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DIMERIZATION: An Emerging Concept for G Protein–Coupled Receptor Ontogeny and Function

Stephane Angers,[1] Ali Salahpour,[1] and Michel Bouvier
Department of Biochemistry and Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, Montréal, H3C 3J7, Canada; e-mail: michel.bouvier@umontreal.ca, stephane.angers@umontreal.ca, ali.salahpour@umontreal.ca

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■ **Abstract** In the last four to five years, the view that G protein–coupled receptors (GPCRs) function as monomeric proteins has been challenged by numerous studies, which suggests that GPCRs exist as dimers or even higher-structure oligomers. Recently, biophysical methods based on luminescence and fluorescence energy transfer have confirmed the existence of such oligomeric complexes in living cells. Although no consensus exists on the role of receptor dimerization, converging evidence suggests potential roles in various aspects of receptor biogenesis and function. In several cases, receptors appear to fold as constitutive dimers early after biosynthesis, whereas ligand-promoted dimerization at the cell surface has been proposed for others. The reports of heterodimerization between receptor subtypes suggest a potential level of receptor complexity that could account for previously unexpected pharmacological diversities. In addition to fundamentally changing our views on the structure and activation processes of GPCRs, the concept of homo- and heterodimerization could have dramatic impacts on drug development and screening.

INTRODUCTION

According to the most recent predictions, GPCRs represent the third largest family of genes present in the human genome. Their physiological and pharmacological importance can be easily appreciated when we consider the wide variety of stimuli that use these transmembrane proteins as signal transducers. Although scattered studies throughout the 1970s and 1980s had proposed that GPCR could exist as dimer or higher-structure oligomers [for a review see (1)], the preponderant models

[1]Have contributed equally to the manuscript.

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depicted them as monomeric entities that interact with hetero-trimeric G proteins upon ligand activation. More recently, an increasing number of studies have suggested that they do exist and may function as oligomeric complexes, frequently referred to as dimers. Here, we review the most recent biochemical and biophysical evidence supporting their existence with a special emphasis on techniques that allow their assessment in living cells. The proposed roles of dimerization in receptor ontology and signaling properties as well as the potential implications of heterodimerization and its regulation on receptor pharmacology are also discussed. Because most techniques used cannot easily distinguish between dimers and larger oligomers, the term dimer is used in this review as the simplest form of oligomer for sake of simplicity.

COIMMUNOPRECIPITATION AS A TOOL TO DETERMINE HOMO- AND HETERODIMERIZATION

In recent years, one of the most common biochemical approaches used to investigate GPCR dimerization has been coimmunoprecipitation of differentially epitope-tagged receptors. In the first study using such an approach, Hebert et al. used coexpression of HA and MYC-tagged β2-adrenergic receptors (B2AR) where detection of HA immunoreactivity in samples immunoprecipitated with an anti-MYC antibody was taken as evidence of intermolecular interactions between the two polypeptide chains (2). The selectivity of the interaction was controlled using an epitope-tagged MYC-M2-muscarinic receptor, another GPCR that could not be coimmunoprecipitated along with the HA-β2-adrenergic receptor. Since then, similar strategies have been used to document homodimerization of the dopamine (3), the mGluR5 (4), the δ-opioid (5), the calcium (6) and the M3 muscarinic receptors (7). More recently, coimmunoprecipitation experiments were also used to demonstrate the existence of heterodimers between closely related receptor subtypes. These include: GABAb R1 and GABAb R2 receptors (8–10), the δ-opioid and κ-opioid receptors (11), the δ-opioid and μ-opioid receptors (12, 13), and the SST3 and SST2a somatostatin receptors (14). Heterodimerization among more distantly related receptors such as the adenosine A1 and dopamine D1 receptors (15), the angiotensin AT1 and bradykinin B2 receptors (16), and the δ-opioid and β2-adrenergic receptors (17) has also been observed using coimmunoprecipitation protocols. These studies led to the suggestion that GPCRs exist both as homo- and heterodimers. The possible functional implications of these interactions are discussed in later sections.

Although commonly used to study protein-protein interactions, coimmunoprecipitation of membrane receptors requires their solubilization using detergents, and it may be problematic when considering highly hydrophobic proteins such as GPCRs that could form artifactual aggregates upon incomplete solubilization. Despite this possible caveat, two lines of evidence have been used to validate coimmunoprecipitation results. First, treatment of cells with hydrophilic cross-linking

agents before lysis and solubilization was shown to stabilize dimers, which suggests that preformed complexes were present at the surface of the cells (2, 5). Second, no coimmunoprecipitation between differentially tagged receptors could be achieved when membranes derived from cells expressing each receptor individually were mixed and solubilized, which argues that receptor dimerization requires coexpression and that solubilization does not promote spurious dimerization. Nevertheless, the general acceptance of GPCR dimerization still awaited the direct demonstration that these complexes existed in living cells. This was made possible with the development and utilization of biophysical methods based on light resonance energy transfer.

DETECTION OF DIMERS IN LIVING CELLS

Resonance energy transfer approaches are based on the nonradiative transfer of energy between the electromagnetic dipoles of an energy donor and acceptor (18). In the case of fluorescence resonance energy transfer (FRET), both the donor and acceptor are fluorescent molecules, whereas for bioluminescence resonance energy transfer (BRET), the fluorescent donor moiety is replaced with the bioluminescent catalytic activity of an enzyme. Prerequisites for these processes are: (a) the existence of an overlap between the emission and excitation spectra of the donor and acceptor molecules and (b) that the donor and acceptor be in close molecular proximity, typically <100 Å. The critical dependence on the molecular nearness between donors and acceptors for energy transfer (the efficiency of transfer decrease with the 6th power of the distance) makes BRET or FRET systems of choice to monitor protein-protein interactions in living cells. This should be contrasted with confocal colocalization approaches that can provide information about regional but not molecular proximity due the low spatial resolution of light microscopy. Indeed, merging of fluorescent markers will be detected for molecules that can be as much apart as the visible light wavelength ($\sim$4000–7000 Å). BRET and FRET thus offer unique approaches that allow the monitoring of protein oligomerization in living cells without disrupting the natural environment where they occur. The following sections describe how variants of these approaches have been used to document GPCR dimerization.

Bioluminescence Resonance Energy Transfer (BRET)

BRET is a natural phenomenon occurring in many marine organisms such as the sea pansy *Renilla reniformis* and the jellyfish *Aequorea victoria*. In these animals, the bioluminescence resulting from the catalytic degradation of the substrate coelenterazine by the luciferase enzyme (luc) produces resonance energy that can be transferred to the green fluorescent protein (GFP), which, in turn, emits fluorescence at its characteristic wavelength upon dimerization of the two proteins (19).

The first BRET study to document GPCR dimerization in living cells was carried out using fusion proteins that genetically link GFP and Renilla luciferase (Rluc) to the carboxyl terminus of the human β2-adrenergic receptor (β2AR) (20). Transfer of energy between the β2AR-Rluc and β2AR-GFP constructs coexpressed in the same cells was observed under basal conditions, indicating that this receptor forms constitutive homodimers. Stimulation with the selective β-adrenergic agonist, isoproterenol, increased the level of energy transfer detected, indicating that receptor activation is associated either with an increase in dimer formation or else with conformational changes that lead to a closer proximity between the Rluc and the GFP resulting in increased energy transfer efficiency.

Constitutive BRET signals were also detected for the δ-opioid (21) and TRH receptors (22), indicating that the existence of preformed dimers is a common feature among GPCRs. As for the β2AR, agonist stimulation increased the BRET between the TRHR-Rluc and TRHR-GFP. In contrast, the BRET detected for the δ-opioid receptor was insensitive to agonist treatment. Whether this difference reflects meaningful variation in the mechanisms underlying receptor activation or is merely technical remains to be determined. In any case, caution should be exercised when interpreting the effects of ligand treatment on the BRET signals because the current approach does not allow us to easily distinguish between changes in the conformation of pre-existing dimers and an increase in their number.

Fluorescence Resonance Energy Transfer (FRET)

FRET WITH GFP Many different spectral mutants of the jellyfish green fluorescent protein now exist (23). Two of these variants, the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP), have spectral properties that make them a good FRET pair and have been used successfully to measure protein-protein interaction in living cells (24–26).

Using the yeast α-mating factor receptor Ste2 fused to CFP and YFP, Overton & Blumer observed constitutive FRET in both whole yeast cell and in purified plasma membrane fractions (27). Based on the fact that the agonist pheromone and GTPγS could not modulate the FRET efficiencies, the authors concluded that monomer-dimer equilibrium is unlikely to be affected during signaling. It is important to mention, however, that carboxyl terminally truncated versions of the receptor (that are not affected in their signaling properties) were needed to obtain significant transfer of energy, emphasizing the importance of protein conformation in energy transfer experiments.

Using the wild-type GFP and the newly described red fluorescent protein (RFP) as FRET partners that were fused to the carboxyl terminus of the gonadotropin-releasing hormone receptor, Cornea et al. (28) also monitored constitutive homodimerization at the single cell level. The agonist buserelin induced a dose-dependent increase in FRET in a specific area of the plasma membrane, which suggests that dimerization is not a uniform process but rather that it occurs in specific regions,

possibly related to receptor activation. In a more recent study, FRET between the GnRH-CFP and the YFP-GnRH was found to be entirely dependent upon agonist stimulation (29).

PHOTOBLEACHING FRET (pbFRET) Homodimerization of the somatostatin SSTR5 (30) and heterodimerization between the SSTR5 and the dopamine D2 receptors (31) were also recently described by Rocheville et al. using photobleaching FRET (pbFRET). Photobleaching is an intrinsic property of a fluorophore characterized by the fading of the fluorescent signal upon continuous exposure to excitation light. In pbFRET, the close proximity of an acceptor fluorophore offers an alternate route (FRET) for the deactivation of the energetically excited state of the flurophore that results in a slower photobleaching of the donor molecule. Using cells expressing HA-tagged SSTR5 and D2 receptors, and fluorescein and rhodamine-conjugated antibodies, Rocheville et al. observed a slower photobleaching decay of fluorescein when both fluorescein- and rhodamine-conjugated antibodies were incubated with the cells coexpressing either two SSTR5 receptors or the SSTR5 and the dopamine D2 receptors, which suggests dimerization between these receptors. In the case of the SSTR5 homodimers, pbFRET revealed the presence of constitutive dimers in higher expressing cell lines, but agonist stimulation was needed to observe the signal in cells expressing a low number of receptors. For SSTR5-D2 heterodimers, no pbFRET was observed in the absence of ligands, but agonists for either of the receptors led to a significant pbFRET signal that was interpreted as agonist-promoted heterodimerization. Interestingly, an antagonist was also found to promote pbFRET in the case of the SSTR5-D2 heterodimer. Whether the lack of constitutive signal reflects the sensitivity limits of the assay or an absolute ligand requirement for heterodimerization remains to be investigated.

HOMOGENEOUS TIME RESOLVED FRET (HTRF) HTRF takes advantage of the long-lived fluorescence of fluorophores such as the lanthanide chelate europium^{3+} and allophycocyanin that allow delayed FRET measurements, thus reducing the background resulting from the short-lived cell autofluorescence. By conjugating anti-Flag and anti-myc antibodies to europium and allophycocyanin, McVey et al. confirmed the occurrence of constitutive homodimerization of the δ-opioid receptor at the surface of cells coexpressing myc- and Flag-tagged receptors (21). In the same study, heterodimerization between the β2-adrenergic and the δ-opioid receptor was observed using coimmunoprecipitation and, albeit to a smaller extent, by BRET, but no interactions between these two receptors could be monitored using HTRF. Whether this is a reflection of dimerization events occurring in intracellular compartments that are inaccessible to the antibodies or of the inability of HTRF to detect this heterodimer remains to be determined. However, it should be reiterated that resonance energy transfer signals are extremely dependent on the distances between donor and acceptor molecules. It is thus conceivable that, for some interactions, the donor and acceptor probes could reside in spatial arrangements that would not permit energy transfer.

FLUORESCENT LIGANDS Very recently, receptor ligands coupled to fluorophores were also used to monitor GPCR dimerization. Using the peptide agonist luteinizing hormone labeled with fluorescein and rhodamine, dimerization of the LH receptor has been studied in HEK293 cells (32). FRET between the receptor-bound ligands was readily detected, which indicates that the LH receptor exists as a dimer at the cell surface. Interestingly, the FRET is entirely dependent on the signaling integrity of the receptors because there was little or no energy transfer between mutant receptors impaired in signaling activity. These results suggest that LH receptor functionality involves receptor-receptor interactions.

Even if these biophysical techniques have brought important new evidence that strongly supports the hypothesis that GPCRs exist as dimers in living cells, each technique bears its own advantages and disadvantages. For BRET and FRET using GFPs, the signal of energy transfer reflects interactions occurring anywhere in the cell. In some cases, as in the study of the role of dimerization in protein folding and trafficking, this characteristic may be wanted. However, spurious interactions in endoplasmic reticulum (ER) or aggregation leading to proteasome degradation could theoretically interfere with the specificity of the signal. pbFRET and HTRF techniques, using antibody-conjugated probes that are cell impermeable, detect only the interactions at the cell surface. However, the bivalent nature of antibodies that could, on their own, promote dimerization or initiate conformational changes represents a potential drawback of these techniques. The use of fluorescent ligands linked to technological advances in fluorescent microscopy imaging and spectroscopy will certainly help circumvent some of the limitations of the currently used techniques and should facilitate the study of receptor dimerization in tissues and cells that naturally express them, possibly at the single molecule level.

CONSTITUTIVE VERSUS LIGAND-PROMOTED DIMERIZATION

From the preceding section, it is readily evident that the question of whether GPCRs exist as stable preformed dimers or dynamic structures that can be modulated by ligands has attracted considerable attention. Indeed, clarifying the role of ligand in promoting and/or modulating the oligomeric state of the receptors would contribute to understanding the role that dimerization plays in receptor function. This question takes a particular importance when considering the existence of heterodimers. In fact, if heterodimerization is a common phenomenon among GPCRs, the occurrence of ligand-promoted exchange of subunits between dimers would allow combinatorial regulatory possibilities that could underlie a pharmacological diversity and complexity unforeseen until now.

For many other receptor families such as the tyrosine kinase and the cytokine receptors as well as for the receptors from the TNF-α family, agonist-promoted homo- and heterodimerization is generally believed to be the rule, and equilibrium between monomer and dimer is assumed to be part of the receptor activation process

(33, 34). However, this notion has been recently challenged at least for some receptors. In particular, crystallographic and protein-protein interaction studies have suggested that the erythropoitin receptor exists as a preformed dimer and that hormone binding leads to activation by promoting conformational changes within the receptor dimer rather than by modulating the dimerization state (35, 36). For GPCRs, whether dimers were visualized by direct Western blot analysis, coimmunoprecipitation, or energy transfer approaches, evidence exists to support both constitutive and agonist-promoted dimers. As shown in Table 1, three types of scenario emerge when considering the ensemble of the data available: (a) Dimers are detected under basal conditions and no change in their amount is observed upon ligand treatment, indicating that they represent stable preformed complexes; (b) dimers are detected under basal conditions, but ligands can modulate the extent of dimers observed; and (c) ligand treatment is a prerequisite to the detection of dimers.

This diversity of observations may reflect true differences in the receptors considered in each study, but more likely it is a reflection of the interpretational difficulties that are associated with the various techniques used. In all cases, apparent changes in the amount of dimer observed upon ligand treatment could be due to conformational changes of the dimer rather than in the number of dimeric units. For Western blot and coimmunoprecipitation-based approaches, the accessibility of the specific immunogenic epitopes used to detect the dimers could be either favored or reduced by the conformational changes promoted by the ligands. Such changes in accessibility would lead to more or less immunoreactive signals that could mistakenly be interpreted as changes in the amount of dimers. For energy transfer experiments, ligand-induced conformational rearrangements could modify the distance between the energy donor and acceptor. Since, as previously discussed, the efficacy of energy transfer decreases with the 6th power of the distance between the donor and acceptor molecules, significant changes in BRET or FRET could thus be detected as a consequence of subtle changes in conformation within a preformed dimer. It follows that the initial position of the energy donors and acceptors within the receptor dimer relative to the conformational changes imposed by the ligand could explain the difference in basal energy transfer signal and ligand-sensitivity. Depending on the method used to label the desired receptor (antibody vs GFP) and on the region of labeling (N terminus vs C terminus), receptor motion imposed by a specific ligand could determine whether a change in signal is detected. In some cases, the signal could go from undetectable to important, whereas in others the large basal signals would cover changes due to small conformation change.

In our opinion, the interpretational limitations discussed above interdict a definitive and general conclusion about the relative importance of constitutive vs agonist-promoted dimerization. Nevertheless, the recent description of the crystal structure of the amino-terminal domain of the metabotropic glutamate receptor (37) strongly suggests that, at least for this receptor, dimers are preformed and that ligand binding simply changes the conformation of the dimer. Indeed, the large N-terminal ectodomain that provides the ligand-binding pocket of the receptor was found to

TABLE 1 Constitutive versus ligand-promoted dimerization and effect of agonist on the dimers detection

Receptors	Method of detection	Constitutive	Ligand-induced	Effect of ligands	Reference
β2-Adrenergic	Western blot, coimmunoprecipitation	√		Agonist increases higher-molecular-weight species	2
Dopamine D2	Western blot	√		Not tested	3
MGluR5	Western blot, coimmunoprecipitation	√		Not tested	4
δ-Opioid	Western blot, coimmunoprecipitation	√		Agonist decreases higher-molecular-weight species	5
Calcium	Western blot, coimmunoprecipitation	√		Not tested	6
Muscarinic M3	Western blot, coimmunoprecipitation	√		No effect of agonist	7
GABAb	Western blot, coimmunoprecipitation	√		Not tested	8–10
δ-κ opioid heterodimer	Western blot, coimmunoprecipitation	√		Not tested	72
SST5	Western blot	√		Agonist increases higher-molecular-weight species	30
SST5	pbFRET	Depends on expression level	√	Agonist-mediated modulation in pbFRET	30

SST2, SST3a heterodimer	Western blot, coimmunoprecipitation	√	Not tested	14
Adenosine A1, dopamine D1 heterodimer	Western blot, coimmunoprecipitation	√	Dopamine agonist decreases higher-molecular-weight species	15
δ-, μ-Opioid heterodimer	Western blot, coimmunoprecipitation	√	Not tested	12, 13
Angiotensin AT1, bradykinin B2 heterodimer	Western blot, coimmunoprecipitation	√	Not tested	16
δ-Opioid, β2-adrenergic heterodimer	Western blot, coimmunoprecipitation	√	Not tested	17
β2-Adrenergic	BRET	√	Agonist promotes increased of BRET	20
δ-Opioid	BRET, HTRF	√	No effect of ligands on energy transfer	21
TRH	BRET	√	Agonist promotes increased of BRET	22
Ste2	FRET	√	No effect of agonist on FRET	27
GnRH	FRET	√	Agonist promotes increase of FRET	28

(*Continued*)

TABLE 1 (*Continued*)

Receptors	Method of detection	Constitutive	Ligand-induced	Effect of ligands	Reference
GnRH	FRET, FRAP		√	Agonist induces slowing of receptor lateral diffusion, decreases fraction of mobile receptors, and increases FRET	29
GnRH	BRET		√	Agonist-induced BRET	22
SST5, dopamine D2 heterodimer	pbFRET		√	Agonist and antagonist promotes formation of heterodimer	31
LH	FRET with ligands	Cannot be determined with this technique	Cannot be determined with this technique	FRET between LH-fluorescent molecules	32
CCR5	Western blot		√	Antibody and agonist induce dimerization	59
CCR2	Western blot		√	Antibody and agonist induce dimerization	58
CXCR4	Western blot		√	Agonist induces dimerization	70
Metabotropic glutamate subtype 1	X-ray crystallography	√		Agonist induces change of conformation	37

be dimeric whether it was crystallized alone or cocrystallized with glutamate. In fact, two distinct conformations of the dimer corresponding to the ligand-bound and ligand-free receptor were detected indicating that glutamate promoted or stabilized a specific conformation of the dimer.

ROLE OF RECEPTOR DIMERIZATION IN ENDOPLASMIC RETICULUM EXPORT

The existence of constitutive dimers that would be formed soon after biosynthesis raises the possibility of a role for dimerization in receptor ontology. Indeed, assembly of protein subunits in the endoplasmic reticulum is a common quality control strategy used by the cell to permit the export only of the correctly folded complexes (38). By hiding specific ER retention signals, which interdict the export of incompletely folded proteins, oligomerization favors the entry of the completed proteins in the export system, thus allowing its correct trafficking. A role for dimerization in GPCR export from the ER was first suggested by a series of studies carried out on the metabotropic GABAb receptors (8–10, 39, 40). In these reports, in order to obtain functional metabotropic GABAb receptors, it was necessary to coexpress two distinct receptor subtypes, the GABAb R1 and the GABAb R2. When expressed alone, the GABAb R1 receptor is retained in the ER as an immature protein, whereas the GABAb R2 that is properly targeted to the plasma membrane cannot bind GABA. However, when coexpressed, both receptors reach the plasma membrane as a complex that can bind GABA and inhibit cAMP production through its coupling to Gi/Go G proteins. It thus appears that GABAb R2 serves as a molecular chaperone for the proper targeting of GABAb R1 to the plasma membrane. Consistent with this notion, a recent study has shown the existence of an ER retention signal in the C terminus of the GABAb R1 that is responsible for its intracellular retention (41). Since mutation of this signal leads to the proper trafficking of the GABAb R1 subtype to the cell surface, it has been proposed that coexpression of GABAb R2 would mask the ER retention signal of GABAb R1, presumably through their C-terminal coiled-coil domain, thus allowing membrane expression of GABAb R1. Interestingly, the mutation of the GABAb R1 ER retention signal that leads to membrane targeting is not sufficient to restore signal transduction, which argues that the association with GABAb R2 is not only required for ER export but that it also plays a role in receptor function.

Several other studies on various GPCRs suggest that early dimerization occurring in the ER, which could play a role in trafficking, may be a general feature of this receptor family. The majority of V2 vasopressin receptor mutants associated with nephrogenic diabetes insipidus result in ER retention and lack of cell surface expression of the receptor (42). Yet, many of these mutant receptors form dimers, which suggests that dimerization occurs early after receptor biosynthesis (42). Consistent with this notion, Wess & Zhu have described several

mutations of the V2 vasopressin receptor that can act as negative dominant of the wild-type receptor by preventing its cell surface expression, presumably by retaining it in the ER through dimerization (43). This idea that dimerization in the ER may play an important role in the quality control system leading to the retention of misfolded and mutant receptors is further supported by observations made on a clinically relevant mutation of the CCR5 receptors, the Δ32 mutation, which leads to delayed onset of AIDS in Caucasians who harbor one such allele (44, 45). Indeed, this clinical phenotype may in part be due to the fact that the Δ32 truncation mutant of the CCR5 receptor acts as a dominant negative of the wild-type receptor probably by heterodimerizing with it, thus impairing its cell surface expression and function (46). Similarly, an alternatively spliced form of the D3 dopamine, the open reading frame of which is interrupted in the third cytoplasmic loop (D3nf), impairs cell surface expression of the wild-type full-length receptor, most likely as a result of their association in the ER (47). Whether such heterodimerization between splice variants could play physiologically relevant roles in regulating receptor expression remains to be investigated. Nevertheless, an increasing number of converging reports clearly indicate that GPCRs can dimerize as early as the ER and that this process may play an important role in receptor folding and trafficking.

A variation on the theme of a molecular chaperoning role for GPCR dimerization is provided by the discovery of the receptor activity modifying proteins (RAMPs). While attempting to clone the human calcitonin-gene-related peptide (CGRP) receptor, McLatchie et al. (48) identified a single transmembrane protein that is essential for the ER export of a 7 transmembrane domain receptor known as the calcitonin-receptor-like receptor (CRLR). When expressed individually, the two proteins are retained intracellularly. However, when coexpressed, they form a heterodimer that is targeted to the cell surface to form a functional CGRP receptor (48–50a). To date, three distinct members of the RAMP family have been identified. Interestingly, when coexpressed with CRLR, they confer different pharmacological properties to the resulting receptor. While RAMP1 leads to the formation of a CGRP receptor, coexpression with RAMP2 or 3 leads to an adrenomedullin receptor. Recent studies with radio-labeled ligands have shown that CGRP and adrenomedullin can be covalently cross-linked to RAMP1 and RAMP2,3 respectively (50a), indicating that in addition to serve as a chaperone/escort protein, the RAMPs become an integral part of the receptor complex.

PHARMACOLOGICAL PROPERTIES OF RECEPTOR DIMERS

A role for dimerization in quality control and ER export does not exclude additional functional consequences for the oligomerization process. Pharmacologically, dimerization could explain many of the observations reported over the years that suggested the occurrence of positive or negative binding cooperativity that could not be explained by G protein coupling. However, since this aspect was

recently reviewed (1), we restrict our discussion to the intriguing possibility that heterodimerization between distinct receptors could be the source of additional pharmacological diversity. The first evidence suggesting that heterodimerization between distinct receptor subtypes could lead to pharmacological properties that are different from those of the individual receptors came from the work of Jordan and Devi on the opioid receptors (11). They found that coexpression of the δ- and κ-opioid receptor in the same cells leads to an almost complete loss of binding to selective δ- and κ-ligands (both agonists and antagonists) while preserving binding to nonselective ligands. Interestingly, the binding to both δ- or κ-selective agonists could be restored by adding the two ligands simultaneously, indicating that positive cooperativity occurred. Similar data were observed for selective antagonists but no positive cooperativity between agonists and antagonists was detected.

Heterodimerization between the δ- and μ-opioid receptors was also found to lead to cooperative binding. In their report, Gomes et al. (12) demonstrated that treatment of cells coexpressing δ- and μ-receptors with low doses of selective δ-opioid ligands, such as TIPPψ and deltorphin II, results in a significant increase in binding of the μ-opioid agonist DAMGO. Interestingly, while DAMGO is an μ-agonist, TIPPψ is a δ-antagonist indicating that, in this case, positive cooperativity occurs between agonists and antagonists. Reciprocally, the μ-antagonist CTOP could also significantly increase the binding of the δ-opioid agonist [3H]deltorphin II in cells expressing the δ-μ heterodimer. In an independent study, George et al. found that the high-affinity binding of both δ- and μ-agonists to the heterodimer was insensitive to PTX and guanine nucleotide treatments (13). This contrasts with the sensitivity of the high-affinity agonist binding to these treatments when each receptor is expressed individually. This was interpreted as an indication that heterodimerization leads to a different type of interaction with the G proteins with possible implications in signaling. More recently, the purinergic ligand ADPβS was shown to inhibit the binding of the adenosine agonist NECA to the A1 adenosine/P2Y1 purinergic receptor heterodimer (50b). This pharmacological effect was also accompanied by signaling properties that are different from those of the individual receptors. Indeed, the agonist ADPβs that is without effect on adenylyl-cyclase activity in cells expressing either the Gαi-coupled A1R or the Gαq-coupled P2Y1 alone promoted a reduction in cAMP accumulation through the A1R/P2Y1 complex. This effect could be blocked by the A1R antagonist DPCPX, indicating that the heterodimer was indeed the signaling unit. Pharmacological consequences to heterodimerization between muscarinic M2 and M3 receptors have also been suggested (51).

The discovery that GPCR may form heterodimers that display distinct pharmacological properties raises fascinating possibilities concerning the pharmacological plasticity and diversity of these signaling systems. An obvious possible implication is that dimerization between known receptor isoforms could be responsible for the generation of pharmacologically defined receptor subtypes for which no gene has been identified so far. In this vein, Jordan & Devi have proposed that the δ-κ opioid receptor heterodimer could represent the κ2-subtype (11). This

expansion of the diversity by combinatorial arrangements of pre-existing units is a common theme in biology and may become particularly important for GPCRs now that we know not many more than 600 genes encode for hormone and neurotransmitter receptors (52). Despite these exciting possibilities, much work remains to be done to determine the physiological importance of these observations and to assess the generality of the heterodimerization paradigm for GPCRs. If found to be a general feature of physiological significance, then unraveling the rules that determine the selectivity of heterodimerization will become of the foremost importance. In this context, the occurrence of constitutive vs ligand-promoted dimerization takes a special meaning. Are heterodimers preformed during biosynthesis, in which case, transcriptional or translational control would be required to modulate their levels, or else, can there be ligand-promoted exchanges between the constituents of the dimers so to allow for rapid regulation of heterodimer contingents expressed at the cell surface? Obviously, the potential consequences for signal transduction and its regulation are mind-boggling.

DIMERS AS SIGNAL TRANSDUCING UNITS

The first evidence suggesting a role for dimerization in GPCR activation came from work carried out almost 20 years ago using bivalent antibodies directed against a peptide antagonist of the GnRH receptor (53). The observation that the antibody-induced dimerization of the antagonist converted it into an agonist led the authors to conclude that receptor-induced clustering was sufficient to activate the receptor and promote second messenger production. Similarly, dimerization of the occupied luteinizing hormone-releasing hormone (LHRH) receptor was proposed as the mechanism underlying activation (54). In the mid-1990s a similar study using chemically cross-linked dimeric antagonists of the melanocortin-stimulating hormone (MSH) receptor that also behaved as agonists led Carrithers and Lerner to conclude that receptor dimerization may be sufficient to initiate signaling (55). Interestingly, the dimeric ligands presented a bell-shaped dose-response curve, being agonist at low concentration and reconverting to antagonists at higher concentrations, indicating that once all monomeric receptors are bound by a dimeric antagonist, the latter cannot promote dimerization anymore and thus acts as an antagonist.

Additional evidence suggesting that GPCRs could function as dimeric units came from studies showing that disruption of receptor dimers impaired signaling. For the β2AR, a peptide derived from the sixth transmembrane domain inhibited both receptor dimerization and agonist-induced cAMP production (2). Although this observation is consistent with a role of dimerization in the activation process, one cannot exclude that the loss of dimerization and function represent two independent actions of the peptide. Indeed, for the dopamine D1 receptor, it has been reported that a peptide that inhibits receptor function does not affect receptor dimers (56). Nevertheless, the observation that bivalent anti-β2AR antibodies also stimulate the receptor, but that their monovalent FAB fragments cannot, lends

more support to the notion that β2AR dimerization may well be involved in its activation (57).

Interestingly, antibodies against the chemokine CCR2b receptor have also been shown to promote second messenger production (58). In the case of CCR5, an antibody that induces receptor dimerization was shown to inhibit its function as a coreceptor for HIV thus preventing viral entry into cells (59). These observations indicate that receptor dimerization could have distinct effects on different receptor functions. Further indirect evidence of dimerization as a prerequisite for activation comes from FRET studies between two fluorescent LH molecules. Indeed, FRET between fluorescent derivatives of the hormone could be observed with the wild-type LH receptor but not with a mutant receptor that can bind LH but does not signal (32). This suggests that events linked to receptor activation are required to bring two hormone molecules within a FRET permissive distance, indicating that either a conformational change bringing the two ligands closer within a pre-existing dimer or dimer formation is involved in the activation process.

Although the above evidence supports the notion that GPCRs may function as dimeric units, the most direct and convincing evidence come from studies on the metabotropic GABAb receptor. As will be summarized below, the data available clearly demonstrate that this receptor functions as an obligatory constitutive dimer. As mentioned before, heterodimerization of GABAb R1 and GABAb R2, within the ER, is required for the export and cell surface trafficking of GABAb R1 to the cell surface (8–10, 39). Given that GABAb R1 harbors the binding site for GABA (60), it could have been proposed that GABAb R2 (which can reach the cell surface even in the absence of coexpressed GABAb R1) serves only as a chaperone/escort protein to bring GABAb R1 to its site of action. However, the results of several studies (41, 61, 62) showing that mutation or truncation of an ER retention signal, within the carboxyl tail of the GABAb R1, was sufficient to allow cell surface targeting of GABAb R1 even in the absence of GABAb R2, but not to generate a functional receptor seriously challenged this notion.

Using a collection of chimeric receptors, Pin and collaborators elegantly demonstrated that cooperativity between the extracellular domain (ECD) and the heptahelical domain (HD) of the GABAb R1 and GABAb R2 is essential for adequate receptor function (63). Indeed, coexpression of a chimeric receptor formed with the ECD of GABAb R1 and the HD of GABAb R2 (GABAb R1/2) with the wild-type GABAb R2 led to a functional receptor, whereas coexpression of a GABAb R2/1 chimera with the wild-type GABAb R1 did not allow the formation of a signaling receptor even though mutation of the ER retention signal permitted cell surface expression. These results clearly demonstrate that the presence of a GABAb R2 HD is essential to allow G protein coupling. Given that GABAb R1 harbors the GABA binding site, it provides convincing demonstration that the signaling unit truly requires the heterodimer; one subunit recognizes the ligand (R1) while the other (R2) is engaged in G protein activation. The data obtained in this study also provided evidence for the occurrence of binding cooperativity between the two receptor subtypes. Indeed, although GABAb R2 cannot bind GABA, the

presence of one GABAb R2 ECD, within the heterodimer, appeared essential to permit efficient binding to the ECD of GABAb R1 and to induce signaling. This is best demonstrated by the observation that chimeric heterodimers bearing two GABAb R1 ECDs and either one or two GABAb R2 HDs did not confer agonist-promoted signaling, whereas heterodimers with both R1 and R2 ECDs were functional whether they carried one or two GABAb R2 HDs. Taken together, these results led the authors to propose a model in which the binding of GABA to the GABAb R1 ECD is favored by the GABAb R2 ECD and leads to the activation of the GABAb R2 HD that interacts with and activates the G protein as a result of a series of allosteric interactions within the heterodimer.

More recently, Nelson et al. (63a) reported that heterodimerization between the taste receptor T1R2 and T1R3 is required to confer a response to sweet-tasting molecules in an heterologous expression system. The pattern of coexpression of these receptors in a subset of taste receptor cells is consistent with the notion that they do indeed function as heterodimers in vivo. The existence of a third T1R receptor (T1R1) offers possibilities for additional heterodimer combinations that may underlie distinct responsiveness to various sweet molecules.

Although GABAb and T1-taste receptors are the only two cases for which obligatory dimerization between wild-type receptors has been demonstrated, a significant number of studies have reported functional complementation of mutant receptors that is consistent with the notion that many GPCR signaling units may be dimeric. The first such study involved two chimeric proteins between the $\alpha 2$ adrenergic and the M3 muscarinic receptors that were composed of the first five transmembrane domains of one receptor and the last two transmembrane domains of the other (64). When expressed individually, neither of the chimeric receptors provided ligand binding or signaling activities. However, both adrenergic and muscarinic binding as well as muscarinic signaling were partially recovered when the two chimeras were coexpressed. Similarly, coexpression of two angiotensin II receptor point mutants that were deficient in ligand binding restored the affinity for the angiotensin peptide (65), whereas coexpression of two calcium receptors harboring inactivating mutations in distinct domains was shown to partially reinstate calcium-mediated signaling (66). These examples of genetic transcomplementation were interpreted as evidence for the existence of intermolecular interactions between impaired receptors in a way that restores ligand-binding and signaling determinants within a dimeric complex.

In some other cases, heterodimerization between mutant and wild-type receptors, leading to nonfunctional complexes, was invoked to explain dominant negative phenotypes. For example, a mutant yeast α-mating factor receptor with reduced signaling efficacy significantly impaired wild-type receptor signaling upon coexpression (27). This did not result from a sequestration of the G protein by the mutant receptor because overexpression of the $G\alpha\beta\gamma$ subunits did not reverse the dominant negative phenotype. Since the formation of α-mating factor receptor dimer had been shown by FRET in the same study, the authors concluded that the inhibitory effect of the mutant receptor resulted from its direct interaction with the wild-type receptor leading to an inactive heterodimer. Similar dominant

negative effects have also been described for the chemokine family of receptors. Indeed, a loss-of-function mutant of the CCR2b (CCR2bY139F) acts as a dominant negative for the wild-type CCR2 receptor by blocking signal transduction (58). Likewise, the V64I polymorphism of CCR2, which is associated with a delayed development of AIDS in HIV-1-infected individuals, heterodimerizes with WT CCR5 and CXCR4 receptors (67). Although it has been suggested that chemokine receptor dimerization may prevent HIV infection, the exact mechanisms by which the CCR2V64I mutant receptor protects the HIV-infected individuals remain to be elucidated.

Heterodimerization between distinct receptor subtypes has however been suggested as a potential mechanism underlying synergistic and antagonistic signaling cross-talks. For the δ-κ opioid receptor heterodimerization previously discussed, addition of δ- and κ-opioid agonists led to a synergistic activation of the MAP kinase (11). When considering the δ-μ opioid heterodimer, the same group (12) found not only that the δ- and μ-agonists DAMGO and deltorphin II had a synergistic effect on MAP kinase activation, but also that treatment with the μ receptor antagonist, CTOP, enhanced both the potency and efficacy of δ-opioid receptor signaling. Similarly, treatment with the δ opioid-receptor antagonist TIPPψ was found to enhance μ receptor signaling. As indicated previously, these effects on signaling were accompanied by a corresponding increase in agonist binding capacity, which indicates that complex allosteric interactions within the heterodimers are at work. Synergy between the chemokine CCR2 and CCR5 receptors was also observed in HEK-293 cells as well as in peripheral blood mononuclear cells that endogenously express these receptors (67a). In these cases, subthreshold concentrations of MCP-1 and RANTES, which are selective ligands for CCR2 and CCR5 respectively, provoke nearly maximal calcium flux responses in cells coexpressing the two receptors. Moreover, although the response of each receptor alone is sensitive to PTX, the heterodimer activity is resistant to the toxin treatment, indicating that a non-Gαi/o G protein is engaged by the heterodimer. Consistent with this notion is the selective coimmunoprecipitation of Gα11 with the CCR2/CCR5 heterodimer and not with each receptor alone.

Heterodimerization between the AT1 angiotensin II and B2 bradykinin receptors was also suggested to increase the efficacy and potency of angiotensin II signaling, indicating positive cooperativity within the heterodimer (16). However, in contrast to what was observed for the opioid receptors, the cooperativity was not bilateral because the heterodimerization resulted in a reduced efficacy of bradykinin to stimulate inositol-phosphate production. Another example of negative regulation involving heterodimerization is provided by the adenosine A1/dopamine D1 receptor heterodimer (15). In that case, it was reported that pretreatment with both adenosine and dopamine, but not with either ligand alone, reduced the signaling efficacy of the D1 receptor upon subsequent stimulation.

For the somatostatin receptor family, a signaling role for heterodimers was suggested by studies in which coexpression of a signaling deficient mutant of SSTR5 (Δ318 SSTR5) with the wild-type SSTR1 restored signaling in response to the octapeptide SMS, which selectively binds to SSTR5 and not SSTR1 (30).

No signaling rescue was observed when the related SSTR4 receptor, which is also SMS insensitive, was coexpressed with Δ318 SSTR5, suggesting selectivity in the heterodimerization. Functional complementation between the signaling-deficient Δ318 SSTR5 and a chimeric SSTR5 harboring the second extra-cytoplasmic loop of the SSTR2 that shows impaired somatostatin binding indicated that SSTR5 can also form homodimers.

More recently, heterodimerization between somatostatin SSTR2a and SSTR3 has also been documented (14). In this case however, the formation of the heterodimer leads to a nonfunctional SSTR3 receptor as assessed by GTPγS binding, adenylyl-cyclase, and ERK activity upon stimulation with the selective SSTR3 agonist L-796,778.

In another study, coexpression of the D2 dopamine receptor with the Δ318 SSTR5, which can bind somatostatin but cannot signal, imparts somatostatin-induced cAMP inhibition (31). Interestingly, the somatostatin-induced signal could be blocked by dopamine D2 antagonists, suggesting that the heterodimer formed constitutes a functional unit in which both receptors maintained normal responses (ie: activation and inhibition) to their selective ligands.

In a variation on the theme of functional heterodimerization, Nakanishi-Matsui et al. (68) provided evidence for a novel type of interaction between the protease-activated receptors PAR3 and PAR4 in which the N-terminal portion of PAR3 functions as a cofactor for the cleavage and activation of PAR4 by thrombin. However, whether the synergy observed between PAR3 and PAR4 results from their heterodimerization remains to be formally tested. Because a fusion protein between the N-terminal exodomain of PAR3 and the transmembrane domain of CD8 was equally active to promote PAR4 signaling as the full-length PAR3, the N-terminal portion of PAR3 would need to be a major determinant of such heterodimerization.

Internalization

Although the role of dimerization in agonist-promoted internalization was formally tested in only a few studies, the fact that it is intimately linked to the receptor activation process led several groups to use it as a marker to investigate if dimers could represent the functional signaling unit. In particular, investigators took advantage of the differences in the internalization characteristics of receptors that were proposed to be involved in heterodimerization. For example, the δ-opioid receptor undergoes efficient etorphine-mediated internalization,whereas the κ subtype does not. Upon coexpression of the two receptors, the extent of the δ-receptor internalization was found to be significantly less and was interpreted as evidence that heterodimerization with the κ receptor led to the retention of the δ receptor at the cell surface (11). Using a similar approach, George et al. (13) showed that exposure of cells coexpressing the δ- and μ-opioid receptor to the δ-selective agonist DAMGO, resulted in accentuated internalization when compared to that of the δ-opioid receptor expressed alone. On the other hand, exposure of the heterodimer to DPDPE, a μ-selective agonist, led to reduced desensitization and internalization as compared to the μ receptor expressed alone. This fact is consistent with the notion

that the heterodimer undergoes internalization with a profile intermediate between those of the individual receptors. When considering the heterodimer between the δ-opioid and the β2AR, Jordan et al. (17) found that both receptors underwent internalization in response to either opioid or adrenergic ligands, which suggests that the heterodimer represented the substrate for endocytosis. Interestingly, coexpression of the β2AR with the κ-opioid receptor inhibited the agonist-promoted internalization of the β2AR, indicating that the formation of an heterodimer between an internalization-prone (the β2AR) and -resistant (the κ-opioid receptor) receptor led to a complex that is preferentially retained at the cell surface. When coexpressing the internalization-resistant SSTR1 with SSTR5, which undergoes efficient agonist-promoted internalization, Rocheville et al. found that somatostatin stimulation led to the internalization of the two receptor subtypes, indicating that in that case the phenotype of the internalizing receptor prevailed (30). Taken together, these observations suggest the intriguing possibility that heterodimerization could selectively modulate the endocytotic properties of distinct receptors.

In one case, heterodimerization has been proposed to change the endocytotic pathway used for internalization. Indeed, the agonist promoted internalization of both the angiotensin AT1 and bradykinin B2 receptors was inhibited by the K44A dynamin-dominant negative mutant in cells coexpressing the two receptors, while the internalization of each of these receptors is dynamin insensitive when expressed individually (16). However, the functional implications of this observation remains to be investigated.

Consistent with the notion that dimers heterodimer may represent the units undergoing agonist-promoted endocytosis, Overton & Blumer (27) found that coexpression of an endocytosis-defective mutant with the wild-type α-mating factor receptor led to the efficient internalization of the complex.

EVIDENCE OF DIMERIZATION FOR ENDOGENOUSLY EXPRESSED RECEPTORS

In most studies discussed in the previous sections, GPCRs dimers were detected and studied in heterologous systems overexpressing the receptor of interest, mainly to ease the experimental manipulation or to overcome technical limitations. Nevertheless, Western blot analysis revealed higher-molecular-weight species that were interpreted as dimers for the dopamine D2 in human caudate (3), the A1 adenosine receptor in pig cortex (69), the CCR5 in peripheral blood mononuclear cells (59), the CCR2 in the human monocytic cell line Mono Mac 1 (58), and CXCR4 in human T cell lines (70). These data are often used to argue that dimerization does not occur solely in heterologous systems that express large receptor numbers. Additional supporting evidence for dimerization of endogenously expressed receptors comes from a recent immunoelectron microscopy study investigating the subcellular distribution of CCR5 and CXCR4 in human macrophages and T cells (71). Both receptors were found predominantly on microvilli and appeared to form homogeneous microclusters in which receptors were often found in pairs.

Interestingly, the clusters were also observed in small trans-Golgi vesicles, consistent with the notion that dimerization occurs early after biosynthesis and could play a role in ER export.

The most compelling evidence for the existence of dimers in vivo comes from the metabotropic GABAb receptor. Indeed GABAbR1 and GABAbR2 expressed in rat cortex could be coimmunoprecipitated using selective antibodies (10), thus clearly demonstrating intermolecular interactions between endogenously expressed receptors. Functional data also support the existence of dimers in cells naturally expressing receptors. For instance, bradykinin receptor anti-sense blocked the bradykinin-mediated potentiation of angiotensin II AT1 receptor signaling in A10 smooth muscle cells. Given that such potentiation was attributed to the formation of heterodimers between the two receptors when heterologously expressed in fibroblasts, the authors interpreted their data as evidence that endogenous bradykinin and AT1 receptors also heterodimerize in the smooth muscle cells (16). Similarly, binding and signaling potentiation between δ- and μ-opioid selective agonists (DAMGO and deltorphin II) has been observed in SKNSH cells that naturally expressed these receptor subtypes and taken as evidence of heterodimerization (12) in these cells.

PERSPECTIVE

Work carried out in particular during the last five years has provided convincing evidence that, at least in some cases, GPCRs exist and function as dimers. Whereas Table 1 summarizes the various technical approaches that have been used to demonstrate their existence, Table 2 provides a compilation of studies that provide evidence supporting a functional role for dimerization in ER export as

TABLE 2 Proposed functional roles for GPCR dimerization

Receptor	Evidence for function of homo- and heterodimers	Reference
β2-Adrenergic	A peptide inhibiting dimerization, impairs agonist-stimulated adenylate-cyclase activation	2
Calcium	Functional complementation of two inactive mutant receptors	66
α-Mating factor	Rescue of internalization-deficient tail-less receptors by WT receptor; dominant negative effect of a signalling mutant and a G protein–coupling-impaired mutant over WT receptor	27
Angiotensin	Coexpression rescues binding of 2 binding-deficient mutants	65
GnRH	Receptor microaggregation during endocytosis	28

(*Continued*)

TABLE 2 (*Continued*)

Receptor	Evidence for function of homo- and heterodimers	Reference
V2 vasopressin	Dominant negative effect on WT receptor cell surface expression by truncated receptors	43
Metabotropic glutamate	Crystal structure of N-terminal domain is a constitutive dimer; ligand promotes a conformational change	73
GnRH	Conversion of an antagonist to agonist by antibody-promoted dimerization of the antagonist	53
MSH	Conversion of an antagonist to agonist cross-linking of the antagonist	55
CCR5	Antibody-promoted dimerization inhibits HIV entry	59
Somatostatin SST5-dopamine D2 heterodimer	Rescue of SST5 signaling impaired mutant by D2R	31
SSTR5 homodimer SSTR5-SSTR1 heterodimer	Functional complementation of binding-deficient mutant with signaling-deficient mutant; internalization of SSTR1-SSTR5 heterodimer	30
Angiotensin AT1-bradykinin B2 heterodimer	Increase efficacy and potency of angiotensin II but reduced bradykinin efficacy	16
δ-κ–Opioid heterodimer	Distinct pharmacological properties of heterodimers + synergistic activation of MAPK	11
δ-Opioid, β2-adrenergic heterodimer	Cotrafficking + synergistic activation of MAPK	17
PPAR3-PPAR4	Role of PPAR3 in ligand presentation to PPAR4	68
δ-μ–Opioid heterodimer	Distinct pharmacological and signaling properties of heterodimers; no effect of GTPγS on heterodimer	13
Dopamine D3 + D3nf	Truncated splice variant retains WT receptor intracellularly	47
Muscarinic M3 − M2 heterodimer	Distinct pharmacology of heterodimer	51
GABAb heterodimer	GABAR2 coexpression with GABAR1 necessary for cell surface expression of GABAR1 and normal GABA signaling	8–10, 39
GABAb heterodimer	Selective binding of a drug to a heterodimer subtype	74
GABAb heterodimer	Allosteric cooperativity between R1 and R2 subtypes	63
CCR5 + CCR5 Δ 32	Dominant negative retention of WT receptor by deletion mutant	46
Adenosine A1, dopamine D1 heterodimer	Desensitization of heterodimer upon pretreatment with A1 agonist and D1 agonist	15

well as in ligand binding and signal transduction. Although a few reports have confirmed their importance in native tissues, most of the studies have taken advantage of heterologous expression systems to demonstrate their formation and functional implications. One of the most important challenges of the next few years will be to investigate the general occurrence and physiological role of GPCR dimerization in cells, tissues, and organisms in which they are naturally expressed. There is no doubt that the greater availability of fluorescent and bioluminescent probes that can be used to label receptor ligands for FRET and BRET experiments, coupled with the increased sensitivity and sophistication of the fluorescent detection systems, will play a significant role in this endeavor.

One of the most intriguing possibilities raised by the discovery of GPCR dimerization is the amazing pharmacological diversity and potential for regulation that would be offered by the occurrence of heterodimerization. Although, clearly demonstrated for a few receptors, additional studies are needed to determine whether the formation of heterodimers between more or less closely related receptors is a general phenomenon with physiological implications or a curiosity of heterologous expression systems. If validated as a general phenomenon occurring in vivo, heterodimerization will, in addition to opening a number of fundamental questions regarding their regulation, offer a formidable challenge for the design of drug-screening strategies that will be adequate for taking advantage of the potentially expanded pharmacological diversity and selectivity that could be unraveled. Finally, if as has been clearly demonstrated for the GABAb receptor and suggested for several other GPCRs, dimers truly represent the signaling units, identifying the dimerization interfaces and determining whether they are stable complexes once formed in the ER or are they objects of ligand-mediated regulation at the cell surface will become a central question that could lead to the development of new drugs acting allosterically to modulate GPCR functions.

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