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Dimerization in GPCR mobility and signaling

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Many types of cell surface as well as intracellular DNA-binding receptors exist and function as dimers; formation of homodimers or heterodimers appears to not only provide molecular mechanisms for agonist-induced activation but also increase specificity of ligand recognition and versatility of downstream signaling. G-protein-coupled receptors (GPCRs) were long thought to be an exception, but in recent years a lot of evidence has accumulated that GPCRs also *can* form dimers, even though it is far from certain when and where they actually *do* so under physiological conditions. Dimerization of GPCRs does not generally seem to be required for ligand recognition or signaling. However, dimerization may serve to affect receptor mobility at the cell surface and in intracellular trafficking, and may be involved in and affect their signaling functions.

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Introduction

Most receptors exist and function as dimers — this is true for cytosolic/nuclear DNA-binding receptors as well as for many families of cell surface receptors [1,2]. Dimerization can occur between identical (homodimerization) or different (heterodimerization) proteins, and both processes have been observed for receptors. There are many ways in which dimers can be formed. Interactions with entirely different types of proteins have been observed and dimerization may occur with just one or with a variety of partners, leading to a multitude of receptor homomers and heteromers as well as to homomeric and heteromeric receptor complexes [3].

Why would a receptor 'want' to dimerize? It appears that dimerization allows the receptor to acquire a number of properties that a monomer does not have. Some of these are listed in Table 1. For example, both protomers within a dimeric complex can contribute to the binding site for the ligand, and thus the nature of the second protomer can determine ligand specificity of a receptor in a dimer. A prominent case for this are the receptors for transforming growth factor (TGF) superfamily members, which are composed of two classes of serine/threonine kinase receptors, termed type I and type II. The seven type I and five type II receptors together with the many ligands generate a plethora of distinct ligand/receptor complexes [4•].

Moreover, many cell surface receptors have a single membrane spanning domain, and it is difficult to see how this can transduce a signal across the membrane; however, in a dimer the two moieties can change their relative distance or orientation and thus extracellular ligand binding can change intracellular properties of a dimeric receptor. This is the case for many receptor tyrosine kinases that can often be activated not only by their (monomeric or dimeric) ligands, but also by antibodies that cross-bridge two receptor monomers [5•].

And third, an interaction of the intracellular parts of the two protomers may provide an activation mechanism, as in the case of tyrosine kinase receptors where the intracellular tyrosine kinase moiety of one protomer phosphorylates the intracellular part of the other protomer, which can lead to enhanced activity of the tyrosine kinases and to the creation of phosphotyrosines as docking sites for signaling proteins [6].

G-protein-coupled receptors have classically been perceived as receptors that do not 'need' to dimerize [1]: their heptahelical structure allows plenty of movements and rearrangements that permit a transfer of the activation signal across the cell membrane, and in fact such agonist-induced structural alterations have begun to be elucidated both in structural and in kinetic terms [7–11]. The tightly packed structure would also assure ligand specificity, and the docking of G-proteins to the intracellular face of receptors in their activated form provided a suitable transduction mechanism.

And in fact, recent data have confirmed that monomeric signaling of GPCRs is possible and occurs with physiological speed: single (i.e. monomeric) β_2-adrenergic receptors as well as rhodopsin and μ-opioid receptors in small lipid vesicles have been shown to couple to their respective G-proteins [12••,13,14], and monomeric rhodopsin in solution has been shown to activate its G-protein transducin at the diffusion limit [15•]. These

Table 1

Mechanistic effects of receptor dimerization

Mechanism	Properties acquired by dimerization
Ligand recognition	Specificity through interactions of ligands with two subunits in a dimer Increased affinity through multiple contact points Increased ligand repertoire via different binding partners
Receptor activation	Changes in distance between subunits as an activation mechanism Crossphosphorylation as an activation mechanism
Signal transduction	Multiple contact points and specificities for intracellular signaling proteins Crossregulation of activity in protomers
Trafficking	Changes in cell surface delivery Crossregulation in receptor internalization
Cell surface mobility	Crossregulation in cell surface mobility

experiments indicate that indeed GPCRs do not need to dimerize in order to execute their basic function of transducing a signal from ligand binding to G-protein activation.

Evidence for GPCR dimerization

However, during the last two decades, an increasing number of observations have indicated that dimer and presumably higher order oligomer formation occurs in GPCRs. These observations are reviewed in more detail elsewhere in this volume [16]. Early findings have been reviewed in depth by Hébert and Bouvier [17]; they included functional complementation of dysfunctional receptors, coprecipitation of differently tagged GPCRs, dimer visualization in SDS-polyacrylamide gels and their disruption by receptor peptides. Even earlier, studies showing that GPCRs could be activated by antibodies or by antagonists bound to antibodies suggested that inducing GPCR dimer formation *per se* might activate GPCRs [18] — a hypothesis that is remarkably similar to the one made over 20 years later on the basis of class C GPCR crystal structures that moving the transmembrane cores of two receptors closer together constitutes their activation principle [19]. Another line of evidence for GPCR dimers was provided by radiation inactivation studies yielding unexpectedly high apparent sizes [17]. For example, the radiation inactivation size of the A_1 adenosine receptor was determined at ≈60 kDa [20], almost twice the size of the receptor protein determined by photoaffinity labeling at ≈35 kDa [21]. However, later studies showed that the radiation inactivation size was reduced to ≈33 kDa by guanine nucleotides [22], raising — in retrospect — the possibility that the large apparent size might be caused by a tight GPCR/G-protein interaction [23] rather than by a GPCR dimer.

Subsequent support for dimer formation of GPCRs was in particular obtained in FRET and BRET studies, employing either fusions of fluorescent and luminescent proteins to the receptors' intracellular C-termini (reviewed in [24]), or antibodies or tags that could be attached to the extracellular side of the receptors [25••]. While there has been methodological criticism of the interpretation [26] and the experimental techniques [27,28] of these studies, there seems to be over-riding consensus that many GPCRs can form dimers, and that they do not only do this in overexpression systems but also *in vivo* [29]. However, in many instances proof for the physiological occurrence and function needs to be provided, and consensus criteria have been developed how this should be done [29].

Physiologically, the most evident case was the $GABA_B$-receptor, where a dimer between the $GABA_{B1}$ and $GABA_{B2}$ subunits is required both for cell surface targeting and for function [30], since $GABA_{B1}$ binds the agonist (but does not couple) and $GABA_{B2}$ couples to G-proteins (but does not bind agonist). FRET experiments using defined extracellularly labeled $GABA_B$-receptors have indicated that the receptors appear to form tetramers, with two $GABA_{B2}$-protomers being distant from each other, while the $GABA_{B1}$-protomers are close to each other (i.e. they presumably form the center of the tetramer) [25••].

GPCR mobility and assembly

Most early data on GPCR dimers suggested that dimerization of GPCRs occurred early on in the biosynthetic pathway [31]. This was evident, for example, in the case of the $GABA_B$-receptor just mentioned, where the interaction of the C-termini of the B_1-subunit and the B_2-subunit is required for cell surface targeting, and also from studies of mutants of the V_2-vasopressin receptor that cause diabetes insipidus and are retained already as dimers in the endoplasmatic reticulum [32]. This would indicate that such dimers are quite static. A static interaction would require either a very high affinity of two protomers for each other (presumably in the pM range), or a covalent S–S bond, which can be found in some class C GPCRs [33].

In order to address the dynamics of GPCR dimerization, we have employed fluorescence recovery after photobleaching (FRAP) experiments [34••]. These experiments use receptors that carry a yellow fluorescent protein (YFP) at the extracellular N-terminus, or a cyan fluorescent protein (CFP) at the intracellular C-terminus. An area of the cell membrane is bleached, and the subsequent 'return' of fluorescence in this area is then monitored. Although at the microscopic level the mobility of GPCRs appears to be a complex phenomenon across small 'fenced' areas [35•], the overall return of fluorescent receptors into such a bleached area (the 'recovery')

appears to follow an exponential time-course [34••]. Binding of an antibody to the extracellular YFP-tag markedly impaired the return of receptors into the photobleached area — while an unrelated receptor carrying an intracellular CFP returned rapidly. If, however, the YFP-labeled and the CFP-labeled receptors formed a dimer, then restricting the mobility of the YFP-labeled receptor also slowed the recovery of the CFP-labeled receptor. Such experiments cannot prove a direct interaction of the two labeled proteins, but they strongly suggest that the two 'travel together' in a microscopically small microenvironment.

Using such experiments, we observed that β_1-adrenergic receptors (CFP-tagged at their C-terminus) were only moderately retarded in their recovery by an immobilized partner receptor (tagged with YFP at their N-terminus). This suggests that these receptors showed only transient interactions, either by dynamically forming transient dimers or by transiently associating in a larger complex of unknown nature, and challenges prevailing views about the static nature of GPCR dimers.

By contrast, β_2-adrenergic receptors appeared to form large and stable oligomeric complexes. First, there was virtually no recovery of intracellularly CFP-labeled receptors when the extracellularly YFP-labeled receptors were immobilized — indicating a robust and long-lived interaction of the two. And second, a single immobilized β_2-receptor seemed to be able to restrict the mobility of 8 (or even more) β_2-receptors — suggesting that they were part of large complexes. Whether these complexes contained additional proteins or components is currently unknown; they might represent plain oligomers, but they might also contain specific scaffolding proteins or lipids. In this context, an interesting claim is that formation of B_2-bradykinin receptor heterodimers, which have not been observed in most laboratories [36], may be facilitated by the expression of calreticulin, suggesting that GPCR dimerization might be regulated by additional proteins [37].

Using the same antibody-immobilization/FRAP technique, Fonseca and Lambert recently reported that D_2-dopamine receptors also show only transient interactions with each other [38•] — a finding that contrasts with a biochemical report suggesting more stable, tetrameric structures of these receptors [39].

Larger oligomeric GPCR assemblies, initially suggested on the basis of much discussed atomic force microscopy images of rhodopsin 'arrays' in the retina (reviewed in [40]), have also been observed by other techniques, including energy transfer and fluorescence complementation studies, which suggest a tetrameric structure for the D_2-dopamine [39] and also other GPCRs. The tetrameric structure of the $GABA_B$-receptor [25••] has been mentioned above. An interesting hypothesis in the assembly of such larger structures comes from the A_2-adenosine receptor, where it has been suggested that these receptors are synthesized and reach the cell surface as dimers and then form larger assemblies [41].

A recent study on the assembly of purified β_2-adrenergic receptors upon reconstitution into phospholipid vesicles also observed a tetrameric structure [42]; interestingly, this structure was not affected by agonists, but inverse agonists induced tighter packing, raising the possibility that even stable oligomers are dynamic.

Signaling in GPCR dimers

GPCR dimerization and oligomerization have been mostly studied in terms of assembly and intracellular trafficking. A much less studied question concerns the effects that dimerization may have on receptor signaling. The most conclusive evidence in this field comes, again, from the $GABA_B$-receptor. As noted above, this receptor functions only as a heterodimer, where the B_1-protomer is responsible for ligand binding and the B_2-protomer for G-protein coupling and signaling. This indicates two major features: firstly, only one protomer in this dimer needs to be active and secondly, the signal must be able to be transmitted from one (the B_1) protomer to the other (the B_2). The first point appears to be a general one, and full activation by only one protomer in a GPCR dimer has been reported for mGlu receptors [43•], and also for leukotriene B_4-receptors, where it has been suggested that the asymmetry in the dimer (with one protomer binding the agonist) is induced by binding of a single G-protein to the receptor dimer [44•]. On the other hand, in a careful analysis of signaling by mGluR5 homodimers Kniazeff *et al.* [45••] observed that while agonist binding to only one protomer (of mGluR1) induced an active state of the receptor, binding to both protomers was required for full activity. Furthermore, the authors suggested that essentially the two protomers were symmetrical, and that activation and signaling could proceed within a single protomer or from one (ligand binding) protomer to another (G-protein coupling) protomer. This raises the possibility that depending on the specific dimer, only one protomer may be sufficient for ligand binding and signaling, while in other cases both protomers may contribute to signaling in a — more or less — additive manner.

In order to monitor the signal transfer within a heterodimer, we have used a FRET technology that permits the monitoring of conformational changes in a GPCR. Introduction of suitable labels — such as YFP and CFP, or FlAsH and CFP — into the third loop and the C-terminus of GPCRs resulted changes in FRET between the labels, when such receptors, expressed and studied in intact cells, were stimulated by agonists [46•,47]. This was investigated for the α_2-adrenergic/μ-opiate receptor dimer [48••], which has been shown to occur *in vivo* [49].

When a FlAsH/CFP-labeled α_{2A}-adrenergic receptor was coexpressed with a μ-opiate receptor, it acquired sensitivity to morphine [48••]. The labeled α_{2A}-adrenergic receptor alone responded only to noradrenaline (but not to morphine) with a decrease in FRET. However, when coexpressed with a μ-opiate receptor, the responsiveness to noradrenaline was still the same, but now the presence of morphine reduced this responsiveness to noradrenaline. Similar inhibitory effects were seen when G-protein activation was monitored, again with FRET methods. These data suggest that the active μ-receptor blocked the activity of the α_{2A}-adrenergic receptor — in line with the models mentioned above that only one receptor in a GPCR dimer is active. This inhibitory effect was very rapid (occurring with a half-life of less than 500 ms), suggesting that it was due to a direct protein–protein interaction rather than a competition for G-protein subunits [50] or other indirect effects.

Inhibitory interactions within a receptor dimer have very recently also been observed for the dopamine D_2 receptor using a functional complementation assay [51•]. These assays revealed — as in the case of the leukotriene B_4-receptor [44•] — that the minimal functional unit consists of two receptors and one G-protein. Crosstalk between the two receptors was shown by the observations that inverse agonist binding to the second protomer enhanced signaling, whereas agonist binding to the second protomer blunted signaling. Thus, these data support the view that in a GPCR dimer only one of the two protomers is active.

Movements within a GPCR dimer have also been studied using FRET technology by inserting the labels (CFP and YFP) into either the first or the second intracellular loop of the two protomers of the mGluR1 [52••]. Agonists increased this *inter*molecular FRET within between subunits that were labeled in the second loop, while it was decreased when both subunits were labeled in the first loop. These studies illustrated that agonist-induced rearrangement of the two protomers in a GPCR dimer may play a crucial role in the activation and signaling process. By contrast, an *intra*molecular rearrangement (in receptors labeled both in the first or second intracellular loops plus the C-terminus) was not detected. However, labeling mGluR1 receptors in the third intracellular loop — the standard site to detect *intra*molecular changes in class A GPCRs [46•,47,53,54] — disturbed membrane targeting. Therefore it is not clear, whether the lack of *intra*molecular rearrangements is a characteristic of these specific receptors (or more generally of class C receptors), or whether it was just not detected.

While Tateyama *et al.* [52••] reported agonist-induced changes in *inter*subunit FRET that occurred over several seconds, Marcaggi *et al.* [55•] recently reported FRET changes between the same mGluR1 subunits (labeled in the first intracellular loop) that were not only opposite in direction to the changes reported by Tateyama *et al.* (i.e. an increase in FRET), but also faster by two orders of magnitude, with a half-time of less than 10 ms. This is not only about 100 times faster than the *inter*molecular kinetics reported by Tateyama *et al.* [52••], but also considerably faster than the 50–80 ms activation times observed with most class A *intra*molecular GPCR FRET constructs [11,46•,47,53,54,56]. Whether these fast kinetics are a property of the mGluR1, whether they are a characteristic of *inter*molecular FRET, or whether they are due to the fact that all these constructs were deficient in G-protein coupling, remains to be investigated. Interestingly, a noncompetitive inverse agonist caused no effect opposite to that of agonists, whereas competitive inverse agonists have been observed to induce inverse intramolecular FRET signals in class A GPCRs [57].

Taken together, these studies indicate that rapid agonist-induced conformational changes occur in GPCR dimers which permit not only activation of the dimer, but also signal transfer from one protomer to the other — usually apparently in an inhibitory manner. Inhibitory interactions have, furthermore, been uncovered between the dimers in the $GABA_B$-receptor tetramer [25••]. Disruption of these tetramers markedly increased signaling, suggesting that in a $GABA_B$-receptor tetramer only one of the dimers may be signaling. This is quite reminiscent of the situation in GPCR homodimers, where only one of the protomers appears to be signaling.

Conclusion and future perspectives

Although GPCRs can in many cases function as monomers, they often appear to assemble into dimers, and in some cases even tetramers and higher order complexes. Dimer assembly may occur during biosynthesis, as is certainly the case for receptors that only reach the cell surface in a dimeric form. But GPCR assemblies may also be more transient and dynamic — presumably just as a consequence of various affinities that the protomers in such an assembly may have for each other. The regulation and dynamics of these assemblies will be a major topic of future research.

An even more interesting question is how the protomers in such assemblies may affect each other. A pattern seems to be emerging that consists of several inhibitory mechanisms. Thus, these assemblies may represent a 'reservoir' of signaling that can be liberated upon disassembly. When and how this happens, and the molecular mechanisms of these processes, will be major questions for the future.

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