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Heterodimerization and Surface Localization of G Protein Coupled Receptors

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Abstract

G protein coupled receptors (GPCRs) are one of the largest human gene families, and are targets for many important therapeutic drugs. Over the last few years, there has been a major paradigm shift in our understanding of how these receptors function. Formerly, GPCRs were thought to exist as monomers that, upon agonist occupation, activated a heterotrimeric G protein to alter the concentrations of specific second messengers. Until recently, this relatively linear cascade has been the standard paradigm for signaling by these molecules. However, it is now clear that this model is not adequate to explain many aspects of GPCR function. We now know that many, if not most, GPCRs form homo- and/or hetero-oligomeric complexes and interact directly with intracellular proteins in addition to G proteins. It now appears that many GPCRs may not function independently, but might more accurately be described as subunits of large multi-protein signaling complexes. These observations raise many important new questions; some of which include: 1) How many functionally and pharmacologically distinct receptor subtypes exist *in vivo*? 2) Which GPCRs physically associate, and in what stoichiometries? 3) What are the roles of individual subunits in binding ligand and activating responses? 4) Are the pharmacological or signaling properties of GPCR heterodimers different from monomers? Since these receptors are the targets for a large number of clinically useful compounds, such information is likely to be of direct therapeutic importance, both in understanding how existing drugs work, but also in discovering novel compounds to treat disease.

Keywords

GPCRs; dimerization; oligomerization; cross-talk; pharmacology; signaling

FAMILIES OF GPCRs

All known GPCRs have common structural features, including seven transmembrane spanning domains, an extracellular amino terminus, and an intracellular carboxyl terminus. Mammalian GPCRs have been grouped into three major families by sequence homology, called A, B, and C (or I, II, and III) [1]. The vast majority (>90%) belong to Family A, which include rhodopsin; receptors for biogenic amines such as norepinephrine, dopamine, acetylcholine and serotonin; receptors for peptides such as opioids and enkephalins; as well as receptors for odorants. Family B receptors are a much smaller group, and include receptors for large peptides such as secretin, cytokines, thrombin, and glucagon. Family C receptors are the least numerous, with only about a dozen members. They include the GABA_B, the eight metabotropic glutamate receptors, the

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Ca²⁺ sensing receptor, as well as some pheromone and taste receptors. Additional GPCR families can also be found, including those for both frizzled and smoothened receptors [1]. The Family C receptors are the most structurally unique. This family contains slightly more than a dozen members which are uniquely characterized by very large extracellular amino termini consisting of two distinct lobes comparable to those found in ionotropic glutamate receptors [2]. In most cases, it appears the cleft between these extracellular lobes appears to form the ligand binding domains by forming a structure comparable to a venus flytrap [3], closing on agonist binding. The other two families have much shorter amino termini, and bind ligands primarily within their hydrophobic cores or extracellular loops.

OLIGOMERIZATION OF GPCRS

Family C

It is now clear that many, if not all, GPCRs physically associate with other cellular proteins, including both other GPCRs [2,3] and a variety of other membrane proteins such as RAMPs (receptor activity modifying proteins) [4], as well as a large variety of soluble intracellular proteins such as β -arrestins [5] and many others. Data supporting the association of GPCRs was first presented for muscarinic cholinergic and α_2 - and β_2 -adrenergic receptors (ARs) many years ago [6]. However, despite the work of Bouvier's group on β_2 -AR homodimerization [2], due to technical limitations, this concept remained highly controversial until the cloning of the GABA_B receptor. The first GABA_B receptor cloned was found to express and signal poorly [7,8]. After a second GABA_B receptor (GABA_BR2) was discovered by homology cloning, this construct also was unable to form a functional GABA_B receptor [7,8]. It quickly became clear that formation of functional GABA_B receptors required a complex of two distinct GABA_B receptor subtypes (GABA_BR1 and GABA_BR2), and that formation of such a complex was required for surface expression and trafficking, as well as formation of a functional signaling molecule [9]. The GABA_BR2 subunit was found to be required for promoting surface expression of the GABA_BR1 subunit by masking an ER retention motif in the GABA_BR1 C-terminal tail [10,11], allowing translocation of the heterodimeric complex to the cell surface. However, other domains of the two subunits also interact, as C-terminally truncated constructs also form heterodimeric complexes, suggesting that this interaction is not due to a single domain [9]. The final evidence proving the essential nature of heterodimerization of the two GABA_B receptor subunits was the observation that the two native proteins were quantitatively co-immunoprecipitated from brain with an antibody to either protein alone [12], supporting their existence exclusively as complexes *in vivo*. This was the first unequivocal example where coexpression of two distinct GPCR molecules was required for correct assembly and function of a single receptor.

Similar results have now been obtained for the sweet and umami taste receptors. This receptor family contains three receptor genes (T1R1, T1R2 and T1R3) in humans and rodents. It was quickly discovered that individual constructs expressed alone did not form functional receptors, but that obligatory complexes of two of the three subunits are required for formation of functional taste receptors [13]. Interestingly, the combination of T1R1/T1R3 forms an "umami" taste receptor, responsible for the savory taste of amino acids such as monosodium glutamate; while the T1R2/T1R3 complex forms a functional sweet taste receptor which responds to both carbohydrate and artificial sweeteners [14]. Thus, despite sharing a common subunit (T1R3), these complexes form receptors with completely different ligand binding specificities, demonstrating unequivocally that 1) a single subunit can interact with more than one partner; and 2) heterodimerization can dramatically change the ligand binding specificity of a receptor complex. Interestingly, GABA_B, umami and sweet taste receptors belong to the relatively small Family C GPCR family introduced above [1]. The other Family C GPCRs are also thought to form multisubunit complexes which are, however, primarily homomeric [2].

Accumulating evidence now suggests that each individual subunit within such GPCR complexes is responsible for a particular component of the signaling response. Several examples of this can be observed in the Family C family of GPCRs, including the GABA_B [7,8] and taste [14] receptors. Notably, within the GABA_B receptor complex, the GABA_BR2 subunit is at least partially responsible for promoting surface expression of the GABA_BR1 [7–9], by masking an ER retention motif in the GABA_BR1 carboxyl terminal tail [10,11]. Once the complex is properly assembled, the GABA_BR1 subunit appears to be primarily responsible for binding the agonist, although the affinity is higher when complexed with the GABA_BR2 subunit [9]. On the other hand, the GABA_BR2 subunit appears to be required for coupling to G-proteins and activating functional responses within the cell [9].

Family A

Evidence for formation of multi-subunit complexes of many other GPCRs, including those of the Family A rhodopsin family (which comprise most GPCRs) has also been obtained [2,15], although the functional consequences of such interactions are not as clear. These receptors are much smaller than Family C GPCRs, lacking the large N-terminal venus flytrap domain. Unlike many Family C GPCRs however, Family A GPCRs often form apparently normal receptors when a single recombinant subtype is expressed alone. However, both homo- and hetero-dimers or oligomers have been widely reported for Family A GPCRs as well [15], including subtypes within a subfamily, and also with more distantly related GPCRs. These include homodimers of rhodopsin [16] which have been viewed in highly ordered arrays by atomic force microscopy, suggesting that some receptors may form higher order oligomeric complexes as well as simple dimers. These receptors have also been reported to interact with other transmembrane proteins such as RAMPs, sometimes altering their properties [4]. However, until recently, physical association of Family A GPCRs with other GPCRs or accessory proteins has only rarely resulted in striking phenotypical changes. More commonly, small changes in pharmacology, signaling, and/or agonist-induced internalization are observed. The functional significance of this phenomenon remains under active debate.

Family B

Oligomerization of Family B receptors has not yet been extensively studied, but preliminary evidence suggests that these receptors may also form complexes with other types of proteins, such as RAMPs [17].

ADRENERGIC RECEPTORS

Our laboratory has been studying the adrenergic receptors (ARs) for many years. These receptors respond to the neurotransmitters and hormones norepinephrine and epinephrine, which play key roles in regulation of cardiovascular function, energy metabolism, blood pressure, and many other functions. Drugs acting at these receptors are important therapeutic targets for many diseases, including heart failure, angina, hypertension and benign prostatic hypertrophy [18]. The ARs are divided into three major subfamilies (α_1 , α_2 , β) based on their pharmacological properties, amino acid sequences, and signaling mechanisms [19]. Molecular cloning and pharmacological studies have identified three subtypes within each subfamily; making a total of nine human genes ($\alpha_{1A,B,D}$, $\alpha_{2A,B,C}$, $\beta_{1,2,3}$). All of these genes encode GPCRs of the Family A subfamily and are highly conserved in all known mammalian species, suggesting they play important functional roles. Each AR subfamily couples preferentially to a single G protein type; β -ARs activate G_s and activate adenylate cyclase, α_2 -ARs activate G_i and inhibit adenylate cyclase, and α_1 -ARs activate $G_{q/11}$ and increase intracellular Ca^{2+} . Since the ARs were cloned over a decade ago, we have been attempting to better understand the pharmacological properties and signaling mechanisms of the recombinant subtypes

expressed in heterologous systems, and to delineate the different roles played by individual subtypes that respond to the same agonists and activate the same responses. Our goal has been to reconcile these observations with the pharmacological properties and signals activated by ARs in native tissues.

MANY GPCRS, INCLUDING α_{1D} -ARS, DO NOT REACH THE CELL SURFACE UPON HETEROLOGOUS EXPRESSION

GPCRs are known to function primarily at the cell surface in native tissues, where they usually respond to highly hydrophilic ligands which do not easily cross cell membranes. However, an increasingly puzzling and frustrating observation has been that many GPCRs do not reach the cell surface following heterologous expression, making them difficult to study in recombinant systems. Direct physical interactions between receptors has recently been shown to strongly influence surface trafficking of GPCRs [3,14,20–22]. As discussed previously, the first example of this phenomenon was reported for GABA_B receptors, which belong to the Family C family of GPCRs. Analogous to GABA_B receptors, sweet and umami taste receptors also require obligatory assembly of two distinct subunits to form a single functional receptor. In addition, similar complexes occur with other members of the Family C family of GPCRs, including the eight metabotropic glutamate receptors [23]. Therefore, it is becoming increasingly clear that Family C GPCRs exist almost exclusively as dimers or multimeric complexes, which are critical for proper expression and pharmacology [3]. However, the generality of this phenomenon to the large number of Family A GPCRs remains controversial. A large number of Family A GPCRs do not reach the cell surface following heterologous expression, including α_{1D} - [24,25] and α_{2C} -ARs [26], adenosine 2b [27], all of the bitter taste [28], trace amine [29], the huge family of olfactory receptors which comprise almost half of all GPCRs in humans [30]; as well as many others. The problems with heterologous surface expression are still poorly understood, but have greatly hindered their study. Often, introduction of the rhodopsin N-terminal 30 amino acids in a chimeric construct results in surface expression of such receptors [28], suggesting an important role for the N-terminus in surface localization.

Within the α_1 -AR subfamily, recombinant α_{1A} - and α_{1B} -AR subtypes easily reach the cell surface following heterologous expression as shown by various epitope and fluorescent tags [31]. However, recombinant α_{1D} -ARs are characterized by an almost exclusively intracellular localization [24,32], very low densities of binding sites, and very weak coupling efficiencies in most cells where they have been expressed, making them difficult to study [25,33]. Consistent with a role for the N-terminus in surface expression, we showed that truncation of the N-terminal extracellular 93 amino acids causes dramatic increases in expression of receptor binding sites [34]. We subsequently showed that this sequence can be transplanted to other subtypes, rendering them unable to reach the cell surface [32]. This suggests that the relatively long N-terminus of α_{1D} -ARs is primarily responsible for their inability to reach the cell surface.

As discussed above, it is becoming increasingly clear that GPCRs can form homo- and heterodimers, resulting in the formation of unique multi-protein complexes that have altered trafficking, signaling and drug specificities [35,36]. In fact, recent data has raised the possibility that homo- or heterodimerization may be required for proper expression of all GPCRs [37–39]. There are now many examples of this in the literature, including homo- and heterodimerization of thromboxane A₂ receptors [40]. A striking recent example is the heterodimerization of prostaglandin EP₁ receptors with β_2 -ARs [41], where EP₁ receptors do not appear to significantly affect airway tone, but reduce the bronchodilatory function of β_2 -ARs.

Results from our laboratory have recently demonstrated that co-expression with α_{1B} -ARs results in quantitative surface expression of the normally intracellular α_{1D} -ARs, through a direct physical interaction [22,42] (Figure 1). Mutation and truncation studies suggested this interaction did not involve intracellular signaling pathways or the soluble amino- or carboxy-terminal extensions, suggesting the hydrophobic core and/or intra/extracellular loops are responsible [22]. In addition, α_{1D} -ARs were found to be expressed exclusively on the cell surface when transfected into DDT1 smooth muscle cells, which endogenously express α_{1B} -ARs at about 200–300 fmol/mg protein [43–45]. However, they are intracellularly retained in rat aortic smooth muscle cells, which do not endogenously express α_{1B} -ARs. This process was highly selective, as demonstrated by the inability of the closely related α_{1A} -AR subtype (which does not heterodimerize with α_{1D} -ARs [46]) to promote α_{1D} -AR translocation. This was the first example where physical interaction between Family A GPCRs was observed to promote quantitative surface expression of a predominantly intracellular subtype [22].

As discussed previously, there is accumulating evidence with Family C GPCRs that each individual receptor within a GPCR heterodimer is responsible for a particular component of the signaling complex. Interestingly, we have found that the α_{1B} -/ α_{1D} -AR heterodimer is functionally similar to the GABA_B receptor heterodimer. The α_{1B} -AR serves to promote cell surface expression of the α_{1D} -AR [22], possibly by masking an ER retention motif in the α_{1D} -AR N-terminus [34,47,48]. To examine the functional roles of each subunit of the heterodimer in more detail, we used an uncoupled form of the α_{1B} -AR containing a deletion of three amino acids at the N-terminal portion of the third intracellular loop ($\Delta 12\alpha_{1B}$ -AR), which is completely unable to promote signaling responses [49]. When the uncoupled $\Delta 12\alpha_{1B}$ -AR is co-expressed with the full-length α_{1D} -AR, the complex is transported normally to the cell surface [50], suggesting that signaling is not required for this translocation. However, it appears that the $\Delta 12\alpha_{1B}$ -AR is primarily responsible for binding ligand, since it shows pharmacological properties almost identical to those of α_{1B} -ARs expressed alone; while the α_{1D} -AR couples to G protein activation [50] (Figure 2). Individual receptor subunits acting as distinct components within a heterodimer complex have also been previously shown to occur with heterodimers consisting of H1 histamine and α_{1B} -ARs [51], β_2 -ARs and δ -opioid receptors [52], β_2 -ARs and α_{2A} -ARs [53] and β_2 -ARs and β_3 -ARs [54]. In fact, using constructs of transmembrane helices in protein pulldown assays, Carrillo et al [55] have presented evidence that α_{1B} -ARs form complex oligomeric structures in which major symmetrical interactions may facilitate intradimeric interactions. Taken together, these findings suggest that GPCR heterodimers form functional complexes with distinct drug specificities and signaling properties, in which each individual receptor subunit may be responsible for specific functions. Most of these studies have been done, by necessity, in heterologous expression systems where receptor density is difficult to control and are therefore subject to many potential artefacts. However, the functional significance of Family A GPCR heterodimers has recently been demonstrated *in vivo* for opioid receptors using a heterodimer-selective agonist [56], consistent with the hypothesis that these complexes occur in native tissues.

OLFACTORY RECEPTORS DO NOT REACH THE CELL SURFACE FOLLOWING HETEROLOGOUS EXPRESSION

As discussed above, olfactory receptors (ORs) comprise about half of the entire human GPCR family, but like many other Family A GPCRs do not reach the cell surface following heterologous expression. This problem has prevented characterization of the pharmacological properties and signaling pathways of this large GPCR family [57]. Following heterologous transfection of receptor cDNAs, essentially all ORs remain trapped intracellularly in the endoplasmic reticulum and are non-responsive to their hydrophilic agonists. Receptor mutations or chimeras, such as C-terminal transmembrane truncation, N-terminal addition of

rhodopsin sequences, N-terminal addition of glycosylation sites, or construction of OR/ β_2 -AR chimeras [58–63] have been used to obtain OR surface expression in heterologous cell systems. Although these techniques can be useful for specific applications, the inability to examine wild type ORs limits their utility. The mechanisms involved in such intracellular retention remain unresolved, but present significant obstacles in trying to characterize the properties of these receptors in recombinant systems.

As discussed above, the first examples of receptor heterodimerization serving to translocate intracellular GPCRs to the cell surface were restricted to the small Family C subfamily. However, increasing evidence now supports the concept of physical interactions between the much larger Family A family, as discussed above. We recently reported that physical interaction with β_2 -ARs drives OR expression to the cell surface. ORs are the smallest GPCRs (~300–320 aa), consisting almost exclusively of a seven transmembrane domain hydrophobic core and associated loops [43]. Given that the hydrophobic core of the α_{1B} -AR appeared to be responsible for α_{1B}/α_{1D} -AR heterodimer formation and trafficking [22], we hypothesized that OR surface expression might also be controlled by GPCR heterodimerization. We used the mouse M71-OR as our model OR, because it is one of the few ORs with a known agonist and has been reported to be exclusively intracellular [64] following heterologous expression. Since it was known that olfactory neurons express β -ARs [65], we screened all nine AR subtypes for their ability to promote M71-OR surface expression. Using various tags, confocal microscopy and a luminometer-based cell surface assay, we found that co-expression with β_2 -ARs results in a profound translocation of functional M71-ORs to the cell surface in HEK293 cells. This process was highly selective, as none of the other eight AR family members were capable of promoting M71-OR surface expression. A persistent physical interaction between M71-ORs and β_2 -ARs (but not α_{1B} -ARs) was shown by co-immunoprecipitation and co-internalization of the two receptors in response to their specific ligands. Also, co-expression of wild type M71-ORs with β_2 -ARs resulted in cyclic AMP responses to the M71 agonist ligand acetophenone. Finally, *in situ* hybridization studies demonstrated extensive co-localization of M71-OR and β_2 -AR mRNA expression in mouse olfactory epithelium [66]. This was the first report of heterologous surface expression of a functional wild-type OR, and suggested that heterodimerization with other Family A GPCRs, such as β_2 -ARs, can control OR surface expression. Interestingly, recent studies have shown that, unlike mammals, all *Drosophila* olfactory neurons express two ORs. One is specific to a particular olfactory neuron, while the other seems to be expressed in almost all olfactory neurons. This OR, named OR83b, is highly atypical and has little sequence homology with, and is significantly larger than, the other 60 *Drosophila* ORs [67]. Amazingly, flies lacking OR83b are unable to properly localize specific ORs to normal dendritic compartments or respond to odorant stimuli in electrophysiological assays. In addition, OR83b deficient flies do not demonstrate odor-evoked behaviour in the larval or adult stages. These experiments suggested that OR83b acts an accessory protein in *Drosophila*, whose function is to heterodimerize with other ORs and ensure their appropriate neuronal localization and function [68]. This report provides *in vivo* functional data in support of our hypothesis that OR heterodimerization is required for appropriate localization and function of these receptors. However, other reports suggest that other RAMP-like proteins may be involved in olfactory receptor surface expression [69], and this field is still under active investigation.

SUMMARY

It appears that many different GPCRs of the Family A family can be added to the growing list of GPCRs that require specific GPCR partners for chaperoning to the cell surface. Interestingly, a recent report suggests that β_2 -ARs must first form multimeric complexes in the endoplasmic reticulum to reach the cell surface [37]. This raises the possibility that many, if not all, GPCRs must form such multiprotein complexes to facilitate surface expression. For some receptors,

such as β_2 -ARs, homomeric associations may be sufficient, whereas for other receptors, such as ORs, interactions with other specific receptor types may be required.

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Nonstandard abbreviations

GPCR	G protein coupled receptor
RAMPS	receptor activity modifying proteins
AR	adrenergic receptor
ORs	olfactory receptors
PLCβ	phospholipase C β
PIP₂	phosphatidylinositol 4,5 bisphosphate
IP₃	inositol 1,4,5 trisphosphate
IP3R	

DAGIP₃ receptor

diacylglycerol

Figure 1.

Heterodimerization with α_{1B} -ARs is required for surface localization of α_{1D} -ARs. TOP: Newly synthesized α_{1D} -ARs are trapped in the endoplasmic reticulum (internal oval) by association of a specific retention protein (jagged circle) with the N-terminal domain, preventing translocation to the cell surface. MIDDLE: Synthesis of α_{1B} -ARs causes heterodimerization with the α_{1D} -ARs, displacing the endoplasmic reticulum retention protein and allowing the heterodimeric complex to be transported to the cell surface. LOWER: The heterodimeric $\alpha_{1B/D}$ -AR complex reaches the cell surface where it can then result in physiological responses to agonist activation.

Figure 2.

Specific domains of the $\alpha_{1B/D}$ -AR complex appear to perform specific functions. The α_{1B} -AR subunit (left, darker) appears to be primarily responsible for binding agonists and competitive antagonists, since the complex exhibits pharmacological properties that are characteristic of α_{1B} -ARs. However, the α_{1D} -AR subunit appears to be responsible for signaling, since complexes of this subunit with a signaling deficient α_{1B} -AR show robust responses to norepinephrine. This subunit appears to interact directly with G_q and activates phospholipase $C\beta$ (PLC β). PLC β hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP_2) into inositol 1,4,5 trisphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ then diffuses to the endoplasmic reticulum where it binds to a specific IP $_3$ receptor (IP $_3$ R) to promote release of stored Ca^{2+} , which is the primary signal generated by this receptor complex.