutant constructs. For transient expression, COS cells were plated in 10-cm tissue culture dishes at a density of 0.5 million cells/dish in Dulbecco’s modified Eagle’s medium supplemented with 5% Fetal Clone II 24 h before transfection. The cells were typically transfected with
~3 μg of total DNA (either a single construct or a combination of two constructs)/dish using the diethylaminoethyl-dextran method (3). Other series of experiments varied this DNA concentration as noted. The assays were performed in cells or membranes prepared from cells lifted 48–72 h after transfection. For morphological FRET studies, transfected COS cells were lifted and allowed to attach to coverslips 24 h before study.

Receptor Binding Assays—Radioligand binding studies were performed either with receptor-enriched CHO-HSecR cell membranes prepared as we have previously described (21) or with COS cells transiently expressing mutant tagged receptor constructs. Receptor preparations were mixed with 1–2 pM (125I-Tyr10)secretin-27 radioligand (~20,000 cpm/tube, specific radioactivity 2,000 Ci/mmol) in the absence or presence of increasing concentrations (1 pM to 1 μM) of unlabeled secretin for 1 h at room temperature in KRH medium, pH 7.4, containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. The membranes were routinely preincubated with TM peptides at a concentration of ~40 μg/ml for 2 h at 4 °C prior to initiating the receptor binding assays. This standard condition was established in concentration-response studies with the TM IV peptide, where disruption of the BRET signal was half-maximal at ~20 μg/ml and became maximal at 40 μg/ml, with no additional disruption with concentrations up to 200 μg/ml (data not shown). For receptor binding assays utilizing cell membranes, bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with 0.3% polybrene-soaked receptor-binding filtermats. For COS cell binding assays, bound and free radioligand were separated by repeated washing with ice-cold KRH medium followed by centrifugation. The cells with bound radioligand were lysed using 0.5 n sodium hydroxide. Receptor-bound radioactivity was quantified using a γ spectrometer. Nonsaturable binding was determined in the presence of 1 μM secretin and represented less than 15% of total cpm bound. Saturable radioligand binding data were analyzed using the LIGAND program (22) and were plotted using the nonlinear least squares curve-fitting routine in Prism (GraphPad 3.0, San Diego, CA).

Biological Activity Assays—Secretin stimulation of cAMP accumulation in cells was used as an indication of receptor-mediated biological activity. Assays were performed with CHO-HSecR cells and NG108-15 cells in the absence or presence of TM segment peptides, as well as with mutant secretin receptor constructs expressed in COS cells. Assays were performed according to the manufacturer’s instructions using 384-well white Optiplates, LANCE kits, and the 2103 Envision plate reader from Perkin-Elmer Life Sciences. This immunoassay is based on the competition between a europium-labeled cAMP tracer complex and sample cAMP for binding sites on cAMP-specific antibodies labeled with Alexa Fluor® 647. Detection was based on time-resolved FRET.

CHO-HSecR cells grown in a 24-well plate to 80–90% confluence were incubated with TM peptides at a concentration of 40 μg/ml for 2 h at 4 °C prior to the assay. For mutant HSecR constructs, ~40,000 transfected COS cells were plated in each well of 96-well plates 24 h after transfection. On the day of the assay, the cells were carefully washed with phosphate-buffered saline before being stimulated for 30 min at 37 °C with increasing concentrations of secretin (1 pM to 1 μM) in KRH medium containing 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. The reaction was stopped by adding ice-cold 6% perchloric acid and adjusting the pH of the supernatant to 6.0 using saturated KHCO3. Equal volumes of sample (6 μl of supernatant from each well) and Alexa Fluor® 647-labeled cAMP antibodies in KRH medium containing 0.1% bovine serum albumin (1:100 dilution) were incubated for 30 min at room temperature. After further incubation for 1 h with 12 μl of detection mixture containing the europium-labeled streptavidin and biotin-cAMP, the time-resolved FRET signals were measured using excitation at 340 nm and monitoring emission at 615 and 665 nm.

BRET Studies—Bioluminescence and fluorescence measurements were performed with aliquots of ~25,000 receptor-bearing COS cells in suspension. 48 h after transfection, the cells were lifted using nonenzymatic cell dissociation solution (Sigma) and were washed with KRH medium before the assay. The BRET assay was initiated by adding the cell-permeant Renilla luciferase-specific substrate, coelenterazine h, to the cell suspension to yield a final concentration of 5 μM in a 96-well white Optiplate. This results in a luminescence peak at 475 nm that is capable of stimulating a fluorescence emission peak from YFP at 525 nm, the same wavelength as the BRET signal in this experimental system (see supplemental material). The BRET signal was collected by using the protocol designed for BRET studies in the 2103 Envision fluorescence plate reader (Perkin-Elmer Life Sciences). The Envision plate reader was set up with a mirror (~700 nm) and two emission filter sets for luminescence (460 nm, bandwidth 25 nm) and for fluorescence (535 nm, bandwidth 25 nm). The BRET ratio was calculated based on the ratio of emission (3). For examination of the effects of the peptides, the transfected COS cells were incubated with 40 μg/ml of the TM segment peptides for 2 h at 4 °C before performing the BRET assays.

Saturation BRET experiments in which the donor/acceptor ratio is varied were performed as described previously (19). For these, COS cells were transiently cotransfected with a fixed amount of Rlu-tagged wild type or mutant receptor constructs (1.0 μg DNA/dish) and with increasing amounts of YFP-tagged wild type or mutant receptor constructs (0.3–6 μg of DNA/dish). 48 h after transfection, the cells were detached using cell dissociation medium and were used in BRET assays, as described above. Fluorescence and luminescence intensities were collected from the same populations of cells used for the BRET studies. Background-subtracted fluorescence and luminescence intensities were collected to calculate the acceptor-to-donor ratios that were plotted against the BRET ratios. The total fluorescence intensities and luminescence intensities were measured using specified filter sets. Luminescence was measured after the addition of 5 μM coelenterazine h. Curves were fit to these data and evaluated for quality-of-fit based on R2 values using Prism 3.0. When a single phase exponential curve was significantly better than a linear function (F test determination with p value <0.05), it was utilized to calculate BRETmax and BRET50 values.

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Type-2 experiments, as described by James et al. (7), in which the donor/acceptor ratio was held constant at a ratio of 1:1 while reducing the amount of receptor that would still provide a signal were also performed. The data were plotted as BRET signals relative to the amount of DNA construct utilized. The smallest amount of DNA (0.1 μg of total DNA/dish) yielded 4,630 receptor binding sites/cell. The data were fit to a linear equation, and the intercept with the ordinate was determined. Random interactions are believed to converge near a zero intercept, whereas significant molecular interactions are proposed to yield a positive value (7).

Morphological FRET Assays—FRET microscopy was performed as described previously (10). In brief, COS cells were plated at a density of 0.5 × 10^6 cells/dish in a 10-cm tissue culture dish 24 h before transfection. These cells were transfected using 1.5 μg each of CFP- and YFP-tagged wild type or mutant secretin receptor constructs. Twenty-four h after transfection, the cells were washed, lifted from the dishes, and allowed to grow on 25-mm sterilized coverslips. FRET imaging was performed using an Axiovert 200M inverted epifluorescence microscope (Carl Zeiss, Thornwood, NY) having a fixed filter set (Chroma Technology Corp., Brattleboro, VT) for CFP (excitation, 436/20 nm; dichroic mirror, 455 dclp; and emission, 480/40 nm), YFP (excitation, 500/20 nm; dichroic mirror, Q515 lp; and emission, 535/30 nm), and FRET (excitation, 436/20 nm; dichroic mirror, 455 dclp; and emission, 535/30 nm). Raw images were collected separately for each of the channels with constant exposure times using a monochromatic ORCA-12ER CCD camera (Hamamatsu, Bridgewater, NJ) with automated QED-InVivo 2.039 acquisition software (Media Cybernetics Inc., Silver Spring, MD). Donor or acceptor bleed-through into the FRET channel was corrected (10) using the sensitized-emission method provided by Metamorph version 6.3 (Molecular Devices, Sunnyvale, CA). Corrected FRET represents $F_{RET} = FRET - (B \cdot CFP) - (A \cdot YFP)$ where FRET, CFP, and YFP represent blank (background signal within the respective images)-subtracted images collected in the corresponding channels. Coefficients $B$ (0.54) and $A$ (0.14) represent signal bleed-through into the FRET channel of the donor (CFP) and acceptor (YFP), respectively. Final 16-bit images were converted into 8-bit images using Metamorph 6.3, then assembled, and organized using Adobe Photoshop version 7.0.

RESULTS

Competition BRET Assays—We previously utilized BRET to demonstrate that the secretin receptor forms constitutive homo-oligomers in the cell membrane (9). In the current work, we have attempted to identify the region of this receptor that is responsible for the oligomerization. This was done utilizing a strategy originally applied to the β2-adrenergic receptor in which the TM segment peptide involved in its dimerization interface was used as competitor to inhibit dimerization (12). We have examined peptides corresponding to each of the predicted TM segments of the secretin receptor (Fig. 1A), exploring whether any would interfere with secretin receptor oligomerization, as reflected by its BRET signal. As previously reported, in the absence of competing peptides, the Rlu- and YFP-tagged secretin receptor constructs gave a BRET signal of $0.28 \pm 0.01$, significantly greater than the signal from coexpression of complementary-tagged structurally unrelated receptors (secretin receptor and cholecystokinin receptor, BRET signal of $0.06 \pm 0.01$) (Fig. 2A). This provides the signal that can be expected from similar levels of expression of tagged molecules in the plasma membrane compartment of a cell, even if they do not specifically interact. Other controls consisting of the tagged secretin receptor with the complementary tag absent or in a different cellular compartment gave a lower BRET signal ($0.05 \pm 0.01$). Of the seven TM peptides tested individually, only the peptide corresponding to the sequence predicted for TM IV inhibited the BRET signal (Fig. 2B). Of note, incubation of the secretin receptor simultaneously with all of the other TM segment peptides (TM I, II, III, V, VI, and VII) also had no effect on the receptor BRET signal (Fig. 2B).
To examine which face of TM IV might be important for oligomerization, variant TM IV peptides having their predicted interhelical or lipid-exposed faces modified by alanine replacements were prepared and utilized in secretin receptor BRET assays (Fig. 2B). The TM IV-Ala241,244,248,252 peptide in which the interhelical face was modified inhibited the BRET signal, suggesting that this face is the critical interface for receptor oligomerization. In contrast, the TM IV-Ala243,247,254,257 peptide that had its lipid-exposed face modified had no effect on the secretin receptor BRET signal, suggesting that this face is the critical interface for receptor oligomerization.

Saturation BRET Assays—Saturation BRET studies were performed to distinguish a significant molecular interaction from random approximation of donor and acceptor that might be expected by their being laterally mobile within the same cellular compartment. Fig. 3 shows that COS cells expressing Rlu- and YFP-tagged secretin receptor after transfection using a fixed amount of donor construct (1.0 μg of DNA/dish) and increasing amounts of acceptor construct (0.3–6 μg of DNA/dish) produced the expected increase in BRET ratio that then reached an asymptote. These data support the saturable nature of this molecular interaction. The asymptotic phase of the saturation BRET curve was typically reached with 1.2–1.5 μg of acceptor DNA/dish, representing approximate molar ratios of acceptor:donor of 1.2–1.5:1. Additionally, the expression of these tagged secretin receptor constructs in the presence of peptides representing TM IV or TM IV-Ala241,244,248,252 gave only a low level signal that was not saturable, with best fit of the data not different from a linear regression that was parallel to the asymptotic phase of the saturation curve. This is consistent with random interactions taking place in the plasma membrane.

Functional Role of Secretin Receptor Oligomers—The demonstrated ability of the TM IV peptide to disrupt secretin receptor oligomers provided an important tool to examine the functional importance of the oligomerization of this receptor. Studies were performed to examine this effect. Disruption of oligomerization of wild type secretin receptors did not have any effect on the ligand binding ability of this receptor (Fig. 4, top panel), whereas it reduced agonist-stimulated cAMP accumulation (Fig. 4, middle panel). This was also true of the TM IV-Ala241,244,248,252 peptide. Binding affinities (K_i values in nM) were not different when the wild type secretin receptor-bearing
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FIGURE 4. Effect of disruption of receptor oligomerization with TM segment peptides on the binding and biological activity of the secretin receptor. Shown are competition curves for secretin binding to CHO-HSecR membranes (top panel) and secretin-stimulated cAMP responses in CHO-HSecR cells (middle panel) and in NG108-15 cells (bottom panel) in the absence or presence of noted secretin receptor TM segment peptides. Incubations with the peptides had no effect on secretin binding to its receptor, whereas incubation with the TM IV peptide or its variant significantly reduced the potency of secretin-stimulated cAMP accumulation, whereas the negative control TM VI peptide had no effect on receptor signaling (Fig. 4, bottom panel). Basal levels of cAMP in these cells were 1.9 ± 0.5 pmol/million cells, and maximal levels achieved in response to secretin were 105 ± 14 pmol/million cells.

Mutant Receptor BRET Studies—To confirm the data described above in peptide competition and saturation BRET and functional studies, as well as to refine our understanding of the critical residues responsible for oligomerization and to establish that receptor oligomerization was present within the plasma membrane, mutant intact secretin receptors were prepared. These represented Rlu-, CFP-, and YFP-tagged mutant secretin receptors having their proposed oligomerization interface modified. In addition to the modification of the four residues that had been changed to alanines in the TM IV peptides that had been studied (HSecR-Ala243,247,254,257 with G243A, I247A, I254A, and H257A mutations), constructs were prepared having two of these four residues modified at a time (HSecR-Ala243,254,257 with G243A and I254A mutations or HSecR-Ala243,247 with I254A and H257A mutations). Fig. 5 demonstrates that these modifications had no significant effect on secretin binding ($K_I$ values in nM for wild type secretin receptor (4.7 ± 0.6), and its alanine mutants, HSecR-Ala243,247,254,257 (3.8 ± 1.8), HSecR-Ala243,247,254,257 (4.9 ± 0.2), and HSecR-Ala243,254,257 (4.4 ± 0.3), were not different from each other and not affected by the presence of Rlu, YFP, or CFP at their carboxyl terminus. Receptor densities (thousand sites/cell) were not different for any of the constructs, with wild type HSecR, 78 ± 15; HSecR-Ala243,247,254,257, 77 ± 10; HSecR-Ala243,247,254,257, 71 ± 10; and HSecR-Ala243,254,257, 80 ± 9.

Fig. 5 illustrates the biological responses to secretin of these receptor constructs expressed in COS cells. Basal and maximal levels of cAMP (pmol/million cells) were similar for all of the constructs and for each of their tagged forms: wild type HSecR 1.6 ± 0.5 basal, 141 ± 30 maximal; HSecR-Ala243,247,254,257 1.9 ± 0.5, 155 ± 19; HSecR-Ala243,247,254,257 1.2 ± 0.2, 156 ± 17; and HSecR-Ala243,254,257, 1.7 ± 0.3, 144 ± 13. However, potencies did vary among the constructs. $EC_{50}$ values (in nM) for wild type secretin receptor (0.03 ± 0.01) were similar to HSecR-Ala243,254,257 (0.06 ± 0.03) and significantly different from those for HSecR-Ala243,247,254,257 (0.48 ± 0.15) and HSecR-Ala243,247,254,257 (0.53 ± 0.11) ($p < 0.01$).

BRET studies were performed on COS cells coexpressing Rlu- and YFP-tagged versions of HSecR-Ala243,247,254,257 or HSecR-Ala243,247 or HSecR-Ala254,257 constructs alone or in combination with Rlu- or YFP-tagged wild type HSecR con-
HSecR-Ala243,247 constructs yielded a signal that extrapolated to zero, consistent with absence of a significant molecular interaction, thought to reflect random interactions based on concentration.

Saturation BRET assays were also performed with these constructs. Fig. 8 shows that BRET values in these experiments using intact wild type and HSecR-Ala254,257 receptors increased significantly and reached an asymptote, with curves significantly different from a linear function. In contrast, HSecR-Ala243,247 and HSecR-Ala254,257 yielded little increase in BRET, with the curves not significantly different from a linear fit that was parallel to the asymptotic phase of the saturation curve. Those curves with exponential shapes increasing to an asymptote had their BRET_max and BRET_50 values calculated. For the wild type secretin receptor, the BRET_max value was 0.25 ± 0.01, and BRET_50 was achieved at a YFP/Rlu ratio of 6.5 ± 0.6. These values were similar for the wild type receptor in the presence of the TM VI peptide (BRET_max value was 0.24 ± 0.01, and BRET_50 was achieved at a YFP/Rlu ratio of 5.7 ± 0.5). The values were also similar for the HSecR-Ala254,257 mutant receptor (BRET_max value was 0.27 ± 0.01 and BRET_50 was achieved at a YFP/Rlu ratio of 5.8 ± 0.8). These data continue to support the interpretation that residues Gly243 and Ile247 are critical for the secretin receptor dimerization interface.

**DISCUSSION**

The molecular basis and functional implications of dimerization or oligomerization of G protein-coupled receptors are most extensively described for Class I receptors. Transmembrane segment VI of the β2-adrenergic receptor (12) and of the cholecystokinin receptor (19) have been shown to represent an important determinant of their oligomerization, with competitive incubation with TM VI peptides disrupting BRET signals
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for those receptors. Although disruption of oligomerization of the β2-adrenergic receptor resulted in inhibition of agonist-stimulated cAMP production (12), disruption of oligomerization of the cholecystokinin receptor resulted in no effect on ligand binding or agonist-dependent calcium release (19). It is quite interesting that despite having the analogous regions of both of these Class I G protein-coupled receptors responsible for their oligomerization, the functional effects have not been consistent.

FIGURE 6. Effect of TM IV lipid face mutations on secretin receptor BRET. Shown are the BRET ratios obtained from COS cells expressing the Rlu- and YFP-tagged secretin receptor constructs: HSecR-Ala243,245,254,257 (A), HSecR-Ala243,247 (B), and HSecR-Ala254,257 (C). Here, we have used wild type and mutant receptors in which groups of four or two amino acids on the predicted lipid-exposed face of TM segment IV were mutated to alanine residues. Both HSecR-Ala243,245,247,254 and HSecR-Ala243,247 constructs exhibited significant reductions in BRET ratio, whereas the HSecR-Ala254,257 construct showed a similar BRET ratio to that of wild type receptor. The reduced BRET ratio observed with two mutant receptors was not different from that of other nonspecific interactions (shown in the shaded area). **, \( p < 0.001; * , p < 0.05 \) as compared with the BRET signal obtained from tagged wild type secretin receptor. The data are presented as the means ± S.E. of five independent experiments.

FIGURE 7. BRET analysis with fixed donor/acceptor ratio while varying the level of receptor expression. Shown are the BRET signals plotted relative to the total DNA utilized with a fixed 1:1 ratio of donor/acceptor constructs, as noted. The data were fit to linear equations. The data for wild type secretin receptor and for HSecR-Ala243,247 extrapolated to the ordinate significantly above zero, whereas those for HSecR-Ala243,245,254,257 and HSecR-Ala254,257 constructs extrapolated to zero. These data are consistent with oligomerization for wild type secretin receptor and HSecR-Ala243,247, whereas HSecR-Ala243,245,254,257 and HSecR-Ala254,257 are most consistent with absence of significant oligomerization and likely reflect random interactions based on concentration.

FIGURE 8. Saturation BRET analysis of secretin receptor mutants. Shown are the BRET saturation curves plotted as a ratio of YFP fluorescence to Rlu luminescence that were obtained for pairs of YFP- and Rlu-tagged secretin receptor constructs studied with a fixed amount of donor (1.0 μg of DNA/dish) and increasing amounts of acceptor (0.3–6 μg of DNA/dish). HSecR-Ala254,257 mutant generated an exponential curve that increased until values reached saturation, reflected as an asymptote. This was similar to data for wild type receptor. In contrast, HSecR-Ala243,247,254,257 and HSecR-Ala243,245 constructs yielded curves not statistically different from a linear fit to the data that was parallel to the asymptote in the saturation curve. The saturation curve obtained using the Rlu-tagged secretin receptor with the structurally unrelated YFP-tagged type B CCK receptor yielded a similar linear fit. The data are represented as the means ± S.E. of three independent experiments.
sequence analysis and conservation of patterns suggests substantia differences in the helical bundle of Class II G protein-coupled receptors (13). This absence of understanding of the structure of the helical bundle of these receptors makes it impossible to predict what helical segment might be most exposed and might be most important for receptor-receptor contact in this receptor family. Previous studies have eliminated the amino-terminal and carboxyl-terminal tail regions of the secretin receptor as having any significant effect on its oligomerization (11). The possible impact of its loop regions on the secretin receptor as having any significant effect on its oligomerization have not yet been directly studied.

In the current work, we have demonstrated the importance of TM segment IV of the secretin receptor for its constitutive oligomerization. This was achieved using the competitive TM segment peptide approach (19), with no other segment peptide having any effect on secretin receptor BRET either alone or in aggregate with all of the other segments being studied. This may support a very important role of a possible TM IV-TM IV interaction to yield a secretin receptor dimer. Whether this BRET signal comes from a dimer or from a higher order receptor oligomer will need to be better defined.

BRET assays with intact secretin receptor mutants supported the conclusion based on the peptide competition BRET assays. This approach was also able to confirm the importance of the predicted lipid-exposed face of TM IV. It is noteworthy that we were also able to extend the insight to focus on two key residues within this TM segment, Gly\(^{243}\) and Ile\(^{247}\). Importantly, this was confirmed by studies with mutated intact receptor constructs. The use of these two complementary approaches helps to assure that the effect is direct and does not represent an indirect effect of the mutation disrupting a key helical structure. Although this is quite possible for the intact receptor mutation, similar disruption of structure of the isolated peptide would make its ability to compete for a natural face of the TM segment very unlikely. We also know that these mutations in TM IV did not affect secretin binding and were compatible with a full biological response, although potency was reduced. The identification of these particular residues should act as a useful experimentally derived constraint to help refine our understanding of the helical bundle structure of this receptor. This provides the first such data to help model this helical bundle domain.

The functional studies reported in the current work were also very instructive. Disruption of secretin receptor oligomerization using either competition with the TM IV segment peptide or modifying the lipid face of this TM segment in the intact receptor by mutagenesis both resulted in normal secretin binding. This is fully consistent with our current understanding of the molecular basis of secretin receptor binding, with the extracellular amino-terminal tail believed to provide the site of docking with a one-to-one stoichiometry (25). This is based on receptor mutagenesis and chimeric receptor studies (25, 26), as well as a series of photoaffinity labeling studies using a variety of photolabile probes having sites of covalent attachment distributed throughout the pharmacophore (27–29). This is also consistent with molecular models of peptide docking to Class II G protein-coupled receptors that have been proposed to date (30, 31).

In contrast to the normal secretin peptide binding to receptors that had their constitutive oligomerization disrupted, this experimental manipulation resulted in reduced secretin-stimulated cAMP signaling. Again, this was true both of wild type secretin receptors expressed with competing TM IV peptide as well as mutant secretin receptors in which the lipid-exposed face of TM IV was modified. Both experimental approaches yielded reduced cAMP responses, suggesting less-than-optimal G protein coupling. It is of interest that there has been discussion of the stoichiometry of G protein coupling with G protein-coupled receptors (32). It has been postulated that some receptor dimers can couple to a single heterotrimeric G protein, with a stoichiometry of 2 receptor molecules/G protein (32). There

FIGURE 9. FRET imaging of secretin receptor oligomerization. Shown are representative corrected microscopic images of fixed COS cells expressing CFP- and YFP-tagged wild type secretin receptor (A), CFP- and YFP-tagged HSecR-Ala\(^{243,247,254,257}\) (B), CFP- and YFP-tagged HSecR-Ala\(^{245,247}\) (C), CFP- and YFP-tagged HSecR-Ala\(^{254,257}\) (D), and CFP-tagged wild type secretin receptor along with YFP-tagged structurally unrelated type B CCK receptor (E). The images shown in the left, middle, and right columns represent background-subtracted CFP, background-subtracted YFP, and corrected FRET signals, respectively. Wild type secretin receptors and the HSecR-Ala\(^{234,237}\) receptors showed an apparent FRET signal at the cell surface (arrowheads) and in intracellular compartments. This was abolished by mutation of residues in positions 243, 247, 254, and 257, as well as by mutation of residues in positions 243 and 247 of the secretin receptor. The scale bar represents 25 μm.
is currently no direct basis to understand the stoichiometry of coupling of the secretin receptor with its G protein. Similarly, there is no clear molecular explanation for the negative impact on secretin signaling of disruption of the constitutive receptor oligomers.

This report represents the first insights into the dimerization interface of a Class II G protein-coupled receptor, as well as the first clear evidence of the function of homodimerization of a member of this receptor family. It will be important to understand whether this structural theme will be shared by other members of this family. It will also be of interest to understand whether the structurally related receptors shown to form hetero-oligomers with the secretin receptor (10) utilize the same structural determinants. If such an interface differs in distinct pairs of receptors, it could provide a target for allosteric modulators of secretin function.

Acknowledgments—We thank Laura-Ann Bruins and Renee M. Happs for excellent technical assistance and Evelyn Posthumus for secretarial assistance.

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