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The role of dimerization in the cellular trafficking of G protein-coupled receptors

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Abstract

The concept that G protein-coupled receptors can exist as homo- and/or hetero-meric complexes is now well established. Despite this, how dynamic such interactions are and if this may be modulated during receptor trafficking remain topics of debate. Use of endoplasmic reticulum trapping strategies and the generation of asymmetric homo-mers have started to provide information on the contribution of protein-protein interactions to receptor maturation, cell surface delivery and ligand-mediated endocytosis. Although dimer/oligomer formation appears to be essential for cell surface delivery of class A and class C GPCRs, this may not be the case for class B receptors.

Abbreviations

BRET, bioluminescence resonance energy transfer; CFP, cyan fluorescent protein; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptors; TMD, transmembrane domain; YFP, yellow fluorescent protein;

Introduction

Following reconstitution of single, purified monomeric class A G protein-coupled receptors (GPCRs) into high-density lipoprotein phospholipid bilayer particles agonist ligands are able to activate added G protein [1-2]. Although such observations demonstrate clearly that monomeric GPCRs are able to initiate canonical, G protein-mediated signal transduction, and there are suggestions that certain observations consistent with GPCR quaternary structure have been over-interpreted [3], substantial evidence indicates that GPCRs are able to form and exist as dimers and/or higher-order oligomers [4-5]. The class C, metabotropic glutamate receptor-like $GABA_B$ receptor was the first GPCR recognized to require quaternary structure for cell surface delivery and function (see [6] for review). The functional $GABA_B$ receptor is a constitutive hetero-dimer (or possibly a hetero-tetramer [7]) formed by direct interactions between the polypeptide products of two distinct, but highly related genes. When expressed alone, the $GABA_B$R1 polypeptide is retained in the endoplasmic reticulum (ER) due to the presence of an ER-retention sequence in the intracellular C-terminal tail. Co-expression of the $GABA_B$R2 polypeptide generates protein-protein interactions that result in the retention motif being masked and allows cell surface delivery of the hetero-complex. Despite there being no substantial sequence conservation between class C GPCRs and members of other GPCR families, models that explore the importance of successful pre-assembly of dimers/oligomers in the ER have been explored recently for other classes of GPCRs. The proportion of GPCR populations that exist as dimeric/oligomeric complexes has also been a topic of recent debate, as has whether GPCRs internalize as dimers/oligomers following exposure to agonist ligands. These topics will form the basis of the current review.

Where do GPCR dimers/oligomers form?

Based on understanding the ER assembly of the heteromeric $GABA_B$ receptor complex Salahpour et al., [8] replaced the C-terminal tail of the rhodopsin-like, class A β_2-adrenoceptor with the equivalent region of the $GABA_B$R1. This generated a form of the β_2-adrenoceptor that was trapped inside transfected cells. This construct was also able to prevent cell surface delivery of co-expressed wild type β_2-adrenoceptor and retained the wild type receptor in the ER [8]. These observations were consistent with the C-terminally modified receptor interacting with the wild type receptor but with an inability of wild type β_2-adrenoceptor to mask the $GABA_B$R1 ER-retention motif introduced into the modified β_2-adrenoceptor. Furthermore, these studies also indicated that homo-dimerization of the β_2-adrenoceptor was unlikely to be governed largely or exclusively by interactions involving the C-terminal tail. Although other GPCRs closely related to the β_2-adrenoceptor were not tested, cell surface delivery of an N-terminally epitope-tagged form of the β_2-adrenoceptor was substantially higher when co-expressed with the full length $GABA_B$R1 polypeptide than with the β_2-adrenoceptor containing the $GABA_B$R1 C-terminal tail and ER-retention motif [8]. Importantly, bioluminescence resonance energy transfer (BRET) studies indicated little interaction between the β_2-adrenoceptor and $GABA_B$R1 [8], consistent with the concept that physical interactions between the wild type and the ER-retained, modified form of the β_2-adrenoceptor, rather than simply the presence of an ER-retained protein, was the basis for poor cell surface delivery of the wild type receptor. This general approach has the potential to be used to explore the selectivity and location of GPCR dimerization but is not restricted to use of the $GABA_B$R1 C-terminal ER trapping sequence. A number of other GPCRs, for example the α_{2C}-

adrenoceptor [9], contain what appear to be arginine-based ER-retention motifs. Indeed, transient expression of the α_{2C}-adrenoceptor in HEK293 cells results in a pattern of expression consistent with predominant ER retention [9]. After addition of this ER-retention motif to the C-terminal tail of the chemokine CXCR1 receptor, Wilson et al., [9] noted both that the modified chemokine receptor was unable to reach the cell surface and that the ER-trapped CXCR1 receptor was able to limit cell surface delivery of co-expressed, wild type CXCR1. Furthermore, the ER-retained CXCR1 receptor also prevented cell surface delivery of the closely-related chemokine receptor CXCR2 [9], providing a clear example of the generation within the ER of a heteromer between closely related class A receptors. By contrast, co-expression of the ER-trapped CXCR1 receptor did not modulate cell surface delivery of the α_{1A}-adrenoceptor [9]. This was an important control because there are often concerns about the specificity of GPCR dimerization, particularly in transient transfection studies where it is difficult to define expression levels in individual cells. Because parallel saturation BRET studies [10] indicated that CXCR1 generated homomers and CXCR1-CXCR2 heteromers with equal efficiency, and also confirmed that the CXCR1 receptor did not interact with the α_{1A}-adrenoceptor [9], such studies also suggested that the ‘dominant-negative’ effects of the ER-retained receptor to prevent surface delivery were correlated with, and might reflect, dimerization potential. Since these studies a number of other reports have generated data consistent with physical interactions between GPCR monomers being initiated within the ER. For example, an ER-retained mutant of the α_{2B}-adrenoceptor is able to cause ER-retention of wild type α_{2B}-adrenoceptor [11] as well as of the α_{2A}-and α_{2C}-adrenoceptors [11]. Furthermore, introduction of the ER-retained α_{2B}-adrenoceptor mutant into cells that express various α_2-adrenoceptor subtypes endogenously resulted in reduced cell surface delivery of the endogenous α_2-adrenoceptor [11]. Fluorescence resonance energy transfer (FRET) measurements performed in cells co-expressing C-terminally cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged serotonin 5-HT_{2C} receptors also identified interactions within the ER and, via time-lapse miscoscopy, such complexes were followed through the Golgi apparatus and to the plasma membrane [12]. As FRET efficiencies measured in the ER, Golgi, and plasma membrane compartments were similar, this suggested that the 5-HT_{2C} receptor remained a dimeric or oligomeric complex as it trafficked to the cell surface after synthesis [12]. The ability to measure GPCR-GPCR interactions in cell fractions isolated from sucrose density and other gradient systems, initially employing time-resolved FRET measurements [13] and, more recently BRET studies [14], has also allowed demonstration of receptor homomers in distinct cellular compartments [13-14]. Although not providing direct evidence on the location of biogenesis of GPCR dimers, such studies have confirmed populations of receptor homomers to co-migrate with ER markers, such as the protein-folding chaperone calnexin, as well as plasma membrane markers such as the Na^+/K^+ ATPase [13-14]. Co-localization with calnexin and related chaperone proteins is of particular relevance as direct interactions of a number of GPCRs with calnexin has been shown [15-16] and relate to the progress of receptors from the ER if they are correctly folded.

It is unclear how, subsequently, variation in identity of the final transport vesicle populations for different GPCRs is determined, but a series of elements, often in different regions of the receptor sequence, can modulate export from the ER [17-18], and in many GPCRs there may be multiple such elements. However, recently, a single, highly conserved Leu residue in the first intracellular loop has been suggested to play an important role in ER export for a large number of GPCRs, including a range of adrenoceptors and the angiotensin AT_1 receptor [19] and mutation of this residue may offer a general means to generate ER-retained mutants that could be used in the types of studies described above.

Pathways of cell surface delivery of GPCRs are less well studied than pathways of receptor internalization from the cell surface, but despite clear evidence of distinct vesicular pools being favoured by individual receptors [18, 20], the early stages of ER-quality control involving interactions with ER-resident chaperone proteins, and the subsequent processing of N-linked glycosylation, as GPCRs move from the ER to the Trans-Golgi Network are similar to other proteins that are destined to be trafficked via the secretory pathway. These ensure that only properly folded and assembled proteins proceed. In this regard it is important to note that it is well established that the quaternary structure of many other classes of cell surface receptors, such as ion channels and transforming growth factor receptors, as well as certain ATP binding cassette transporters, are pre-fabricated into the correct quaternary organization before cell surface delivery [21-22]. It is, therefore, hardly surprising that this general rule should also apply to GPCRs, and there is growing evidence that such quaternary complexes may contain G protein subunits as well the GPCR(s) [23]. There have been sporadic reports of the inability to replicate specific examples of GPCR heteromerization and, clearly, it is important to examine potential reasons for these discrepancies. For example, although the proclivity of the angiotensin AT_1 receptor to form heteromers with the bradykinin B2 receptor has recently been questioned [24], it has also recently been suggested that the expression level of the chaperone protein calreticulin defines the effectiveness of these interactions [25] as only the properly folded and fully mature bradykinin B2 receptor is reported to interact with the angiotensin AT_1 receptor [25]. If this is a general feature, it may be helpful in defining the earliest stages of GPCR heteromer generation and, potentially, also homomer formation. In this context it is also interesting to note that cell surface delivery of a DOP-MOP opioid receptor heteromer can be controlled by a Golgi chaperone named RTP4 [26], potentially via RTP4 protecting the DOP-MOP heteromer from ubiquitination and degradation [26]. This can lead to an increase in surface heteromer levels [26].

Receptor co-expression can enhance cell surface delivery of GPCRs

In contrast to the 'dominant negative' effects of certain GPCRs and mutants thereof, there are also a number of reports, apart from the $GABA_B$ receptor, in which co-expression of a second GPCR promotes cell surface delivery of the GPCR being studied [27-28]. For example, a number of adrenoceptor subtypes have been shown to both interact with the α_{1D}-adrenoceptor and to promote its cell surface delivery and function [28-29]. Although the physiological significance remains unclear, interaction with certain GPCRs has also been suggested as a means to promote cell surface delivery in heterologous systems of a number of olfactory receptors to allow their characterization [30]. Although these examples do not require the co-presence of receptor ligands, the concept that cell permeant, small molecule ligands can promote cell surface delivery of a number of GPCR mutants by altering their conformation such that they can now (by)pass the quality control systems of the ER has been widely explored in recent times and such 'pharmacological chaperones' [27, 31-32] have been discussed widely in terms of their clinical potential. Furthermore, extensions of the ability of such ligands to recover the structural organization and trafficking of newly synthesized GPCRs have recently been used to provide novel insights into the location and relevance of GPCR dimerization.

Do mutations and manipulations that interfere with GPCR dimerization modulate ER release and cell surface trafficking?

Many GPCRs that are associated with disease lack function because they fail to pass ER export quality control and reach the cell surface [31-35], rather than being inherently unable to bind ligands and generate signals. This lack of cell surface delivery results in the protein being routed for degradation. In many cases such mutants can also act as ‘dominant-negatives’ as they also prevent the cell surface delivery of a co-expressed wild type form of the GPCR and, in physiological settings, this can account for function being reduced by more than the anticipated 50% in individuals heterozygous for the mutation. For example, for the class C Ca^{2+} sensing receptor a series of mutants including Arg^{66}His and Arg^{66}Cys, that are associated with familial hypocalciuric hypercalcemia/neonatal severe hyperparathyroidism, lack mature glycosylation, and are localized within the ER but not within the Golgi apparatus [34]. Photo-bleaching FRET microscopy showed that these mutants, as well as the wild type receptor were dimerized in the ER [34]. Equally, a number of mutants of the melanocortin 1 receptor are retained intracellularly and have ‘dominant negative’ effects on the function of the wild type receptor [35]. However, although it remains to be investigated fully, it is unlikely that many of these or related mutations are located directly at key GPCR dimeric interfaces (see next paragraph). Despite this, if GPCR mutants that are limited in their ability to dimerize are ER-retained they may be useful in understanding some aspects of the role of dimerization in GPCR assembly.

Canals et al., [36] took advantage of the disrupted oligomeric organization of an α_{1B}-adrenoceptor that contains pairs of point mutations in transmembrane domains (TMD)

I and IV. This mutant was ER-retained when expressed either transiently [37] or stably [36] in HEK293 cells and prevented cell-surface delivery of co-expressed wild type α_{1B}-adrenoceptor [36-37]. Although mutated at 4 positions the modified α_{1B}-adrenoceptor clearly retained core structure because it was able to bind α_1-adrenoceptor ligands, such as [^{3}H]prazosin, with affinity akin to the wild type receptor [36-37] and, although ER-retained, did retain some capacity to generate oligomeric contacts because expression of pairs of this mutant that were C-terminally tagged with bi-molecular fluorescence complementation-competent fragments of YFP [38], resulted in generation of an ER-restricted fluorescent signal [37]. However, sequential three-colour FRET measurements indicated that the detailed organizational quaternary structure of the mutant was different from wild type $_{1B}$-adrenoceptor [37]. The TMD mutant α_{1B}-adrenoceptor was transported to the surface in cells that were exposed to prazosin and other ligands with affinity for the α_{1B}-adrenoceptor [36-37] and in the presence of such a 'pharmacological chaperone' the quaternary structure of the TMD mutant α_{1B}-adrenoceptor, monitored by 3 colour FRET, was restored to something akin to that of wild type α_{1B}-adrenoceptor [37]. Interestingly, prazosin is not a highly cell permeant ligand and the EC_{50} to promote cell surface delivery of the TMD mutant α_{1B}-adrenoceptor was some 50-100 fold higher than the K_d for binding in broken cell preparations [36-37]. This was interpreted as indicating the poor access of prazosin to the ER-retained receptor as the ligand would be required to equilibrate across not only the plasma membrane but the ER membrane as well. The studies indicated that binding of an appropriate ligand was able to either assist folding and/or engender a conformational alteration that enhanced dimerization. To assess if the pharmacological chaperone caused cell surface delivery of a quaternary homomeric complex rather than simply monomers of the α_{1B}-adrenoceptor, Canals et al., [36] also generated a mutant α_{1B}-adrenoceptor that was unable to bind prazosin. This mutant $Asp^{125}Ala$ α_{1B}-adrenoceptor, as for the wild type receptor, was both successfully delivered to the cell surface when expressed in isolation in HEK293 cells and became ER-trapped when co-expressed with the TMD mutant α_{1B}-adrenoceptor [36]. Importantly, addition of prazosin to cells co-expressing $Asp^{125}Ala$ α_{1B}-adrenoceptor and the TMD mutant α_{1B}-adrenoceptor resulted in their co-delivery to the cell surface [36]. As a further control, addition of the $Asp^{125}Ala$ mutation into the TMD mutant α_{1B}-adrenoceptor generated a form of the receptor that was retained in the ER when expressed but which could not be recovered and trafficked to the cell surface by prazosin or other ligands with α_{1B}-adrenoceptor affinity, demonstrating that interaction of the pharmacological chaperone at the orthosteric binding site was required. As the $Asp^{125}Ala$ α_{1B}-adrenoceptor cannot bind ligands, the results of the co-expression studies indicated that it must have been carried to the cell surface in a homomeric complex with the TMD mutant α_{1B}-adrenoceptor [36]. Previous studies had shown that the α_{1B}-adrenoceptor exists as an oligomer rather than a strict dimer [37]. However, these trafficking studies were not appropriate to usefully define whether the overall size of the complex changed in response to binding of the antagonist chaperone ligand, as has recently been suggested for the muscarinic M1 acetylcholine receptor upon binding of the antagonist pirenzepine [39].

In support of a model in which receptor homomers are pre-assembled in the ER and trafficked subsequently to the cell surface, Kobayashi et al., [40] noted that mutation of residue Val 179 of the β_1-adrenoceptor (position 4.46 in the Ballesteros and Weinstein nomenclature, in which the most conserved amino acid in each transmembrane domain across the class A GPCR family is designated X.50 and other amino acids are then related to this position based on the primary amino acid sequence. Hence in TMD 4 of the β_1-adrenoceptor the Trp residue at position 183 in the primary sequence is residue 4.50 and residue 179 is 4 amino acids earlier in the primary sequence) or of Trp 183 (position 4.50) resulted in intracellular retention of the modified receptor. In both cases, cell surface delivery was enhanced by treatment of cells with antagonists, such as alprenolol, that bind the β_1-adrenoceptor. The Val 179 and Trp 183 mutants also displayed increased $BRET_{50}$ values in 'saturation' BRET [10] studies designed to explore the avidity of protein-protein interactions, consistent with a reduced interaction affinity and propensity to dimerize. The $BRET_{50}$ value for $Trp^{183}Ala\beta_1$-adrenoceptor was reduced to close to the value observed for the wild type β_1-adrenoceptor following treatment of cells with alprenolol, but whilst the EC_{50} for alprenolol was considerably greater than the anticipated K_d at the wild type β_1-adrenoceptor, direct estimates of the affinity of ligands for the mutated receptor were not reported. Despite this, these studies are entirely consistent with a model in which correct interactions to generate an appropriately folded receptor homomer are required for the receptor to be allowed to traffic to the cell surface. In a rather different, but conceptually related approach, Kong et al., [41] demonstrated that although when expressed individually, both the wild type dopamine D1 receptor and an $Asp^{103}Ala$ mutant that is anticipated to be unable to bind catecholamine ligands, were delivered to the cell surface, their co-expression resulted in intracellular trapping of both. The asymmetric homomer so produced could be recovered to the cell surface only with the use of cell-permeant agonists and not antagonists [41], suggesting that a conformational change associated with receptor activation was required to generate trafficking-competent quaternary structure. Few studies have attempted to define whether the overall molecular organisation of GPCRs alters during trafficking from ER/Golgi to the cell surface but Vidi et al., [42] have used combinations of bi-molecular fluorescence complementation and FRET to explore this issue for the adenosine A2a receptor, concluding that dimers may be trafficked from the ER to the plasma membrane with subsequent higher-order oligomerization occurring at the plasma membrane. These are challenging experiments, not least because the essentially irreversible formation of the bi-molecular fluorescence complementation signal defines that interactions identified in this way are likely to remain at least in the dimeric state, and further work is required to explore this topic. It is of considerable interest that in the studies of both Canals et al., [36] and Kobayashi et al., [40] mutation of amino acids within TMD IV resulted in ER-trapping, and inefficient, but not elimination of, homomerization. Although other regions of GPCRs have been implicated as being dimer interfaces (see [4] for review), many recent studies have indicated elements of TMD IV to be important for homo-dimeric interactions [37, 43-44]. It is further of interest that in the β_1-adrenoceptor one of these amino acids is the Trp residue that is the most highly conserved residue in TMD IV and, therefore, must play an important role because it is so highly conserved in class A GPCRs. However, at least in the adenosine A1 receptor, mutation of this residue to Ala does not alter homomer formation or cellular trafficking [45]. As such, a general role for this residue in dimerization remains unproven and probably unlikely. Moreover, in a number of X-ray structures of GPCRs a molecule of cholesterol is bound at this location (see [46] for review) and the implications of this for GPCR quaternary structure are unclear. Furthermore, it is even more difficult to extrapolate predictions to GPCR heteromers. For example, although TMD IV mutations modulated homo-dimerization of the β_1-adrenoceptor they did not alter interactions between the β_1-adrenoceptor and the β_2-adrenoceptor [40].

Because single (and even the combination of multiple) point mutants have failed to result in the generation of GPCRs that are obvious monomers, other approaches have been used to try to interfere with GPCR dimerization and hence to define its relevance. For example, addition of peptides that correspond to individual TMDs of GPCRs have been used both in intact cell and membrane-based studies. For the chemokine CXCR4 receptor a synthetic peptide corresponding to TMD IV reduced oligomerization as reported by a reduction of FRET signals in cells co-expressing CFP and YFP- tagged forms of the receptor. It also inhibited ligand-induced actin polymerization, and blocked chemotaxis of malignant cells [47]. These results have been interpreted as evidence for specific functional roles of CXCR4 dimers and build on other studies that have attempted to disrupt chemokine receptor homo- and hetero-mers (see [47-48] for details).

Although many fewer studies have examined homomer and heteromer formation for the class B receptor family, there is good evidence for such interactions (see [49] for review). Although there is no sequence similarity between class B and class A GPCRs, at least for the class B secretin receptor, TMD IV again appears to be a key element in allowing production of the homomer [50], but unlike many class A receptors, to date there is no evidence to support the formation of higher order multimers [51]. It remains to be established if this will be the pattern for other class B receptors and evidence suggests that this may vary between different class C GPCRs. Here the $GABA_B$ receptor can exist as at least a tetramer, whilst for metabotropic glutamate receptors only strict dimers have been detected [7]. Interestingly, by using a peptide corresponding to TMD IV of the secretin receptor Gao et al., [52] were able to apparently monomerize this receptor and this was associated with lack of high affinity, guanine nucleotide-sensitive binding of agonist ligand. These observations are at least consistent with the secretin receptor dimer being required for G protein binding [52], whilst monomeric class A GPCRs are sufficient to produce such pharmacology and function [1-3]. A further element that may be different between class B and class A receptors is that following secretin receptor dimer disruption produced by the TMD IV peptide, the apparently monomeric receptor and various TMD IV point mutants were still able to traffic to the surface of cells [52].

Do GPCRs internalize as dimers?

Once at the cell surface many GPCRs become internalized via endocytosis, either spontaneously or in response to the binding of agonist ligands. This topic has been reviewed extensively and is beyond the scope and capacity of this article. However, the physical organization of internalized GPCRs, and if their oligomeric status is altered during this process, has been the subject of considerable debate. The capacity of a selective ligand of one GPCR to cause internalization of both its cognate GPCR and a second co-expressed GPCR has been used as evidence to favor the presence and internalization of intact GPCR heteromers (see [4] for review). This is more challenging to approach, however, for GPCR homomers. A developing strategy in this area is to generate asymmetric homomers following co-expression of a wild type GPCR and a variant of the GPCR that is unable to bind and respond to the same ligand(s) as the wild type receptor. For GPCRs with catecholamine ligands this can be achieved simply by alteration of the Asp residue at position 3.32 that is required to provide high affinity interaction with the amine headgroup of the ligand. Furthermore, in a limited number of cases ligands able to act as agonists at such mutated receptors but not at the corresponding wild type receptor have been synthesized. In the case of the β_2-adrenoceptor, regulated co-expression of a wild type and an Asp3.32Ser mutant resulted in co-internalization of both forms of the receptor in response to isoprenaline, despite the mutant having no significant affinity for this ligand and not being internalized upon addition of isoprenaline when expressed in the absence of wild type β_2 adrenoceptor [53]. Furthermore, a synthetic ligand that is a full agonist at the mutant receptor but has little affinity for the wild type also caused co-internalization of both forms of the receptor [53]. This basic approach requires to be extended to other GPCRs to test the generality of these observations and the expanding range of GPCRs for which variants that are activated solely by synthetic ligands are available offers a means to do so.

These range of approaches utilized in the studies discussed in this review are starting to unravel the basis and importance of GPCR-GPCR interactions in receptor synthesis and cell surface trafficking. As with other aspects of the importance of GPCR dimerization, key studies need to be translated to physiologically relevant cells and tissues before clear understanding will be achieved.

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