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PERSPECTIVE

The Ants Go Marching Two by Two: Oligomeric Structure of G-Protein-Coupled Receptors

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ABSTRACT

A number of class C G-protein coupled receptors (GPCRs) have been shown to form dimers in the plasma membrane, and mounting evidence supports the hypothesis that many, if not all, class A rhodopsin-like receptors also form dimers or higher-order oligomers. Evidence for this hypothesis has come from SDS-polyacrylamide gel electrophoresis, coimmunoprecipitation, resonance energy transfer, atomic force microscopy, and cross-linking studies, approaches that are reviewed in this article. Like any method, each has its strengths and limitations, and these must be kept in mind when interpreting the data for oligomerization. Recent experimental evidence supports the hypothesis that class A receptors may exist as higher-order oligomers, or even as arrays, with distinct symmetrical interfaces in both the first and fourth transmembrane segments.

G-protein-coupled receptors (GPCRs) comprise a large superfamily of receptors that couple binding of a diverse group of ligands to activation of heterotrimeric G-proteins (Gether, 2000). A number of the class C GPCRs have been shown to form dimers in the plasma membrane, including the calcium-sensing receptor (Bai et al., 1998), the $GABA_B$ receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998), and the metabotropic glutamate receptors (Romano et al., 1996). Mounting evidence also supports the hypothesis that, in the plasma membrane, many if not all of the class A rhodopsin-like receptors are dimeric (or oligomeric) as well (reviewed in Rios et al., 2001; Angers et al., 2002; George et al., 2002; Bai, 2004).

The data that have supported a dimeric or oligomeric structure for class A GPCRs have come from the application of a number of different biochemical and biophysical approaches, several of which have been used by Carillo et al. (2004) in this issue of *Molecular Pharmacology*. They used a combination of coimmunoprecipitation, single-cell fluorescence resonance energy transfer (FRET), and cell surface time-resolved FRET to explore systematically the helix interactions that lead to the quaternary structure of the α1b adrenergic receptor. These studies explored the self-association of a large number of N-terminal fusions containing only 1 or 2 of the transmembrane (TM) segments and also the ability of these fragments to interact with the full-length receptor. As discussed below, the results are remarkably consistent with a model of the rhodopsin quaternary structure based on the results of atomic force microscopy studies of native mouse rhodopsin in the membrane (Fotiadis et al., 2003; Liang et al., 2003).

One of the earliest pieces of evidence that GPCRs might exist as higher-order complexes was the appearance of multiple bands on SDS-PAGE. Such bands have been observed for many membrane proteins, hydrophobic proteins that are susceptible to aggregation and SDS resistance (Soulie et al., 1996). Thus, the appearance of SDS-resistant bands on immunoblots must be interpreted with caution. The higher-order bands seen with transient transfection may result, in part, from the fact that GPCRs exist in the membrane as dimers or oligomers and are prone to aggregate upon removal

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; FRET, fluorescence resonance energy transfer; TM, transmembrane; AR, adrenergic receptor; BRET, bioluminescence resonance energy transfer; D2R, dopamine D2 receptor; mAchR, muscarinic acetylcholine receptor; TMD, transmembrane domain; LH, luteinizing hormone.

from the membrane into detergent-resistant multimeric forms, thus reflecting their state in the membrane. Indeed, the presence of SDS-resistant oligomeric species in native tissue (Nimchinsky et al., 1997; Zawarynski et al., 1998) suggests that such bands are not merely an artifact of heterologous expression.

Bovine rhodopsin, the only GPCR for which a high-resolution structure is available, was crystallized as a nonphysiological dimer with the extracellular end of one receptor and the cytoplasmic end of its "dimer" partner facing the same direction (Palczewski et al., 2000). Thus, in the detergent and crystallization conditions used, rhodopsin is a monomer, although recent evidence suggests that rhodopsin is oligomeric in the native membrane (Fotiadis et al., 2003; Liang et al., 2003) (see below).

The β2 adrenergic receptor (β2AR) has been inferred to be a monomer when solubilized in the detergent dodecyl maltoside, although the receptor binds ligands normally in this detergent and seems to be functionally intact (Gether et al., 1995, 1997). Moreover, we have found that a β2AR cysteine mutant is efficiently cross-linked in the plasma membrane, but when this mutant receptor is extracted in dodecyl maltoside, no cross-linking is observed (W. Guo and J. A. Javitch, unpublished observations), consistent with the detergent-induced disruption of an oligomeric interface. Nonetheless, bioluminescence resonance energy transfer (BRET) (Angers et al., 2000; Mercier et al., 2002) and cross-linking studies (Guo et al., 2002) have shown that the β2AR and the dopamine D2 receptor (D2R) are almost exclusively dimeric or oligomeric in the plasma membrane. Thus, the native oligomeric structure that exists in the membrane seems to be disrupted in detergent.

Likewise, the M2 muscarinic acetylcholine receptor (mAchR) that can be coimmunoprecipitated from a detergent extract of Sf9 cells is almost completely nonfunctional, whereas the material that binds ligand in detergent seems to be almost exclusively monomeric, consistent with its being a monomer in detergent (Park and Wells, 2003). Thus, the material that can be coimmunoprecipitated is either misfolded in the membrane or becomes misfolded during extraction from the membrane. It is possible, however, that in a different detergent and/or in the presence of ligand, it might be possible to preserve higher order structure in the soluble state. Consistent with this, more active M2 mAchR could be coimmunoprecipitated when the receptor was solubilized in the presence of ligand, which presumably partially stabilizes the oligomeric structure in detergent (Park and Wells, 2003).

Data derived from the use of coimmunoprecipitation have been used as support for an oligomeric structure of these and other receptors. Given the large amount of SDS-resistant material present on immunoblots of transiently transfected GPCRs and the findings discussed above for the M2 mAchR, data from the use of coimmunoprecipitation must be interpreted with caution and the aid of extensive controls (see below). Indeed, it was reported that *any* of a large number of GPCRs coexpressed with the 5HT-1A receptor could be coimmunoprecipitated (Salim et al., 2002).

Much useful information has come from FRET and BRET studies, which have the advantage that they can be applied to receptor in the membrane of living cells rather than to detergent extracts. Although the application of these techniques is complex and potentially rife with artifacts if not carried out appropriately, FRET and BRET have provided evidence that many GPCRs are homodimers and/or heterodimers in the membrane (reviewed in Angers et al., 2002). Most of these studies have employed receptors fused at their C-termini to different variants of green fluorescent protein or to luciferase. With standard FRET or BRET it can be difficult to establish the cellular compartment in which the FRET or BRET is being observed, and, given the caveats described above for transient transfection, it is difficult to rule out a contribution to the FRET or BRET signal of intracellular misfolded or aggregated material. FRET microscopy approaches, including ratio imaging, donor photobleaching, and donor recovery after acceptor photobleaching (Schmid and Sitte, 2003), have been developed to visualize the localization of protein-protein interactions at the plasma membrane or in intracellular compartments.

The use of fluorescently labeled antibodies to two different coexpressed N-terminal extracellular epitope tags has allowed the application of time-resolved FRET to GPCRs in the plasma membrane (McVey et al., 2001). Because only cell surface receptors have their N termini accessible to antibodies in an intact cell, only cell surface interactions should be observed. A potential problem of this approach is that the antibodies used to identify the epitope-tagged receptors are bivalent and thus might be expected to cluster receptors. Thus, appropriate controls must be performed to establish that this is not the cause of the FRET signal observed. In addition, the size of the antibody is rather large compared with the receptor, making it difficult to assess distances between the epitopes to which these antibodies are attached.

A caveat related to the interpretation of FRET/BRET, as well as cross-linking studies, is the possibility that crowding of receptors in the membrane as a result of very high-level expression in heterologous cells could lead to artifactual FRET/BRET signals or to cross-linking. This was elegantly addressed by Bouvier and colleagues, who showed that the BRET signal produced by β2AR homodimers was unaffected over nearly a 100-fold level of expression (Mercier et al., 2002). Only at extraordinarily high levels of expression was "bystander BRET" observed, indicating that at more typical levels of expression, as well as at very low levels of expression, the receptors were almost exclusively dimeric or oligomeric (Mercier et al., 2002). This conclusion is in full agreement with cross-linking studies in the D2R (Guo et al., 2002).

The introduction of quantitative BRET (Mercier et al., 2002) has also facilitated measurements of apparent affinity between the dimer partners and thereby demonstrated a specificity of GPCR interactions that is often not apparent by the coimmunoprecipitation methods described above or even in some cases by nonquantitative BRET.

Unraveling a structural mechanism for cross-talk between receptors in a dimeric complex must start with the identification of the dimer interface. Although extracellular, transmembrane, and/or C-terminal regions have been proposed as possible sites of receptor interactions in the dimerization of GPCRs in some classes (George et al., 2002), given the limitations imposed by the large size of the probes used in BRET and FRET and limited sites of attachment, it has not been possible to identify the interaction surface using these techniques. A number of studies have employed cross-linking to demonstrate that GPCRs in the membrane are dimeric or oligomeric (Hebert et al., 1996; Cvejic and Devi, 1997;

AbdAlla et al., 1999; Rodriguez-Frade et al., 1999; Vila-Coro et al., 1999, 2000). These studies have used relatively long lysine-reactive bifunctional cross-linking reagents, and it has not been possible to infer the specific residues or regions that were cross-linked.

We have demonstrated that D2R can be oxidatively cross-linked via $Cys168^{4.58}$ at the extracellular end of TM4 at a symmetrical interface (Guo et al., 2002). Mutation of $Cys168^{4.58}$ to Ser abolishes cross-linking but does not disrupt the D2R dimer interface, based on the cross-linking of this construct observed when another residue at the interface is simultaneously mutated to cysteine (W. Guo and J. A. Javitch, unpublished observations).

Because cross-linking requires that only one of the two cysteines involved is modified initially by the reagent, and the derivatized cysteine then reacts by collision with the second unmodified cysteine, the rate of collision must be much faster than the rate of initial modification. This is consistent with the idea that the cysteines are initially very close. The actual distance between the α-carbons of disulfide-linked cysteines is 5.6 $\pm$ 0.6 Å (Aziz et al., 2002). The very high fraction of receptor that can be cross-linked, the apparent specificity of the cross-linking (based on the appearance of a single homodimer band), and the lack of cross-linking of $Cys56^{1.54}$ (which, based on the bovine rhodopsin structure, has a similar lipid accessibility as $Cys168^{4.58}$), all argue for the proximity of the TM4 cysteines in the native state. Thus, it is likely that in the membrane, untreated with copper phenanthrolene, D2R exists as a homodimer but that this dimer does not survive detergent solubilization.

Our finding that the site of cross-linking in D2R is $Cys168^{4.58}$ at the extracellular end of TM4 is consistent with the hypothesis that TM4 forms a symmetrical dimer interface. Fragment coexpression studies were also consistent with the existence of a symmetrical TM4 interface in D2R (Lee et al., 2003). In contrast, in a study that led to the study in this issue, a truncated fragment containing only TM1 of the α1AR showed time-resolved FRET with the full-length receptor, suggesting the involvement of TM1 in the dimerization of this receptor (Carrillo et al., 2003) Studies on yeast α factor receptor (Overton et al., 2003) have also suggested that TM1 plays a central role in dimerization.

Based on analysis of correlated mutations, it has been hypothesized that various rhodopsin-like GPCR subtypes may differ in their contact interfaces (Filizola and Weinstein, 2002; Filizola et al., 2002), consistent with either TM1 or TM4 as dimer interfaces in different receptors. However, given the possible organization of GPCRs into higher order oligomers, it is important to note that these predictions may in fact include information about conserved interfaces other than dimers.

It is interesting that cross-linking studies of the C5a receptor provided evidence for cross-linking of a symmetric dimer interface in TM4 as well as in TM1/TM2, but it was difficult to incorporate both of these symmetrical cross-links into a model of dimer formation. These authors proposed the existence of a higher-order oligomeric structure as most consistent with these data (Klco et al., 2003).

The organization of rhodopsin into two dimensional arrays of dimers has been supported by atomic force microscopy analysis of native mouse retinal membranes (Fotiadis et al., 2003; Liang et al., 2003) (Fig. 1) (but see also Chabre et al., 2003). Although these images are not of sufficient resolution to allow identification of individual helices, based on molecular modeling, TM4 and TM5 were proposed to be involved in symmetric intradimeric contact, whereas an asymmetric interface between the TM5-TM6 loop and the TM1-TM2 loop formed dimeric rows (Fig. 2). A row of dimers was proposed to interact with an adjacent row of dimers at symmetric TM1 interfaces (Fig. 2).

In this issue, Carrillo et al. (2004) reproduce their earlier finding of the interaction of a TM1 fragment with the full-length receptor both by coimmunoprecipitation and by time-resolved FRET at the cell surface. In addition, these methods demonstrated self-association of a TM1 fragment. Fragments comprising TM3–4 and TM5–6, but not TM7, were also able to interact with full-length receptor based on coimmunoprecipitation and time-resolved FRET. Symmetrical interactions were noted between fragments containing TM4, but this fragment did not interact with TM1–2 or TM5–6. It is noteworthy that control experiments failed to show interaction of the TM1 fragment with the full-length histamine H1 receptor, suggesting a specificity of interaction, although related H1 receptor controls were not reported for the other fragment interactions.

These findings are consistent with the model proposed for the packing of mouse rhodopsin into an array (Liang et al., 2003); a symmetrical TM4–5 interaction forms the dimers, an asymmetrical TM5-TM6 interaction with TM1-TM2 forms the interdimer contacts, and there is a symmetrical TM1 interface between the dimer rows (see Fig. 2). It is remarkable that these receptor fragments (except for TM7) are expressed at the cell surface, where they can show time-re-

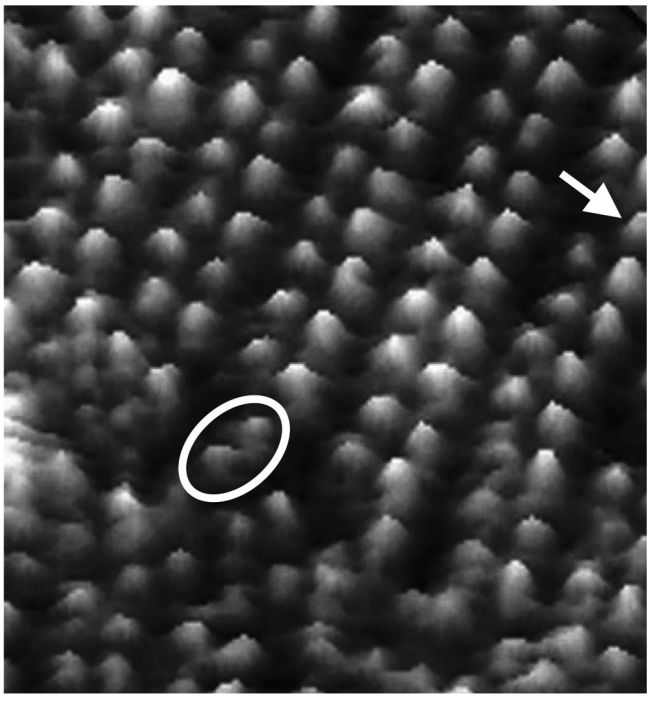

Fig. 1. Organization and topography of the cytoplasmic surface of rhodopsin. A topograph obtained using atomic-force microscopy (see Fotiadis et al., 2003) shows the paracrystalline arrangement of rhodopsin dimers in the native disc membrane. Individual dimers, presumably broken away from one of the rows, are shown inside an ellipse, and an occasional rhodopsin monomer is shown by an arrow. [This figure was kindly provided by K. Palczewski and collaborators.]

solved FRET and that specific interactions seem to be maintained in detergent extracts. Additional methods, such as cysteine cross-linking, will be useful to map the exact interfaces and to investigate further the physical basis of these interactions and the determination of their specificity.

Although most GPCRs studied have been inferred to be dimers or oligomers, the role of GPCR oligomerization remains enigmatic. Most importantly, it has not been established thus far whether receptor activity relies on its organization in a quaternary structure or whether the primary function of oligomerization is to enable an additional criterion for the endoplasmic reticulum quality control mechanism. Multiple experiments suggest a functional interaction between the binding sites in heterodimeric receptors, from the perspective of novel pharmacology and synergistic or antagonistic effects on signaling (Jordan and Devi, 1999; Gomes et al., 2000; Jordan et al., 2001, 2003). These findings are very provocative, but for those receptors, which are coupled to multiple G-proteins and to multiple signaling and regulatory pathways, it has been difficult to rule out indirect effects and to build a mechanistic understanding of receptor cross-talk.

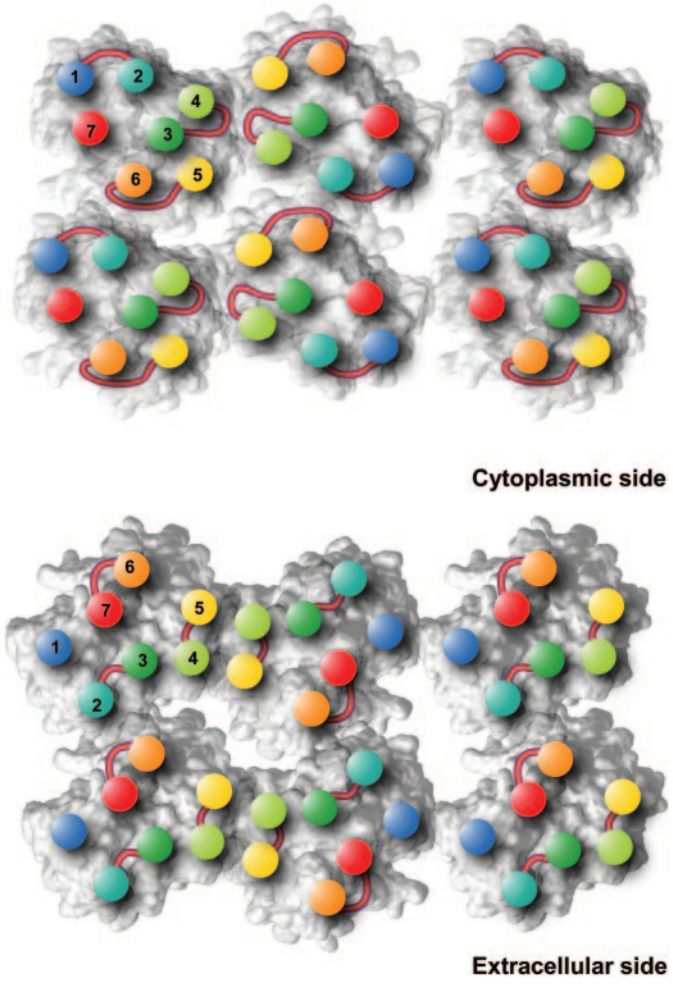

Fig. 2. Model for the packing arrangement of rhodopsin molecules within the paracrystalline arrays of native disc membranes. Cytoplasmic side (top) and extracellular side (bottom) of rhodopsin oligomers. Positions of helix ends are marked by colored discs and the corresponding helix numbers. Extracellular and cytoplasmic loops are drawn schematically at the corresponding locations. TM4 and TM5 form contacts between monomers. Most contacts between dimers are created by the intracellular loop between TM5 and TM6 from one monomer with the loop between TM1 and TM2 and the C-terminal residues from the partner monomer. Only half of a second row of rhodopsins is shown. Hydrophobic residues from TM1 near the extracellular side create the contact between double rows. [Reprinted from Fotiadis D, Liang Y, Filipek S, Saperstein DA, Engel A, and Palczewski K (2004) The G protein-coupled receptor rhodopsin in the native membrane. *FEBS Lett* **564:**281–288. Copyright © 2004 Elsevier Science. Used with permission.]

Because of their heterodimeric nature, the $GABA_B$ receptors have been enormously important in pointing out that a number of functions previously thought to occur in the monomer actually require a dimeric structure. Whether these lessons from a class C GPCR can be generalized to class A GPCRs is not yet known but merits serious consideration and experimental analysis. Cell-surface trafficking (Kaupmann et al., 1998; Margeta-Mitrovic et al., 2000) is not the only role of $GABA_B$ receptor heterodimerization. Another role became apparent upon investigation of the mechanism of GABA binding and receptor-G protein coupling. The N terminus of GB2 differs from that of GB1; it does not bind GABA or any other known ligand (Galvez et al., 2001). It is surprising, however, that mutations in GB1 of intracellular residues predicted to be critical for coupling to G-protein [by analogy with the closely-related metabotropic glutamate receptors family (Gomeza et al., 1996; Francesconi and Duvoisin, 1998)] had no effect on G-protein activation by the $GABA_B$ heterodimer (Robbins et al., 2001; Duthey et al., 2002). In contrast, analogous mutations within the second or third intracellular loops (IL2 and IL3) of GB2 alone led to a loss of G-protein signaling by the heterodimer (Robbins et al., 2001; Duthey et al., 2002; Havlickova et al., 2002). Thus the $GABA_B$ receptor functions through a form of trans-activation, wherein GB1 binds agonist and GB2 couples to G-protein.

It is interesting that coexpression of a chimeric construct containing the N terminus (NT) of GB1 and the transmembrane domain (TMD) of GB2 (GB1/2) with a chimeric construct containing the N terminus of GB2 and the TMD of GB2 (GB2/1) resulted in induction of inositol phosphate formation in response to GABA application, whereas the individual chimeras did not (Galvez et al., 2001). If the structure and function of the chimeric GB1/2 and GB2/1 receptors are not significantly different from those of wild-type receptors, the ligand-induced signaling (i.e., *cis*-activation) of this GB1/2+GB2/1 heterodimer suggests the possibility that both *cis*- and *trans*-activation may occur in homodimeric class A GPCRs, in which both "subunits" are capable of binding ligand and signaling to G-protein. In this scenario, the $GABA_B$ receptor might be a relatively unique case in which one subunit is unable to bind ligand and the other is unable to couple to G-protein, so that only *trans*-activation occurs in the wild-type $GABA_B$ heterodimer and only *cis*-activation occurs in the GB1/2+GB2/1 heterodimer.

A recent study has demonstrated that in a heterodimeric complex of GPCRs, each fused to G protein, ligand binding can lead to second messenger activation via an interaction between the "cis" receptor and the "trans" G-protein (Carrillo et al., 2003). This does not represent *trans*-activation but rather *cis*-activation (binding site and intracellular loops that contact G-protein from the same GPCR) of a G-protein fused to the *trans*-receptor. The only demonstration to date of true $GABA_B$-like *trans*-activation in another GPCR comes from a study of the luteinizing hormone (LH) receptor, a class

A GPCR that has a large extracellular N-terminal domain that binds LH (Ji et al., 2002). Through studies of different combinations of coexpressed mutant LH receptors, thereby creating pseudoheterodimers, these authors were able to conclude that both *cis*-and *trans*-activation must occur in the LH receptor dimer. The mechanism for the *trans*-activation of the LH receptor and whether it involves contact between an NT and the *trans*-TMD or propagation of conformational change directly between the TMDs is unknown.

Another important unanswered question is the stoichiometry of the signaling complex. That is, in a homo-dimeric GPCR in which both subunits are capable of interaction with G-protein, does the GPCR dimer signal though two heterotrimeric G-proteins, or does the dimer interact with a single G-protein? Recent evidence from refolded leukotriene B4 receptor reconstituted with G-protein in detergent suggests that only one heterotrimeric G-protein binds to a receptor dimer (Baneres and Parello, 2003), and interactions consistent with such a proposal have been modeled (Filipek et al., 2004). Whether this finding can be generalized to all GPCRs remains an important question for further research, as is elucidation of the possible functional role of the formation of higher-order arrays of GPCRs.

An important step toward this goal is identification of the structural determinants of oligomerization. The physical basis of GPCR interaction in the membrane remains mysterious—a traditional view would be that the largely hydrophobic residues that seem to form these oligomeric interfaces would have a favorable interaction with lipid and would not be driven to associate with other helices, either symmetrically or asymmetrically. Nonetheless, multiple studies suggest a specificity to these interactions that is further supported in Carrillo et al. (2004) by the lack of time-resolved FRET between the α1b receptor and the full-length H1 receptor as well as by the interaction of particular fragments and not others.

Because coimmunoprecipitation data comprise a major component of this manuscript, it must be pointed out that despite the specificity of the data and their consistency with the rhodopsin model, this technique remains problematic. Note in Fig. 2 the large amount of slowly migrating SDS-resistant aggregate present as the major species after immunoprecipitation but present in the initial lysate at a vastly lower ratio. These SDS-resistant aggregates are therefore formed during immunoprecipitation, conceivably in part through the formation of new disulfide bonds during the precipitation process. Enough of the monomer is dissociated in SDS to allow the detection of this species and thereby to establish coimmunoprecipitation, but this provides some warning because the mechanism leading to this aggregation is unclear. Perhaps similar but less stable aggregates form with coexpression of the fragments as well, but these may be more easily disrupted by SDS. Regardless, the authors are to be commended for the systematic application of the approach as well as for validating many of the results with time-resolved FRET measurements in the membrane, the results of which are qualitatively identical to the coimmunoprecipitation data. All methods of course have limitations, but the systematic application and validation through complementary methods make this an important contribution to our evolving understanding of GPCR oligomerization. Further studies must examine other receptors and use other complementary methods to continue to probe the validity and generality of these findings to the entire superfamily of GPCRs.

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