Spatial Intensity Distribution Analysis Quantifies the Extent and Regulation of Homo-Dimerization of the Secretin Receptor

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ABSTRACT

Previous studies have indicated that the G protein-coupled secretin receptor is present as a homo-dimer, organized through symmetrical contacts in transmembrane domain IV, and that receptor dimerization is critical for high potency signalling by secretin. However, whether all of the receptor exists in the dimeric form or if this is regulated, is unclear. We used measures of quantal brightness of the secretin receptor tagged with monomeric enhanced green fluorescent protein (mEGFP) and Spatial Intensity Distribution Analysis to assess this. Calibration using cells expressing plasma membrane-anchored forms of mEGFP initially allowed demonstration that the Epidermal Growth Factor receptor is predominantly monomeric in the absence of ligand and whilst wild type receptor was rapidly converted to a dimeric form by ligand, a mutated form of this receptor remained monomeric. Equivalent studies showed that at moderate expression levels the secretin receptor exists as a mixture of monomeric and dimeric forms, with little evidence of higher-order complexity. However, sodium butyrate induced up-regulation of the receptor resulted in a shift from monomeric towards oligomeric organization. By contrast, a form of the secretin receptor containing a pair of mutations on the lipid-facing side of transmembrane domain IV was almost entirely monomeric. Down-regulation of the secretin receptor-interacting G protein G\(_\alpha_s\) did not alter receptor organization, indicating that dimerization is defined specifically by direct protein-protein interactions between copies of the receptor polypeptide, whilst short term treatment with secretin had no effect on organization of the wild type receptor but increased the dimeric proportion of the mutated receptor variant.
Summary statement

The secretin receptor exists in the cell membrane as a mixture of monomers, dimers and oligomers with the proportions of these defined by receptor expression level: A pair of mutations in transmembrane domain IV limits the receptor to the monomeric state.

Short title

Secretin receptor homo-dimerization

Keywords

secretin receptor, dimerization, SpIDA, G protein-coupled receptor, confocal microscopy, oligomerization

Non-Standard Abbreviations

Bi-FC, Bi-molecular fluorescence complementation; BRET, Bioluminescence Resonance Energy Transfer; CHO, Chinese hamster ovary; EGFR, Epidermal Growth Factor receptor; EGFP, Enhanced Green Fluorescent Protein; GPCR, G protein-coupled receptor; PBS, Phosphate Buffered Saline; QB, quantal brightness; RET, Resonance Energy Transfer; RoI, Region(s) of Interest; SpIDA, Spatial Intensity Distribution Analysis.
INTRODUCTION

The concept that single polypeptide G protein-coupled receptors (GPCRs) can form, exist and may function, as dimers and/or higher-order oligomers, rather than simply as monomers, has steadily gained acceptance [1-2]. In the case of the glutamate-like or ‘class C’ receptors their existence as obligate dimers/oligomers has long been established [3] and is an integral component of their mechanism of signal transduction. In contrast, for the rhodopsin-like or ‘class A’ GPCRs, clear evidence shows that they can function as monomers [4-5], and this has led to debate over the relevance for function and pharmacology of dimers and/or higher-order oligomers, that clearly can be shown to exist [1-2, 6], even if in some cases the stability, and durability of such quaternary structures has been questioned [1, 7-8] or even argued to be incorrect [9]. As with many other areas of study on the structure, function and pharmacology of the over-arching GPCR superfamily, questions relating to the organizational structure of the small group of ‘class B’ receptors, which recognise and respond to sets of peptide hormones that control many aspects of integrative physiology, have attracted rather less attention [10]. Despite this, a number of the class B receptor members have been actively studied in relation to their quaternary structure, with perhaps the greatest attention to date having been directed towards the secretin receptor [11-14]. As for many class A GPCRs the application of resonance energy transfer (RET)-based techniques, in which forms of a receptor labelled with distinct, RET-competent tags are co-expressed, has been central to analysis of ‘dimerization’ and for the secretin receptor, combinations of bioluminescence resonance energy transfer (BRET) and bi-molecular fluorescence complementation (Bi-FC) have been used to suggest that this receptor exists as dimers but not as higher-order oligomers [13]. Moreover, by employing a series of peptides corresponding to individual transmembrane domains of the secretin receptor to disrupt potential receptor-receptor interactions Harikumar et al., [14] were able to provide evidence of a key role of transmembrane domain IV in forming the dimer interface. Moreover, mutation of residues of transmembrane domain IV predicted to be on the external, lipid-facing region of this sequence resulted in abrogation of receptor-receptor interactions measured by RET techniques [14]. The minimum set of
mutations required to produce this effect was conversion of both residues Gly\textsuperscript{243} and Ile\textsuperscript{247} to Ala [14]. In addition to the effects upon dimerization it was observed that these mutations also reduced the potency of secretin to stimulate intracellular cAMP production [14].

RET-based techniques have provided enormous insight into potential protein-protein interactions in living cells and how these may be disrupted [15-17] but clear limitations apply for RET, not least the very specific effects of distance and dipole orientation on signals observed. Moreover, although widely used in transfected cell systems, the production of BRET- or FRET-competent partners generally requires molecular manipulation to introduce fluorescent proteins and/or a bioluminescent enzyme into the sequence of the protein being studied, and as such would require complex genetic engineering and crossing of transgenic lines to generate animals in which such studies could be performed with receptors expressed at endogenous levels [15]. Moreover, as RET-techniques require close proximity or direct interaction between copies of the differentially labelled variants of the protein of interest then, whilst suitable to define that some proportion of the protein is dimeric or oligomeric, it is not well suited to assess what this proportion may be and as such, whether a significant fraction of the secretin receptor is monomeric remains undefined. In recent years there have been efforts to employ mathematical analysis of the distribution of fluorescent signal generated from a single fluorophore-tagged protein to gain insight into quaternary organization of such-tagged proteins. Spatial Intensity Distribution Analysis (SpIDA) has been suggested to be able to do so [18-19], and in recent times we have shown that SpIDA can indeed identify both monomeric versus dimeric/oligomeric organization, and ligand regulation of such organizational structure, simply by appropriate mathematical analysis of Regions of Interest (RoI) within laser scanning confocal images from cells expressing a single protein construct tagged with a suitable fluorophore [20-22]. In the current studies we have applied SpIDA to re-assess the basal organizational structure of the secretin receptor at different expression levels and the effects of mutations within transmembrane domain IV on this, as well as to probe the contribution of G protein to the stability of secretin receptor organization and the potential of the ligand secretin to alter this. To enhance confidence in the quantitative analyses we also generated and tested a variety of other protein constructs, labelled
with the same monomeric form of enhanced Green Fluorescent Protein (mEGFP), that are known to exist as monomers or dimers or are known to interconvert between these states in a ligand-dependent manner [20-22]. The results indicate that at moderate expression levels the basal state of the secretin receptor is predominantly dimeric, although a significant proportion of monomers co-exist with the dimers, and that increasing expression level both decreases the proportion of monomeric species whilst leading to the detection of oligomeric forms that are larger than dimers. Mutation of key residues in transmembrane domain IV alters this equilibrium to greatly favour the monomeric state. Extensive downregulation of G\(\alpha_s\), the key signal transducing G protein associated with secretin receptor function, had no effect on the distribution of receptor quaternary structure whilst, although without effect on the structure of the wild type receptor, short term addition of secretin increases the organizational structure of the mutationally modified form of the receptor that compromises basal dimerization.

**EXPERIMENTAL PROCEEDURES**

**Materials**

General laboratory chemicals were from Sigma-Aldrich (Poole, UK) or Fisher Scientific (Leicester, UK). Otherwise, DNA restriction endonucleases, calf intestinal alkaline phosphatase, T4 DNA polymerase and T4 ligase were from New England Biolabs (Hitchin, UK). Wizard Plus SV Miniprep kit was from Promega (Southampton, UK). NuPage Novex pre-cast 4-12 % Bis-Tris gels, NuPage MOPS SDS running buffer, NativePAGE™ Novex® 3–12% Bis-Tris Gels and associated reagents were from Invitrogen (Paisley, UK). QIAfilter Plasmid Maxi Kit, PCR purification kit and QIAquick gel extraction kit were from Qiagen (Crawley, UK). Agarose was from Flowgen Biosciences (Nottingham, UK). Secondary horseradish peroxidase conjugated antibody was from Sigma-Aldrich or GE Healthcare Life Sciences. ECL reagent was purchased from Pierce (Tattenhall, UK). Polyethylenimine (PEI) was from Polysciences Inc (Warrington, PA, USA). Protease inhibitor cocktail tablets and N-glycosidase F were from Roche Diagnostics, (Mannheim, Germany). Antibodies were either generated “in house” (anti-GFP, anti-G\(\alpha_s\)), or
were from Santa Cruz Biotechnology (Insight Biotechnology, Middlesex, UK) (anti-EGF-R), Sigma-Aldrich (anti-tubulin) or Abcam (Cambridge, UK) (anti-EGF-R (phospho-Y1068), anti-Na+/K+ATPase). Cholera toxin and n-dodecyl β-D-maltoside were from Sigma-Aldrich (Poole, UK). Secretin was from Tocris Bioscience (Bristol, UK).

DNA constructs

PM-1-mEGFP, PM-2-mEGFP and EGFR-mEGFP were made as described in [20]. A C-terminally truncated form of Robo 1-mEGFP was made as described in [21]. Tyr251Ala,Arg285Ser EGFR-mEGFP [23] was a modification of the EGFR-mEGFP construct, made by 2 rounds of Dpn1 mutagenesis (QuikChange, Agilent Technologies/Stratagene, Santa Clara, USA). Human secretin receptor-mEGFP and human Gly243Ala,Ile247Ala secretin receptor-mEGFP constructs were made from constructs as described in [14] by inserting fragments, cut with HindIII (then blunted, using T4 DNA polymerase) and XhoI, corresponding to secretin receptor and secretin receptor Gly243Ala,Ile247Ala into pEGFP-N1 (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) at NheI (blunted, as above) and XhoI. All constructs containing mEGFP incorporated the Ala206Lys mutation to ensure that the results were not influenced by any tendency of the fluorescent protein to aggregate [24] and were verified by sequencing.

Cell lines

All cells were maintained in a humidified incubator with 95% air and 5% CO2 at 37°C. Parental Flp-In™ T-REx™ 293 cells (Invitrogen, Paisley, UK) were maintained in DMEM (high glucose) supplemented with 10% (v/v) fetal bovine serum, 100 U.ml⁻¹ penicillin, 100 μg.ml⁻¹ streptomycin, 10 μg.ml⁻¹ blasticidin and 100 μg.ml⁻¹ zeocin. Cell lines generated that used Flp-In™ T-REx™ 293 cells as the base were maintained in DMEM (high glucose) supplemented with 10% (v/v) fetal bovine serum, 100 U.ml⁻¹ penicillin, 0.1 mg.ml⁻¹ streptomycin, 10 μg.ml⁻¹ blasticidin and 200 μg.ml⁻¹ hygromycin B. Chinese hamster ovary (CHO) secretin receptor-mEGFP and CHO Gly243Ala,Ile247Ala secretin receptor-mEGFP
cell lines were maintained in Hams F-12 Nutrient Mix (Invitrogen, Paisley, UK) supplemented with 5% (v/v) fetal bovine serum, 100 U.ml⁻¹ penicillin, 100 μg.ml⁻¹ streptomycin and 500 μg.ml⁻¹ zeocin.

**Stable cell line generation**

Inducible Flp-In™ T-REx™ stable cell lines able to express PM-1-mEGFP, PM-2-mEGFP, EGFR-mEGFP, Tyr²⁵¹Ala,Arg²⁸⁵Ser EGFR-mEGFP and the Robo 1-mEGFP construct were generated as described [20-22]. After 48 h medium was changed to that with no zeocin, but supplemented with 200 μg.ml⁻¹ hygromycin to initiate selection of stably transfected cells. Pools of cells were established (10 to 14 days for resistant colonies to form) and tested for inducible expression by the addition of 100ng.ml⁻¹ doxycycline for 48 h followed by screening for fluorescence corresponding to mEGFP and by immunoblotting.

mEGFP-tagged wild type and Gly²⁴³Ala,Ile²⁴⁷Ala secretin receptor-bearing CHO cell lines were prepared by transfection of Chinese hamster ovary cells known to not express any secretin receptors, following the approach we previously described [25]. Stable, receptor-bearing cells were enriched using zeocin (500 μg.ml⁻¹) and clonal cell lines were selected utilizing limiting dilution techniques. These were shown to exhibit fluorescence at the cell surface and levels of receptor expression were quantified using secretin radioligand binding and by quantification of mEGFP fluorescence.

**Cell membrane preparation**

Cells induced with the required concentration of doxycycline to express PM-1-mEGFP, PM-2-mEGFP, Robo1(truncated)-mEGFP, or other constitutively expressing constructs, were washed and then harvested with ice-cold PBS. Pellets of cells were frozen at -80°C for a minimum of 1h, thawed and resuspended in ice-cold 10mM Tris, 0.1mM EDTA, pH 7.4 (TE buffer) supplemented with Complete protease inhibitors cocktail. Cells were homogenized on ice by 40 strokes of a glass-on-teflon homogenizer followed by centrifugation at 1000 × g for 5 min at 4°C to remove unbroken cells and nuclei. The supernatant fraction was removed and passed through a 25-gauge needle 10 times before being transferred to ultracentrifuge
tubes and subjected to centrifugation at 50,000 × g for 30 min at 4°C. The resulting pellets were resuspended in ice-cold TE buffer, the protein concentration was assessed and membranes were stored at -80°C until required.

**Generation of cell lysates and immunoblotting**

Cells were washed once in cold Phosphate Buffered Saline (PBS) (120 mM NaCl, 25 mM KCl, 10 mM Na₂HPO₄ and 3 mM KH₂PO₄, pH7.4) and harvested with a minimum volume ice-cold lysis buffer, (150 mM NaCl, 0.01 mM Na₃PO₄, 2 mM EDTA, 0.5 % n-dodecyl β-d-maltoside, and 5% glycerol plus Protease inhibitor cocktail tablets, pH 7.4). Extracts were passed through a 25-gauge needle and incubated for 30 min at 4°C while on a rotating wheel, centrifuged for 10 min at 21000 × g and the supernatant recovered to fresh tubes. Samples were prepared by the addition of SDS-PAGE sample buffer and heated to 65°C for 5 min before being subjected to SDS-PAGE analysis using NuPAGE 4 to 12% Bis-Tris gels and MOPS buffer. After separation, the proteins were electrophoretically transferred to nitrocellulose membrane, which was then blocked (5% fat-free milk powder in PBS with 0.1% Tween-20) at 4°C on a rotating shaker overnight. The membrane was incubated for 3 h with primary antibody (1:10000 sheep anti-GFP, 1:10000 rabbit anti-Gas, 1:1000 rabbit anti-EGFR, 1:10000 rabbit monoclonal anti-EGFR (phospho-Y1068), as indicated) in 2% fat-free milk powder in PBS-Tween, washed (3 × 10 min PBS-Tween) and then incubated for 3 h with appropriate secondary antibody (horseradish peroxidase-linked rabbit anti-goat IgG or donkey anti-mouse, diluted 1:10000 in 2% fat-free milk powder in PBS-Tween). After washing as above, signal was detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL) according to the manufacturer’s instructions. Alternatively, some blots were detected using fluorescently labelled secondary antibodies (IRDye® 800CW donkey anti-goat and IRDye® 680RD donkey anti-mouse (Li-Cor Biotechnology, Cambridge, UK). These were used at a dilution of 1:20000 in Odyssey® PBS blocking buffer (Li-Cor Biotechnology) according to the manufacturer’s instructions.

Secondary antibodies were detected using an Odyssey Sa Infra-red Imaging System (Li-Cor Biotechnology).
Native Blue polyacrylamide gel electrophoresis

Flp-In™ T-REx™ 293 cells induced with doxycycline to express constructs as indicated and subject to treatments as indicated, were harvested in 1 × PBS and lysed in lysis buffer (150 mM NaCl, 0.01 mM Na₃PO₄, 2 mM EDTA, 0.5 % n-dodecyl β-d-maltoside, and 5% glycerol supplemented with Protease inhibitor cocktail tablets, pH 7.4) on a rotating wheel for 30 min at 4°C. Samples were then centrifuged for 30 min at 100,000 × g at 4°C and the supernatants collected. 16 µg of solubilized supernatant plus 4µl G250 additive was loaded on to each lane of NativePAGE™ Novex® 3–12% Bis-Tris Gels. In some samples, SDS was added to 1% final concentration 10 minutes prior to loading. After electrophoresis at 0°C (using buffers and conditions indicated by the manufacturer), proteins were transferred (90 min at 25 V) on to a PVDF membrane which had been pre-wetted for 30 seconds in methanol and then soaked for several minutes in transfer buffer. The membrane was then fixed in 8% acetic acid, shaking for 15 minutes, stained with Ponceaux S, (Sigma-Aldrich (Poole, UK)), (0.2% in 1% acetic acid) to allow the markers to be visualized, rinsed to remove the Ponceaux S and immunoblotted with anti-GFP antiserum as described above.

Cyclic AMP assays

cAMP assays were performed using a homogeneous time-resolved fluorescence (HTRF®) cAMP dynamic kit (CisBio Bioassays). Cells were detached by incubation at 37°C for 5-10 minutes with Versene (Invitrogen), counted and added at 5000 cells/well to low-volume 384-well plates (Proxi-plate™ 384 Plus, Perkin Elmer). Cyclic AMP production was stimulated by the addition of varying concentrations of secretin followed by a 30 min incubation at room temperature. Outputs were measured by using a PHERAstar FS plate reader (BMG Labtech).

Determination of relative expression levels by mEGFP fluorescence

CHO cell lines expressing secretin receptor-mEGFP or Gly²⁴²Ala,Ile²⁴⁷Ala secretin receptor-mEGFP were seeded at 25,000 cells per well in 96-well solid black bottom plates (Greiner Bio-One) pre-coated with 0.1
mg.ml\(^{-1}\) poly-D-lysine. After 24 h growth, cells were washed 3 times in HBSS buffer (Invitrogen, Paisley, UK). 100 µl of HBSS was added to each well and the plates were read using a CLARIOstar fluorescence-compatible reader (BMG Labtech Ltd, Buckinghamshire, UK) using an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The HBSS was then replaced with a further 100 µl / well of HBSS containing a 1:1000 dilution of 10 mg.ml\(^{-1}\) Hoechst 33342 stain (Molecular Probes, Oregon). The plate was incubated at 37°C for 15 min, the HBSS and stain were then removed and the wells washed with 2 × 100 µl HBSS, 100 µl / well of HBSS was added and the plate read using an excitation wavelength of 355 nm and an emission wavelength of 455 nm. The second reading was then used to correct the first for any variations in cell number.

**Deglycosylation reactions**

Cells were washed once and harvested in cold PBS. Pellets were incubated in lysis buffer (150 mM NaCl, 0.01 mM Na\(_2\)PO\(_4\), 2 mM EDTA, pH 7.4, 0.5% n-dodecyl β-D-maltoside and 5% glycerol, supplemented with Complete protease inhibitor cocktail tablets, Roche Diagnostics), on a rotating wheel for 1 hour at 4°C. Samples were then centrifuged at 4°C for 15 min at 14,000 × g and the supernatant aliquoted and its protein concentration assessed, before storage at -80°C. De-glycosylation reactions were set up by adding 1 unit N-glycosidase F to 15 µg protein lysate and 1 × N-glycosidase F buffer (20mM phosphate buffer, pH 7.2) in a final volume of 15 µl. This was then incubated for 2 hrs at 37°C before being analysed by SDS-PAGE.

**Spatial Intensity Distribution Analysis (SpIDA)**

SpIDA was carried out essentially as described in [20]. All regions of interest (RoI) measurements were selected from the basolateral membrane surface. Monomeric equivalent unit (MEU) values for EGFR-mEGFP, Tyr\(^{251}\)Ala,Arg\(^{285}\)Ser EGFR-mEGFP, Robo1-mEGFP, secretin receptor-mEGFP and Gly\(^{243}\)Ala,Ile\(^{247}\)Ala secretin receptor-mEGFP were measured by normalizing their assessed quantal
brightness (QB) values with an average QB value measured from the PM-1-mEGFP construct (all measurements were made using 2% laser power to ensure consistency). To distinguish between monomeric and dimeric/oligomeric species, PM-1-mEGFP MEU occurrence/frequency x-y graphs (MEU bin size = 0.2) were plotted for each MEU value measured during excitation. Such plots revealed a symmetrical distribution of the values and Graphpad Prism normality tests indicated the distributions were Gaussian (see Results and Statistical analyses). The data from each frequency x-y plot of EGFR-mEGFP, Tyr^{251}Ala,Arg^{285}Ser EGFR-mEGFP, Robo1-mEGFP, secretin receptor-mEGFP and Gly^{243} Ala,Ile^{247}Ala secretin receptor-mEGFP were then divided at an MEU value of 1.48, (which represented 75% of the data set, falling within the mean ± 1.5 standard deviations), which was set as the border to distinguish between monomeric and dimeric species in studies where individual MEU values exceeded 1.48.

In the case of EGFR-mEGFP and EGFR-mEGFP, Tyr^{251}Ala,Arg^{285}Ser cells were grown in Lab-Tek 4 well chambered cover-glasses (Thermo Fisher Scientific, Paisley, UK), treated (or not) with Epidermal Growth Factor (EGF) for 10 minutes and then fixed with 4% paraformaldehyde. The cells were kept under PBS at 4°C prior to imaging. Similarly, secretin receptor-mEGFP and Gly^{243} Ala,Ile^{247}Ala secretin receptor-mEGFP cells were also grown in 4 well chambered coverslips prior to treatment (or not) with secretin (concentration and times as indicated) before fixation and storage as above.

**Calculation of receptor density at the cell surface by SpIDA**

SpIDA software also reports the mean fluorescence intensity for each RoI analyzed. The number of EGFR-mEGFP, Tyr^{251}Ala,Arg^{285}Ser EGFR-mEGFP, Robo1-mEGFP, secretin receptor-mEGFP and Gly^{243} Ala,Ile^{247}Ala secretin receptor-mEGFP molecules.μm^{-2} (density) was measured by dividing the mean fluorescence intensity value by the quantified monomeric QB value.

**Statistical analyses**
Variation in receptor number or mean/median of QB produced by treatment with either ligands or with varying concentrations of doxycycline was assessed by either Students t-test or 1-way ANOVA, with the use of Bonferroni’s or Dunnett’s test for multiple comparisons as appropriate. Normality distributions of recovered QB values defined as MEUs were assessed by D’Agostino and Pearson, normality tests (at p > 0.05) and by skewness and Kurtosis assessments. Distributions that failed the normality assessment (at p < 0.05) were considered to be non-Gaussian.

RESULTS

Spatial Intensity Distribution Analysis (SpIDA) can sample confocal laser scanning images to discriminate between distributions of monomers and dimers/oligomers of proteins tagged with Enhanced Green Fluorescent Protein (EGFP) within defined Regions of Interest (RoI) [18-22]. We initially employed Flp-In™ T-REx™ 293 cells stably harboring at the Flp-In™ T-REx™ locus either a single copy of monomeric Ala206Lys EGFP (mEGFP) [24], or a linked tandem of this polypeptide, each form containing an introduced, plasma membrane-anchoring, palmitoylation + myristoylation (P-M) sequence taken from the N-terminus of the Lyn non-receptor tyrosine kinase (Figure 1A). In each case, addition of the antibiotic doxycycline allowed expression of the corresponding protein (Figure 1B) and imaging of the cells confirmed location of the polypeptides at the plasma membrane (Figure 1C (i, ii)). RoI within confocal images of the coverslip attached, basal surface of such cells (Figure 1C (iii, iv)) were subjected to quantal brightness (QB) measurement and SpIDA (Figure 1D). By varying the concentration of the inducer doxycycline a range, from 32-214 molecules.μm⁻² (mean 107.9 ± 2.1, n = 270) (Table 1), of amounts of the PM-1-mEGFP construct was observed (Figure 1D). Importantly, analysis of the PM-1-mEGFP construct across this range of expression levels indicated that the QB values (mean 11.89 ± 0.23 units, n = 270) were normally distributed (Figure 1E (i)) and, therefore, as a monomeric protein, this mean QB value was defined as corresponding to 1.00 monomeric equivalent units (MEU) (Figure 1E (i)). Analysis of the full data set indicated that mean + 1.5 standard deviations of the QB values for PM-1-mEGFP corresponded to 1.48 MEU. In subsequent studies QB values > 1.48 MEU were therefore defined
as ‘larger than monomer’ (see Experimental for details). Equivalent studies were also performed on RoI selected from confocal images of the basolateral surface of cells induced to express PM-2-mEGFP, i.e. the construct containing the tandemly linked pair of mEGFP molecules. This resulted in expression levels (38-224 molecules/μm², mean 101.4 ± 1.5) that were equivalent to those of PM-1-mEGFP (Figure 1D). Now, however, the absolute QB (mean 23.74 ± 0.42 units, n = 360) was 1.99 ± 0.04 times the mean QB recorded for PM-1-mEGFP. This indicates that the tandemly-linked pair of mEGFP molecules is identified in this approach, on average, as corresponding to a ‘dimer’. Analysis of the full data set showed that once more the QB values were normally distributed (Figure 1E (ii)). Here mean + 1.5 standard deviations of the QB corresponded to 3.00 MEU and, therefore, in subsequent studies QB values > 3.00 MEU were considered to be ‘larger than dimer’ (i.e. oligomeric) (Figure 1D). Using this analysis, for PM-1-mEGFP 93.7% of the observations corresponded to ‘monomer’ and 5.9% to ‘dimer’, with less than 0.5% identified as ‘oligomer’. For PM-2-mEGFP 71.1% of the observations corresponded to ‘dimer’, 7.8% ‘oligomer’ and 21.1% ‘monomer’ (Figure 1D).

To provide further confidence in the capacity of SpIDA to define monomeric, dimeric and oligomeric populations of a true transmembrane receptor protein we turned to the wild type Epidermal Growth Factor receptor (EGFR) and a Tyr²⁵¹Ala,Arg²⁸⁵Ser mutant of this receptor [23]. The wild type EGFR is known to move rapidly from being largely monomeric to being predominantly dimeric upon binding of the ligand EGF, whilst the Tyr²⁵¹Ala,Arg²⁸⁵Ser mutant fails to dimerize or signal in response to ligand [23] (Figure 2A). To confirm this expectation, we generated Flp-In™ T-REx™ 293 cells stably harboring either wild type EGFR-mEGFP or Tyr²⁵¹Ala,Arg²⁸⁵Ser EGFR-mEGFP. Following induction of expression with doxycycline cells were treated with or without 100 nM EGF for 10 minutes, cell lysates were generated, resolved by non-denaturing Blue Native PAGE and immunoblotted with an anti-GFP antiserum (Figure 2B). As shown previously [20], addition of EGF to the wild type receptor resulted in a substantial proportion of this polypeptide now migrating as a dimer, whilst in the absence of EGF all the detected receptor was monomeric. However, although expressed at similar levels as the wild type receptor, Tyr²⁵¹Ala,Arg²⁸⁵Ser EGFR-mEGFP remained monomeric after addition of EGF (Figure 2B).
Importantly, the dimeric form of EGFR-mEGFP did not represent a covalently-linked adduct. Addition of SDS to samples prior to resolution by Blue Native PAGE resulted in all of both wild type and Tyr^{251}Ala,Arg^{285}Ser EGFR-mEGFP migrating as monomeric species. The lack of activation of Tyr^{251}Ala,Arg^{285}Ser EGFR-mEGFP by EGF was also highlighted following resolution of untreated and EGF-treated samples by denaturing SDS-PAGE where, although the wild type receptor became phosphorylated on residue Tyr^{1068} in response to the ligand, Tyr^{251}Ala,Arg^{285}Ser EGFR did not (Figure 2C). ROI of confocal images from the basolateral surface of cells induced to express wild type EGFR-mEGFP (Figure 2D) were analysed via the SpIDA software. This showed that across expression levels of between 29-135 molecules,μm^{-2}, whilst this construct was detected predominantly as a monomer (79.2%) in the absence of EGF, it was largely dimeric (87.1%) in the presence of EGF (Figure 2E, Tables 1 and 3). By contrast, although as expected, Tyr^{251}Ala,Arg^{285}Ser EGFR was indeed largely monomeric (94.2%) in the absence of EGF, this distribution was unaltered (monomer 91.7%) following addition of the ligand (Figure 2F, Tables 1 and 3). These results are entirely consistent with the biochemical studies and demonstrate both that ligand-induced receptor dimerization and the lack of capacity of a mutationally modified transmembrane receptor to reach the dimeric configuration can be observed and quantified directly by SpIDA performed on simple confocal images.

To extend these studies we considered the axonal guidance receptor Robo-1. We have previously argued, based largely on analysis of fluorescence resonance energy transfer (FRET) studies that, in the basal state the full length version of this receptor is largely dimeric [21]. In these previous studies we also examined a C-terminally truncated version of Robo-1 (Figure 3A) which although remaining largely dimeric also displayed a monomeric fraction. When expressed, the full length form generated a basolateral expression pattern that was difficult to assess via SpIDA (not shown) we, herein, therefore used the C-terminally truncated form, which did not present this issue. When induced from the Flp-In™ T-REx™ locus of Flp-In™ T-REx™ 293 cells harboring a C-terminally mEGFP tagged form of this construct (Figure 3A), it was expressed predominantly as an apparently 165kDa polypeptide (Figure 3B) located at the plasma (Figure 3C(i)) and basolateral membranes (Figure 3C(ii)). QB analysis indicated
this construct to display mean MEU = 2.04 ± 0.05, n = 128) with particle number ranging from 20-128 μm⁻² (mean 48 ± 1.2) (Table 1). SpiDA analysis indicated this receptor construct to be present predominantly as dimers (79.7%), with smaller proportions of monomer (14.8%) and potential oligomers (5.5%) (Figure 3D).

Harikumar et al., [13] have previously used combinations of bioluminescence resonance energy transfer (BRET) and bi-molecular fluorescence complementation (Bi-FC) to argue that the secretin receptor, a class B GPCR, forms dimers but not higher-order oligomers. Furthermore, they generated evidence that residues within transmembrane domain IV, specifically Gly²⁴³ and Ile²⁴⁷ provide key determinants for this interaction [14]. Following in-frame linkage of mEGFP to the C-terminal tail of either the wild type secretin receptor or a Gly²⁴³Ala,Ile²⁴⁷Ala mutant, CHO-K1 cell lines stably expressing each form were generated (Figure 4A). Images of the basolateral surface of these lines suggested similar distribution of the wild type and mutant receptor (Figures 4B (i, ii)) and direct measures of mEGFP fluorescence indicated each form of the secretin receptor to be expressed as similar levels per cell (wild-type = 1.09 ± 0.04 fluorescence units, Gly²⁴³Ala,Ile²⁴⁷Ala mutant = 1.00 ± 0.03 fluorescence units).

Studies on a number of class A GPCRs, using either SpiDA [20] or single molecule analysis [26], have indicated that oligomeric organization may increase with receptor density. This issue has not been assessed for any class B GPCR. Therefore, to consider this for both wild type and the Gly²⁴³Ala,Ile²⁴⁷Ala secretin receptor mutant, and because these cells lacked a means to regulate receptor expression in an antibiotic-dependent manner, we treated cells expressing either form of the secretin receptor with sodium butyrate, which can activate viral promoters and enhance protein expression in stably transfected cells, when a viral promoter is used to express the protein, and has been shown in a number of cases to produce upregulation of a GPCR of interest [27-28]. Over-night treatment of cells with 5mM sodium butyrate, resulted in extensive upregulation of each form of the receptor, as shown using imaging studies that focused on the basolateral surface of the cells (Figure 5A). This effect was concentration-dependent over a broad range of sodium butyrate concentrations (10 μM to 5 mM) when assessed in immunoblotting studies using an anti-GFP antiserum (Figure 5B) or by employing QB and SpiDA to quantify receptor
levels $\mu$m$^{-2}$ (Figure 5C). Both the wild type secretin receptor-mEGFP and the Gly$^{243}$Ala,Ile$^{247}$Ala secretin receptor- mEGFP mutant were detected as two resolvable species in SDS-PAGE (Figures 5B, 5D). These reflected differentially N-glycosylated species as treatment with N-glycosidase F increased mobility of both forms and favored production of the more rapidly migrating of these species (Figure 5D).

In the cells expressing wild type secretin receptor but not treated with sodium butyrate, QB and SpIDA analysis on RoI selected from confocal images indicated that at the basolateral surface the receptor was expressed at modest levels ($63.6 \pm 1.1$ receptors $\mu$m$^{-2}$, $n = 240$, mean ± SE) whilst equivalent analysis indicated Gly$^{243}$Ala,Ile$^{247}$Ala secretin receptor-mEGFP to be present at $56.3 \pm 1.1$ receptors $\mu$m$^{-2}$, $n = 240$, mean ± SE) (Table 2, Figure 6). Although expressed at similar levels, when calculated and expressed as MEU, data derived from QB analysis indicated that dimeric/oligomeric organization of wild type secretin receptor-mEGFP (MEU = $1.92 \pm 0.05$ (n = 240) (Table 2, Figure 6A) was markedly greater than for Gly$^{243}$Ala,Ile$^{247}$Ala secretin receptor-mEGFP (MEU = $1.07 \pm 0.02$ (n = 240) (Table 2, Figure 6B). SpIDA performed on these data sets indicated the wild type receptor to be predominantly dimeric (59.2%) but a substantial fraction was identified as being monomeric (33.8%), whilst a small proportion (7.1%) was scored as oligomeric (Figure 6A). By contrast, the Gly$^{243}$Ala,Ile$^{247}$Ala mutant secretin receptor was assessed to be predominantly monomeric (90.0%) with the remaining 10.0 % scored as dimeric (Figure 6B). Treatment with 5 mM sodium butyrate increased levels of the wild type secretin receptor by some 3.15 fold (to $200.5 \pm 2.7$ receptors $\mu$m$^{-2}$, $n = 240$, mean ± SE) (Table 2). It also increased receptor organizational complexity (MEU = $2.42 \pm 0.06$, mean ± SE) (Table 2) and this corresponded to a diminution in the proportion of monomeric receptor species (to 14.6%) and an increase in the proportion of oligomeric forms to 22.1% (Figure 6A). By contrast, although treatment with 5 mM sodium butyrate produced an even greater extent of upregulation of Gly$^{243}$Ala,Ile$^{247}$Ala secretin receptor-mEGFP (3.69 fold) (Table 2) this produced only a small increase in calculated population MEU ($1.19 \pm 0.02$) (Table 2), consistent with the bulk of this variant remaining monomeric. A modest increase in the proportion of assessed dimers was also observed at the elevated expression level (Figure 6B) but only a
single isolated observation was consistent with an oligomeric arrangement of the mutant receptor (Figure 6B).

The secretin receptor couples predominantly to the G protein Gs to cause elevation of cAMP levels upon addition of the hormone secretin, and in these cells did so with pEC50 10.63 ± 0.10 (mean ± SEM, n = 5) for the hormone (Figure 7A). As Gs is clearly able to interact with the secretin receptor as part of a signalling complex we then considered whether the G protein might contribute to stability of the dimeric form of the receptor. Although cholera toxin is most often considered as an activator of Gs and therefore of adenylyl cyclase activity and cAMP levels, sustained treatment of cells with this toxin is well known to cause a marked down-regulation of Gαs levels [29-30]. After treating cells expressing the wild type secretin receptor with cholera toxin (5 μg.ml-1, 24 h), extensive downregulation of Gαs was confirmed in immunoblotting studies (Figure 7B). This did not intrinsically appear to alter the basolateral distribution of the receptor (Figure 7C). QB analysis and SpiDA confirmed that treatment with cholera toxin did not affect levels of the receptor (untreated 83.5 ± 2.6 molecules.μm-2, treated 75.1 ± 3.1 molecules.μm-2) (Table 2) or its distribution profile (Figure 7D). Moreover, although treatment with cholera toxin did appear to result in small shifts in the dimer/monomer proportions of the secretin receptor to favour the monomeric state, this did not reach statistical significance (Figures 7E, 7F). These studies indicate that although it would certainly have been a reasonable prediction that availability of cognate G protein could contribute to dimer stability, in the case of the secretin receptor this plays a minimal role and again suggests that key interaction affinity is provided by residues of transmembrane domain IV, with particular roles for Gly243 and Ile247.

Finally we assessed if treatment with secretin might alter the organizational structure of the secretin receptor-mEGFP constructs. A concentration of secretin (100 nM) substantially greater than needed to fully activate cAMP production (Figure 7A) was added to cells expressing either wild type secretin receptor-mEGFP or Gly243Ala,Ile247Ala secretin receptor-mEGFP and the cellular distribution of the mEGFP tag was monitored over time. Substantial levels of internalization of both forms of the receptor were observed at time points beyond 15 minutes (Figure 8A). To assess if secretin produced
rapid effects on receptor organizational structure we therefore fixed cells and employed SpIDA 10 minutes after addition of secretin (Figure 8B). No statistically significant effect of the ligand was observed on the proportions of monomer, dimer or oligomer forms for the wild type receptor (Figure 8B(i)) whilst a statistically significant increase in mean MEU (p = 0.0004) (Table 2) was detected for Gly243Ala,Ile247Ala secretin receptor-mEGFP, corresponding to an increase in dimeric, and corresponding reduction in monomeric, forms (Figure 8B(ii)) of the mutated receptor.

DISCUSSION

The tendency for GPCRs to associate with themselves or with other family members is broadly aligned with receptor family, with the class C, glutamate-like GPCRs forming the most stable, essentially obligate dimers/oligomers [3], the class A GPCRs often associating quite transiently [7] and via diverse interfaces, and the class B GPCRs believed to be intermediate between the other two classes [1-2]. The prototypic class B secretin receptor has been reported to associate along the lipid face of transmembrane domain IV to form homodimers, with no contribution of either extracellular or intracellular regions of this receptor [13-14]. These dimeric complexes can be destabilized or disrupted by competition with a peptide corresponding to the sequence of transmembrane domain IV or by mutating at least two residues along the lipid face of this helix in an intact secretin receptor construct [14]. Thus, whilst it is known that the secretin receptor is capable of forming and existing as homo-dimeric complexes, the percentage of receptors involved in such complexes relative to those in monomeric form has not been established, and the techniques used to date to examine the quaternary organization of the secretin receptor are poorly suited to assess this.

In the currently reported studies, we clearly demonstrate the power of SpIDA [18-19] to determine the percentage of a population of cell surface receptors at steady state that remain free as monomers versus those present as dimeric complexes. As proof of concept this was very clearly validated using the EGFR, known to form dimeric complexes in an agonist-dependent manner that are required for
biological activity. The ability to disrupt agonist-induced formation of such complexes using a well-established EGFR mutant [23] was similarly demonstrated.

In applying the SpIDA methodology to a class B GPCR receptor for the first time, we demonstrate that under stable, equilibrium conditions and with modest expression levels, some 60% of the secretin receptors on the cell surface are involved in homo-dimeric complexes, with the remainder predominantly in monomeric form. This is substantially higher than has been reported for a number of class A GPCRs [7, 22, 26], but lower than for the class C GPCRs where dimerization/oligomerization appears to be an intrinsic and integral feature of their structure and mode of action [3]. Few, if any higher-order secretin receptor complexes were identified using this technique at lower expression levels and this may reflect that the interface appears to be formed specifically by symmetrical transmembrane domain IV-transmembrane domain IV interactions [14] and that this organizational structure may well be common amongst class B GPCRs [11, 31-32]. Whilst larger oligomeric complexes of many class A receptors have been observed [1-2] this appears to be highly receptor concentration-dependent [20, 26] and that for different members of the family distinct transmembrane domains, and even regions out-with the core seven-transmembrane domain structure of the receptors, may contribute to such interactions [1-2]. Like the class A GPCRs, induction of high levels of expression of the secretin receptor by treatment of cells with a maximally effective concentration sodium butyrate did result in the appearance of a significant proportion of oligomeric species, but the molecular basis for this organization remains to be explored. It was also shown that mutating residues along the lipid face of transmembrane domain IV of the secretin receptor was capable of disrupting the dimeric complexes, resulting in almost exclusive presence of monomeric forms of this receptor. This, too, provides support for a single, structurally well-defined interface determining the production of secretin receptor homodimers, with the ability to disrupt such complexes by engineering changes in that single region [14]. Little is currently understood about the dynamics of complex formation for the class B GPCRs. The current demonstration of an equilibrium between secretin receptor monomers, homo-dimers and oligomeric forms suggests that there is likely a
stable rate of association and dissociation of these complexes. However, it remains unclear what these rates are or what might influence them.

An unanswered issue in many studies of GPCR organizational state, despite a large number of reports on the topic [1] is the effect of ligands on the distribution between these forms. Recently, for both serotonin 5-HT2c receptor [20] and the muscarinic M1 acetylcholine receptor [22] we have used SpIDA to show that certain, but not all chemical classes, of antagonists at these receptors can either increase [22] or decrease [20] the proportion of receptors within such complexes. There are no useful small molecule antagonists of the secretin receptor. However, although sustained treatment with the agonist ligand secretin causes internalization of both the wild type and Gly243Ala,Ile247Ala mutant secretin receptor, and this can confound analysis and interpretation of SpIDA we show that, at least prior to receptor internalization, interaction with secretin does not alter the structural organization of the wild type receptor. Interestingly, we did note a significant increase in the proportion of the Gly243Ala,Ile247Ala mutant detected as being dimeric. Such data must be interpreted with caution, however. Although it is clear from studies with the Gly243Ala,Ile247Ala mutant that the secretin receptor can internalize from the cell surface as a monomer, the observed increase in ‘dimeric’ species may reflect clathrin-induced receptor proximity rather than genuine enhanced protein-protein interactions between the receptor monomers.

It is important to appreciate that unlike methods based on single molecule detection [7, 26, 33-36], SpIDA provides a statistical sampling of fluorescent properties of suitably labelled molecules within individual RoI [18], and in so doing indicates the likelihood of monomers, dimers or oligomeric predominating in each RoI. As such there is a need to sample many RoI and to apply statistical inference to reach conclusions. As noted in Results, analysis of the distribution of measured QB values for both PM-1-mEGFP and PM-2-mEGFP showed these to be normally distributed. As such, definition of ‘larger than monomer’ in the experimental studies, as those observations having QB > 1.48 MEU, and > 3.00 MEU as being ‘oligomeric’ reflect selection above 1.5 standard deviations greater than the mean QB for the fluorescent molecule used for calibration. This gate could have been set at an even more stringent
level, e.g. 2.0. standard deviations above the mean value i.e. > 1.64 MEU, to be scored as dimeric. This would have altered absolute estimates of percentages of monomer versus dimer, i.e. for the EGFR the same data set would have returned 85% monomer and 15% dimer in the basal state and only 78.3% dimer and 20.8% monomer in the presence of the agonist ligand (Table 3), this would not change the conclusion that binding of EGF moves the monomer-dimer equilibrium substantially towards the dimer. Similarly, although providing slightly different percentages of monomer to dimer for the secretin receptor this would have not altered, and indeed would further strengthened, the conclusion that the Gly\textsuperscript{243}Ala,Ile\textsuperscript{247}Ala is largely monomeric.

Possible contributors to the stability of GPCR complexes are GPCR-interacting proteins, with the most obvious being the predominant G protein that interacts with the receptor to allow signal transduction to occur. In the case of the secretin receptor this is the cAMP-stimulatory G protein \( G_s \). By treating cells with cholera toxin for a sustained period \( G_s \) is downregulated [29-30]. Herein, immunoblotting to detect \( G_s \) showed this process to be very effective. Despite this SpIDA was able to demonstrate that this manoeuvre had no substantial impact on the state of association of the secretin receptor. Importantly whilst the homodimeric secretin receptor complex has been reported to be functionally important, contributing to high affinity binding and agonist biological activity [11, 14], this suggests that the G protein association event is not required for formation or stabilization of this complex.

Observations of internalization of the non-ligand-occupied GPCR along with the ligand-occupied GPCR involved in class B GPCR heterodimers suggest a relatively prolonged presence of such structures [37]. In another report, co-expression of GIP receptors that did not internalize in response to GIP with the GLP-1 receptors that did internalize in response to their natural ligand, were able to slow or inhibit the latter process [38]. This also supports the prolonged presence of these complexes, but will need to be studied more extensively for other class B family members. As with other GPCR classes, agonist-induced internalization of class B receptors, including the secretin receptor, appears to occur via clathrin-mediated endocytosis and involves a key role for arrestin isoforms that interact with the receptor in an agonist-dependent manner. As presently implemented, SpIDA effectively samples and assesses the organization
of fluorescent complexes at the basolateral surface of cells in contact with a glass coverslip, then proteins that move away from this surface and internalize within the cell become invisible to the analysis. As such, although we were able to demonstrate that relatively short term addition of a receptor saturating concentration of secretin did not alter the organizational structure of the wild type receptor, it would be of great interest to assess if the secretin receptor remains predominantly dimeric when internalization of the agonist-occupied receptor is achieved.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: GM, LJM and RJW, developed and co-ordinated the project, RJW and JDP performed the experiments, KGH provided novel tools and reagents. GM, RJW and LJM, with the assistance of others, wrote the manuscript.

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Tables

Table 1  Details of analysis of mEGFP-tagged control constructs used

<table>
<thead>
<tr>
<th>Construct and treatment</th>
<th>Number of RoIs analysed</th>
<th>Mean MEU value</th>
<th>Range of expression.μm⁻²</th>
<th>Average expression.μm⁻²</th>
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<tr>
<td>PM-1-mEGFP</td>
<td>270</td>
<td>1.00 ± 0.02</td>
<td>32-214</td>
<td>107.9 ± 2.1</td>
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<td>PM-2-mEGFP</td>
<td>360</td>
<td>1.99 ± 0.04</td>
<td>38-224</td>
<td>101.4 ± 1.5</td>
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<tr>
<td>EGF-R-mEGFP (UT)</td>
<td>240</td>
<td>1.22 ± 0.33</td>
<td>29-135</td>
<td>55.0 ± 1.2</td>
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<tr>
<td>EGF-R-mEGFP (100nM EGF)</td>
<td>240</td>
<td>1.97 ± 0.03</td>
<td>20-121</td>
<td>62.1 ± 1.4</td>
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<tr>
<td>EGF-R Y251A R285S-mEGFP(UT)</td>
<td>240</td>
<td>1.14 ± 0.02</td>
<td>22-156</td>
<td>56.2 ± 1.3</td>
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<tr>
<td>EGF-R Y251A R285S-mEGFP(100 nM EGF)</td>
<td>240</td>
<td>1.11 ± 0.02</td>
<td>24-140</td>
<td>53.1 ± 1.3</td>
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<td>(Truncated) Robo1-mEGFP</td>
<td>128</td>
<td>2.04 ± 0.05</td>
<td>20-128</td>
<td>48 ± 1.2</td>
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UT = untreated
Table 2 Details of analysis of mEGFP-tagged secretin constructs and treatments used

<table>
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<tr>
<th>Construct and treatment</th>
<th>Number of RoIs analysed</th>
<th>Mean MEU value</th>
<th>Range of expression, μm⁻²</th>
<th>Average expression, μm⁻²</th>
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<tr>
<td>hSecR-mEGFP (UT)</td>
<td>240</td>
<td>1.92 ± 0.05 ᵇ</td>
<td>28-124</td>
<td>63.6 ± 1.1</td>
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<tr>
<td>hSecR-mEGFP (5 mM sodium Butyrate)</td>
<td>240</td>
<td>2.42 ± 0.06 ᵃ</td>
<td>76-287</td>
<td>200.5 ± 2.7</td>
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<td>hSecR G²⁴³A I²⁴⁷A-mEGFP (UT)</td>
<td>240</td>
<td>1.07 ± 0.02</td>
<td>22-146</td>
<td>56.3 ± 1.5</td>
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<tr>
<td>hSecR G²⁴³A I²⁴⁷A-mEGFP (5 mM sodium Butyrate)</td>
<td>240</td>
<td>1.19 ± 0.02 ᶜ</td>
<td>95-296</td>
<td>207.9 ± 2.5</td>
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<tr>
<td>hSecR-mEGFP (UT)</td>
<td>240</td>
<td>1.92 ± 0.04</td>
<td>29-206</td>
<td>83.5 ± 2.6</td>
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<td>hSecR-mEGFP (5 μg.ml⁻¹ Cholera Toxin)</td>
<td>240</td>
<td>1.85 ± 0.05</td>
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<td>75.1 ± 3.1</td>
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<td>hSecR-mEGFP (UT)</td>
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<tr>
<td>hSecR-mEGFP (100 ng.ml⁻¹ secretin)</td>
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<td>2.23 ± 0.06</td>
<td>29-159</td>
<td>80.0 ±1.8</td>
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<tr>
<td>hSecR G²⁴³A I²⁴⁷A-mEGFP (UT)</td>
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<td>1.25 ± 0.03</td>
<td>27-200</td>
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<td>hSecR G²⁴³A I²⁴⁷A-mEGFP (100 ng.ml⁻¹ secretin)</td>
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<td>1.43 ± 0.04</td>
<td>9-240</td>
<td>72.5 ± 2.3</td>
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UT = untreated

ᵃ greater than hSecR-mEGFP UT,  p = 0.0005
ᵇ greater than hSecR G²⁴³A, I²⁴⁷A-mEGFP,  p < 0.0001
ᶜ greater than hSecR G²⁴³A, I²⁴⁷A-mEGFP UT,  p = 0.004
Table 3 Analysis of monomer to dimer proportions for the unoccupied and EGF-stimulated EGFR estimated at different statistical stringency

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<th>Cell line</th>
<th>Treatment</th>
<th>Quaternary structure</th>
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<th>2 × Standard Deviations</th>
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<td>EGFR-mEGFP</td>
<td>UT</td>
<td>monomers</td>
<td>79.2% [&lt; 1.48 MEU]</td>
<td>85% [&lt; 1.64 MEU]</td>
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<td></td>
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<td>dimers</td>
<td>20.8% [&gt; 1.48 MEU]</td>
<td>15% [&gt; 1.64 MEU]</td>
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<tr>
<td></td>
<td></td>
<td>oligomers</td>
<td>0% [&gt; 3.00 MEU]</td>
<td>0% [&gt; 3.33 MEU]</td>
</tr>
<tr>
<td>EGFR-mEGFP</td>
<td>100 nM EGF 15 min</td>
<td>monomers</td>
<td>10.8%</td>
<td>20.8%</td>
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<tr>
<td></td>
<td></td>
<td>dimers</td>
<td>87.1%</td>
<td>78.3%</td>
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<td></td>
<td></td>
<td>oligomers</td>
<td>2.1%</td>
<td>0.8%</td>
</tr>
<tr>
<td>EGFR Y\textsuperscript{251}A,R\textsuperscript{285}S-mEGFP</td>
<td>UT</td>
<td>monomers</td>
<td>94.2%</td>
<td>96.7%</td>
</tr>
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<td></td>
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<td>dimers</td>
<td>5.4%</td>
<td>2.9%</td>
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<td>oligomers</td>
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<td>EGFR Y\textsuperscript{251}A,R\textsuperscript{285}S-mEGFP</td>
<td>100 nM EGF 15 min</td>
<td>monomers</td>
<td>91.7%</td>
<td>96.7%</td>
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<td>dimers</td>
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<td></td>
<td>oligomers</td>
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<td>0%</td>
</tr>
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UT = untreated
Figure Legends

Figure 1 Calibration of SpIDA analysis using plasma membrane-attached forms of monomeric EGFP
Cartoon illustration of the plasma membrane interaction of PM-1-mEGFP and PM-2-mEGFP constructs. The green ellipses signify molecules of mEGFP (A). B. Immunoblot analysis of the membrane expression of PM-1-mEGFP and PM-2-mEGFP following doxycycline-induced expression in Flp-In™ T-REx™ 293 cells using an anti-GFP antiserum. Corresponding immuno-detection of tubulin provided loading controls. C. Confocal images of Flp-In™ T-REx™ 293 cells expressing either PM-1-mEGFP (i, iii) or PM-2-mEGFP (ii, iv) across the centre of cells (i, ii) or at the basolateral surface (iii, iv). Scale bar = 20 μm. D. SpIDA analysis displaying the full data set captured from individual RoI presented as Monomeric Equivalent Units (MEU) versus levels per μm². Blue = PM-1-mEGFP, Red = PM-2-mEGFP. Values corresponding to mean + 1.5 SD are shown (1.48 MEU for PM-1-mEGFP, 3.00 MEU for PM-2-mEGFP) as dotted lines. Proportions of each construct scored as monomer, dimer and oligomer are shown. E. Individual QB values, presented as MEU, for both PM-1-mEGFP (Ei) and PM-2-mEGFP (Eii) displayed Gaussian distribution.

Figure 2 The EGFR undergoes ligand-induced dimerization whilst Tyr²⁵¹Ala, Arg²⁸⁵Ser EGFR does not
Cartoon illustrating the potential for the binding of EGF (yellow) to promote dimerization of wild type EGFR, whilst the mutations (red) in Tyr²⁵¹Ala,Arg²⁸⁵Ser EGFR prevent this (A). B. Blue Native PAGE analysis of wild type EGFR and Tyr²⁵¹Ala,Arg²⁸⁵Ser EGFR treated or not with EGF (100 nM, 10 min). In the two right hand lanes samples were treated with 1% (w/v) SDS prior to addition to the gel. Lanes labelled – Dox were not induced to express the receptor constructs. Results show anti-GFP immunostaining. C. EGF promotes phosphorylation of Tyr¹⁰⁶⁸ in wild type EGFR but not in the Tyr²⁵¹Ala, Arg²⁸⁵Ser mutant. Samples resolved by SDS-PAGE and immunoblotted to detect either total
EGFR levels (left hand side) or Tyr\textsuperscript{1068} phosphorylation (right hand side). D. basolateral images of Flp-In\textsuperscript{TM} T-REx\textsuperscript{TM} 293 cells induced to express wild type EGFR (i, ii) or Tyr\textsuperscript{251}Ala, Arg\textsuperscript{285}Ser EGFR (iii, iv) treated with vehicle (i, iii) or EGF (ii, iv). E. SpIDA of RoI from cells expressing wild type EGFR, untreated (green) or treated with EGF (purple). F. SpIDA of RoI from cells expressing Tyr\textsuperscript{251}Ala, Arg\textsuperscript{285}Ser EGFR, untreated (green) or treated with EGF (purple). Proportions of each construct scored as monomer, dimer and oligomer are shown.

**Figure 3 A Robo-1 receptor construct is predominantly dimeric**

Cartoon illustrating the C-terminally truncated form of Robo-1 used in these studies and its relationship to the full length protein (A). B. Immuno-detection of this construct following induction (+ doxycycline) in Flp-In\textsuperscript{TM} T-REx\textsuperscript{TM} 293 cells. C. Cross sectional (i) and basolateral (ii) confocal images of these cells following induction of expression of the Robo-1 construct. Scale bar = 20 μm. D. SpIDA analysis of the organizational state of the Robo-1 construct as in Figure 1. Proportions scored as monomer, dimer and oligomer are shown.

**Figure 4 Expression of wild type secretin receptor and Gly\textsuperscript{243}Ala, Ile\textsuperscript{247}Ala secretin receptor in CHO-K1 cells**

Immunoblotting cell membrane preparations of non-transfected CKO-K1 cells or those expressing either wild type secretin receptor-mEGFP or Gly\textsuperscript{243}Ala, Ile\textsuperscript{247}Ala secretin receptor- mEGFP with an anti-GFP antiserum (A, upper panel). Equivalent detection of Na\textsuperscript{+}/K\textsuperscript{+} ATPase provided loading controls (A, lower panel). B. Representative confocal images of the basolateral membrane of the receptor expressing cells (i) wild type secretin receptor, (ii) Gly\textsuperscript{243}Ala, Ile\textsuperscript{247}Ala secretin receptor. Scale bar = 20 μm.

**Figure 5 Treatment with sodium butyrate upregulates levels of both wild type Gly\textsuperscript{243}Ala, Ile\textsuperscript{247}Ala secretin receptor**
Images of the basolateral surface of CHO cells expressing wild type (Ai,ii) or Gly^{243}Ala, Ile^{247}Ala (Aiii,iv) secretin receptor that were untreated (Ai, iii) or treated overnight with 5 mM sodium butyrate (Aii,iv) are shown. B. Samples of cell lysates generated from CHO cells expressing wild type (i) or Gly^{243}Ala, Ile^{247}Ala (ii) secretin receptor treated with the indicated concentrations of sodium butyrate were resolved by SDS-PAGE and immunoblotted with an anti-GFP antiserum. Corresponding immuno-detection of tubulin provided loading controls. C. Receptor levels assessed via quantal brightness analyses are shown with treatment with varying concentrations of sodium butyrate. D. Samples as in B (treated with 5 mM sodium butyrate) were treated with or without N-glycosidase, resolved by SDS-PAGE and immunoblotted with an anti-GFP antiserum (upper panels). Corresponding immuno-detection of tubulin (lower panels) provided loading controls.

**Figure 6** SpIDA analysis indicates the wild type secretin receptor to be a mixture of monomers and dimers whilst Gly^{243}Ala, Ile^{247}Ala secretin receptor is largely monomeric

QB and SpIDA was performed on CHO-K1 cells expressing either wild type secretin receptor (A) or Gly^{243}Ala, Ile^{247}Ala secretin receptor (B) that were either untreated (red) or treated overnight with 5 mM sodium butyrate (green). Proportions of data corresponding to monomer, dimer or oligomer are shown.

**Figure 7** Substantial downregulation of G{alpha} does effect secretin receptor quaternary organization

CHO-K1 cells expressing the wild type secretin receptor were treated with various concentrations of secretin and cAMP levels measured. A representative example of n = 5 is shown (A). B. CHO-K1 cells expressing the wild type secretin receptor were then treated with vehicle or with cholera toxin for 24 hours. Lysates from these cells were then immunoblotted to detect G{alpha} (upper panel). Immunoblotting for tubulin provided loading controls (lower panel). C. Confocal images of the basolateral surface of cells treated with vehicle (i) or cholera toxin (ii) are shown. Scale bar = 20 μm. D. QB analysis and SpIDA were performed on such samples. Each data point represents a unique RoI. Untreated = red.
Cholera toxin-treated = yellow. Proportions of predicted monomer, dimer and oligomer are shown. E., F. Statistical analysis of the outcomes, E. calculated based on % of monomer, dimer and oligomer. F. calculated based on % of monomer versus dimer + oligomer. ns = not significantly different.

**Figure 8 Short term treatment with secretin does not alter the quaternary organization of the wild type secretin receptor**

CHO-K1 cells expressing the wild type (Ai) or Gly^{243}Ala, Ile^{247}Ala (Aii) secretin receptor were untreated (UT) or treated with 100nM secretin for the indicated times. They were then fixed and imaged to identify receptor attached mEGFP. B. Wild type (Bi) or Gly^{243}Ala, Ile^{247}Ala (Bii) cells were treated for 10 mins with vehicle (green) or 100 nM secretin (red), images were analysed by SpIDA, each data point represents a unique RoI. Proportions of predicted monomer, dimer and oligomer are shown.
FIGURE 1

(A) (B)

(C) (i) (ii) (iii) (iv)

(D)

(E) (i) (ii)
FIGURE 2

(A) mEGFP

(B) EGFR-

Y^{251}A,R^{285}S

100ng.ml^-1
doxycycline
100nM EGF
1% SDS

(C) 100ng.ml^-1
doxycycline
100nM EGF

(D)(i) (ii) (iii) (iv)

(E) Monomeric equivalent

unit (based upon 11.89)

(Untreated) 0%

100 nM EGF

2.1%

20.8%

87.1%

79.2%

10.8%

(F) Monomeric equivalent

unit (based upon 11.89)

(Untreated) 0.4%

100 nM EGF

5.4%

8.3%

94.2%

91.7%
FIGURE 3

(A) Full length mRobo1 → SS C-myc SNAP mRobo1 → mRobo1

Transmembrane region

Truncated mRobo1 → SS C-myc SNAP mRobo1 → mEGFP

(B) **kDa**

- 260
- 160
- 125
- 90
- 70
- 50
- 38
- 30
- 25
- 15

100ng.ml\(^{-1}\) doxycycline

α-GFP

α-tubulin

(C) (i) (ii)

(D) Monomeric equivalent unit (based upon 11.89)

- Digitonin
- MEGFP

Truncated mRobo1

- 5.5%
- 79.7%
- 14.8%

Receptor number. \(\mu m^{-2}\)
FIGURE 4

(A) 

<table>
<thead>
<tr>
<th>kDa</th>
<th>“empty” CHO cells</th>
<th>CHO hSecR mEGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G^{243}A, I^{247}A, WT</td>
</tr>
<tr>
<td>260</td>
<td></td>
<td>243, 247</td>
</tr>
</tbody>
</table>

Expected size of hSecR-mEGFP

α-GFP

α-sodium potassium ATPase

(B) (i) 

CHO hSecR mEGFP

(ii) 

CHO hSecR G^{243}A, I^{247}A mEGFP

Scale: 20 μm
FIGURE 6

(A) 

Receptor number. μm⁻²

Monomeric equivalent unit (based upon 11.89)

0 mM sodium butyrate
7.1%  22.1%

5 mM sodium butyrate
59.2%  63.3%

(B) 

Receptor number. μm⁻²

Monomeric equivalent unit (based upon 11.89)

0 mM sodium butyrate
0%  0.4%

5 mM sodium butyrate
10%  18.3%

90%  81.3%
FIGURE 8

(A) (i) UT 5 min 10 min 15 min 30 min 60 min

(ii)

(B) (i)

Receptor number, μm⁻²

Monomeric equivalent unit (based upon 11.89)

no treatment 10 min 10⁻⁷M secretin

14.8% 15.7%

62.9% 66.2%

22.4% 18.1%

(ii)

Receptor number, μm⁻²

Monomeric equivalent unit (based upon 11.89)

no treatment 10 min 10⁻⁷M secretin

1.4% 2.9%

22.9% 34.8%

75.7% 62.4%