MINIREVIEW

Probing Receptor Structure/Function with Chimeric G-Protein-Coupled Receptors

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ABSTRACT

Owing its name to an image borrowed from Greek mythology, a chimera is seen to represent a new entity created as a composite from existing creatures or, in this case, molecules. Making use of various combinations of three basic domains of the receptors (i.e., exofacial, transmembrane, and cytoplasmic segments) that couple agonist binding into activation of effectors through heterotrimeric G-proteins, molecular pharmacology has probed the basic organization, structure/function relationships of this superfamily of heptahelical receptors. Chimeric G-protein-coupled receptors obviate the need for a particular agonist ligand when the ligand is resistant to purification or, in the case of orphan receptors, is not known. Chimeric receptors created from distant members of the heptahelical receptors enable new strategies in understanding how these receptors transduce agonist binding into receptor activation and may be able to offer insights into the evolution of G-protein-coupled receptors from yeast to humans.

Chimera (chiˈ me ˈra; also chiˈ mae ˈra). Noun. 1. from Greek Mythology: An imaginary she-monster typically displayed as a composite of grotesquely different parts, most often of lion, goat, and serpent.

Few would have suspected that the early identification of a few notable cell-surface receptors that require heterotrimeric G-proteins (GPCRs) for signal propagation (Yarden et al., 1986; Dixon et al., 1987; Hargrave and McDowell, 1992) would ultimately lead to the appreciation of GPCRs as a superfamily that constitutes ~5% of the human genome, making it the most populous family in cell signaling. Since the early characterization, isolation, purification, and molecular cloning efforts, several universal properties of GPCRs, such as their heptahelical nature, have been revealed (Morris and Malbon, 1999). Many important discoveries in the structure, function, and biology of GPCRs are the result of innovative use of chimeric receptors composed of domains of various GPCRs in an attempt to overcome formidable obstacles. The literature highlights more than 100 examples using this strategy to address issues such as the molecular nature of the ligand binding domain, the roles of specific transmembrane spanning segments (TMS), and the nature of the cytoplasmic domains in defining downstream signaling and receptor regulation/trafficking (Table 1). The central thrust of this review is to ascertain how the chimeric receptor strategy has been applied to studies of GPCRs and what we have learned about the values and limitations of the approach.

Organization of GPCRs

The landmarks for GPCRs can be appreciated in the form of the mythological chimera (Fig. 1A). Dominant in the landmarks of a GPCR are the seven TMS or core “heptahelical” structure embedded in the lipid bilayer (here a “lily pond” bilayer). The TMS appear to be composed of 20- to 22-residue stretches of largely hydrophobic amino acids that are predicted to be α-helical in structure. The actual length of TMS might increase by two to three residues in regions of a GPCR that traverse the lipid bilayer obliquely. The N terminus (depicted as the composite of a lion/she-goat) is exofacial and includes the products of post-translational addition of N-linked complex carbohydrates (-CHO). The ligand binding

ABBREVIATIONS: GPCR, G-protein-coupled receptor; TMS, transmembrane segments; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AC, adenyl cyclase; Rfz2, rat Frizzled 2; β2AR, β2-adrenergic receptor; αMFR, yeast α mating factor receptor.
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CT, C terminus; NT, N terminus; mACHR, muscarinic acetylcholine receptor; GnRH, gonadotropin-releasing hormone; TSH, thyroid-stimulating hormone; mGluR, metabotropic glutamate receptor.
domain of GPCRs is not uniform with respect to the role of the N terminus, because for many GPCRs (e.g., adrenergic and serotonergic receptors), the ligand binding occurs somewhere within in the lipid bilayer (Tota and Strader, 1990), although the N terminus and exofacial “loops” must contribute to the forces that organize the binding pocket for the ligand in these receptors. Other families of GPCR make use of large N-terminal domains and exofacial domains (e.g., gonadotropin receptors and Frizzleds) that are essential for signal propagation, whereas some seem to depend mostly on the N-terminal domain itself for receptor binding and activation by agonist (e.g., glutamate metabotropic receptors). The cytoplasmic domains of a GPCR include three or four (in the case of palmitoylated GPCRs such as the β2-adrenergic receptor and rhodopsin) intracellular loops (Loops 1–4) and a C-terminal sequence of variable length (70+ residues for β2-adrenergic, 20+ residues in Frizzled-1, etc.). These cytoplasmic domains are sites of post-translational modifications (including protein phosphorylation, dephosphorylation, palmitoylation, and ubiquitination), interaction with their cognate heterotrimeric G-proteins, and association with scaffolds, cytoskeletal attachments, and other protein targets.

### Probing Ligand Binding Domains of GPCRs with Chimeras

Since 1988, chimeric receptors have been employed to determine which regions of a GPCR are responsible for agonist binding and for activation of the receptor. The simplest demonstration of this principle was the creation of a chimeric receptor composed of the gonadotropic leuteinizing hormone (LH) receptor, which responds poorly to the gonadotropin follicle-stimulating hormone (FSH), and the large, N-terminal exofacial domain of the FSH receptor...
Substitution of the FSH receptor N terminus resulted in a FSH/LH chimera that bound FSH, underwent activation, and signaled similarly to the LH receptor in response to phospholipase C activation and accumulation of inositol 1,4,5-trisphosphate (Hirsch et al., 1996). Because the two gonadotropin hormone receptors do display some homology in the exofacial loops, the character of the binding of FSH and subsequent activation of the chimera cannot be ascribed solely to the presence of the N-terminal domain of the FSH receptor, but the basic observation is compelling.

For receptors with ligand binding domains embedded in the lipid bilayer, studies with chimeric GPCRs revealed new insights into what constitutes a binding site and its intrinsic ability to signal downstream. Detailed pharmacological analysis of $\beta_1$-$\beta_2$-adrenergic receptor chimeras expressed in Escherichia coli revealed complex contributions of TMS and N termini to the binding character for both agonists and antagonist ligands alike (Marullo et al., 1990). Early work on chimeric $\alpha_2$- with $\beta_2$-adrenergic receptor showed that TMS VI and especially VII were essential to agonist and antagonist binding specificity (Kobilka et al., 1988; Eason and Liggett, 1996). The dopamine D1 receptors characteristically activate adenyl cyclase (AC), whereas the D2 receptors inhibit AC (Fig. 1C). Substitution of the region, including TMS VI, VII, and extending to the C-terminal tail of the D2 molecule into the D1 receptor, resulted in a chimera with enhanced binding of D2-agonist, diminished binding of D1 ligands, and a switch from a D1-like to a D2-like response (i.e., stimulation of AC) in response to a D2-agonist (MacKenzie et al., 1993). Many examples of making good use of TMS “swapping” to ascertain binding/activation properties of GPCRs can be found in the literature (Table 1). Most chimeras were constructed between members within a single family (family I), fewer were created from members of families II and III, and there are no reports of chimeras constructed from family IV (Wess, 1998).

**Probing Downstream Signaling with Chimeric GPCRs**

One of the central goals in cell signaling is to understand more completely the basis for receptor “activation” by agonist and how this signal is propagated to the downstream elements in the signaling pathways. In some cases, chimeras of GPCRs proved invaluable in sorting out the roles of specific receptor domains in receptor activation and downstream signaling. Analysis of $\alpha_1$-$\beta_2$-adrenergic chimeras demonstrated, for example, that swapping the third cyto-

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**Fig. 1.** GPCR chimeras and analysis of ligand binding domain. The GPCR in the mythological rendition of a chimera (A). The three domains of a prototypic GPCR. The seven transmembrane segments (labeled in Roman numerals I–VII) of the GPCR constitute the lipid bilayer domain, displayed here as a lily pad. In the case of adrenergic receptors, this 7-TMS domain is the ligand-binding domain. The exofacial domain of GPCR includes the N-terminal region of the receptor (depicted as the lion head of the chimera) that is typically N-glycosylated with complex carbohydrate (CHO). For receptors that interact with peptide/protein ligands, the N-terminal exofacial region can be much larger and include motifs such as cysteine-rich domains (CRDs). The intracellular loops (labeled iLoop1, iLoop2, and iLoop3) and the C-terminal tail (-COOH) of a GPCR (depicted as the serpent head) constitute the cytoplasmic domains that are intimately involved in signal propagation to heterotrimeric G-proteins. In some GPCRs a cysteinyl residue in the C terminus beyond TMS VII is palmitoylated, creating a fourth loop (iLoop4). The intracellular loops are accessible to protein kinases, such as protein kinases A and C and G protein-coupled receptor kinase 2, and are subject to phosphorylation after activation by agonist. The phosphate groups are depicted as green spheres. Exofacial, transmembrane, and cytoplasmic domains of GPCR; swapping of exofacial domains dictates ligand binding/activation of LH/FSH receptors (B). This schematic demonstrates that for the GPCRs that interact with glycoprotein ligands, the exofacial domain is large, complex, and dictates the ligand binding character of the chimera. The cytoplasmic domain dictates the downstream read-outs. The 7TMS domain of many GPCRs constitutes the ligand-binding domain for small molecular weight agonists/antagonists (C). For many small ligands (catecholamines, dopamine, adenosine, etc.) that bind GPCR, the ligand binding determinants are largely transmembrane in nature and the functional nature is dictated by the cytoplasmic domains (loops 1–3 and C-terminal tail). It has been shown that after the receptor has been expressed and incorporated into the cell membrane, exhaustive proteolytic digestion can remove the exofacial loops, whereas the 7TMS remain intact, as does the ligand binding capacity for small molecule ligands (Morris and Malbon, 1999).
plasmic loop of the $\alpha_1$-receptor to the $\beta_2$-receptor resulted in a chimera with $\beta_2$-adrenergic binding character while coupling to phosphatidylinositol signaling as a $\alpha_1$-adrenergic receptor (Cotecchia et al., 1990). Most of the chimeras created were composed of domains taken from closely related members of family I of the V basic families of the GPCRs (Table 1). One very recent example, in sharp contrast, created a chimera from distant members of family I (e.g., $\beta_2$-adrenergic receptor) and family V (e.g., Frizzleds) to eliminate a 20-year obstacle to the detailed analysis of the signaling of the Wnt protein, secreted glycoprotein ligands involved in many aspects of early development. Wnts bind to and activate Frizzleds, heptahelical membrane receptors found in developing embryos, and thereby regulate cell fate, proliferation, and patterning (Malbon et al., 2001). The overarching obstacle to study of the Wnt ligands and the larger Frizzled family for 20 years was the inability to purify biologically active Wnts (Cadigan, 2002). Only recently was it shown that Wnts are palmitoylated (Willert et al., 2003), which would explain the inability of standard biochemistry designed for purification of soluble proteins to succeed at isolating Wnts with full biological activity.

To obviate the need for active ligand, chimera were designed in which the exofacial and TMS of the well-characterized $\beta_2$-adrenergic receptor could be used to drive the activation of the cytoplasmic domains of the Frizzleds, making use of the heptahelical nature and similarities of Frizzleds to GPCRs (Fig. 2A). The 7TMS that constitute the ligand binding domain and the exofacial N terminus and exofacial loops of the $\beta_2$-adrenergic receptor provided the core of this chimeric GPCR. The cytoplasmic domains,
including the intracellular loops 1 to 3 and the cytoplasmic C terminus of the rat Frizzled-2 (Rfz2), were substituted for their corresponding sequences in the β2-adrenergic receptor (β2AR) (Liu et al., 1999b). Frizzled-2 was selected as the prototype for these experiments, because this receptor was shown to couple Wnt-5A signaling to calcium mobilization through a process that is pertussis toxin-sensitive in zebrafish (Slusarski et al., 1997) as well as mammalian embryonic stem cells in culture (Ahumada et al., 2002). The chimera was constructed and expressed in Chinese hamster ovary cells that lack β2AR to ascertain whether the chimera retained the ability to bind agonist and antagonist ligands like the parent β2AR. The affinities of the β2AR/Rfz2 chimera for both β-adrenergic agonist and antagonist were nearly identical to those of the parent β2AR, demonstrating that the essence of the ligand binding domain was not altered by the creation of the chimera (Ahumada et al., 2002). In addition, the β2AR/Rfz2 chimera displayed the agonist-specific, GTP-dependent shift in receptor affinity, a hallmark for GPCRs; in this case, however, the G-proteins with which the Frizzled-2 cytoplasmic domains interact was not that of the parent β2AR (i.e., Gs), but rather the heterotrimeric G-proteins Go and Gt2 (Wang and Malbon, 2003). Go and Gt2 were found to be essential to downstream signaling of Frizzled-2 to phosphatidylinositol signaling, Ca2+ mobilization, and cyclic GMP degradation (Wang and Malbon, 2003).

The success with the β2AR/Rfz2 chimera provoked an analysis of the canonical Frizzled-1 signaling that promotes activation of the phosphoprotein Disheveled, suppression of glycogen synthase kinase-3, stabilization of β-catenin, and activation of the Lef/Tcf-sensitive transcription of key genes in early development (Cadigan and Nusse, 1997), a pathway for which no G-protein had been implicated. The chimera was constructed using the same successful strategy employed for the Frizzled-2 chimera (Fig. 2B). There is no homology between the cytoplasmic regions of the rat Frizzled-1 (Rfz1) and those of the parent β2AR molecule. The β2AR/Rfz1 chimera does not activate AC but was shown to stimulate β-catenin stabilization, accumulation, and activation of the Lef/Tcf–sensitive transcription (Fig. 2C). In mouse F9 totipotent teratocarcinoma cells, activation of the β2AR/Rfz1 chimera leads to an activation of the Wnt-β-catenin pathway and ultimately to formation of primitive endoderm from these stem cells (Liu et al., 1999a, 2001), an early stage in vertebrate development (Fig. 2D). Isoproterenol treatment promotes formation of primitive endoderm as measured by expression of the TROMA-1 antigen, a hallmark of primitive endoderm. This activation through the β2AR elements of the chimera can be blocked by propranolol and is insensitive to KT5720, a potent inhibitor of protein kinase A (Liu et al., 2001). In addition, the β2AR/Rfz1 chimera displays the pharmacological properties of the β2AR, an agonist-specific GTP-dependent shift in affinity dependent on the presence of Gq (the cognate G-protein of Rfz1) as well as sensitivity to blockade by pertussis toxin (DeCostanzo et al., 2002). These data demonstrate the ability of creating chimeric receptors between very distant members of the GPCR superfamily.

Probing Ligands and Downstream Signaling for Orphan GPCRs

The β2-AR/Frizzled chimeras were an important advancement to a field that had labored to better define important signaling pathways in the absence of purified, active ligand. The chimeras were functional, demonstrated that Frizzleds are indeed members of the GPCR superfamily, and enabled new advances in gene profiling in which the outcome of the activation of a single, specific Frizzled could be characterized by downstream signaling and the genes that respond to Wnt activation (Li et al., 2004). A similar challenge has been stimulated by in silico analysis of genomic data searching for new gene products that detect a likely 7TMS motif, characteristic of a GPCR (Shaaban and Benton, 2001). Suspected GPCRs constitute a family of “orphan” receptors whose ligands and downstream signaling is not known. New 7TMS orphan receptors are identified routinely, and strategies are necessary to promote rapid identification of their ligand agonists (and antagonists) as well as the nature of the downstream signaling (Ahumada and Wang, 2002). One proposal is to make use of the chimergic GPCR in combination with the benefits of yeast genetics to screen orphan receptors for agonists (Fig. 3). Chimera can be created between the exofacial and TMSs of the orphan receptor with the cytoplasmic domains of the α-mating factor receptor (Ste2, α-MFR), a GPCR responsible for pheromone-induced mating and “schmoo” formation (Snyder, 1989). These orphan 7TMS/α-MFR chimera can be used in high-throughput screens of existing small molecule libraries (i.e., drug screens) and/or screens of combinatorial libraries of peptides. Schmoo formation can provide a facile read-out for positive clones, accelerating the efforts to “de-orphan” interesting 7TMS receptors.

Orphan receptors can be probed for downstream signaling pathways, in the absence of the knowledge of their ligands, much the way the β2AR/Rfz chimeras overcame the absence of purified Wnt ligand to expand the knowledge of the downstream Wnt-Frizzled pathways. This approach is not as facile and requires that the investigator have available a full battery of potential read-outs, which will enhance but not guarantee the identification of the downstream signaling pathway of the orphan receptor. The downstream signaling properties of the vasopressin-related receptor 1 were elucidated recently through the application of a chimeric receptor approach (Gupte et al., 2004). Positive identification of a downstream signaling pathway for an orphan receptor can enhance ligand screens through use of sensitive downstream signaling assays as read-outs. At this point, the receptors may best be considered “homeless” rather than orphan receptors. Drawing upon information about the structure of the N terminus/exofacial and TMS sequences, the nature of the downstream signaling, and information on the expression of the receptor in vivo, it may be possible to narrow down the possibilities and to identify, with the benefit of pharmacology/physiology literature, some likely candidate ligands.
GPCR chimeras can be viewed in a similar manner. Chimeras have been created successfully from the three major domains borrowed from most of the classes that compose the GPCR superfamily. Each of these chimeras provided important insight into the functional roles of specific domains in ligand recognition, receptor activation, downstream signaling, and receptor trafficking. Perhaps the most remarkable feature to emerge from all of these chimeras is that they are functional, highlighting some nearly universal properties about how GPCRs function. The exofacial and 7TMS core of the β2AR seems to provide a “mechanosensing” device capable of converting the binding of agonist into some physical force on GPCR cytoplasmic domains or those borrowed from a distantly-related 7TMS receptor and to activate the downstream signaling pathway cognate to the donor of those cytoplasmic domains. What the nature of the physical force(s) exerted on the cytoplasmic domains of any GPCR is still largely a matter of speculation, with torsion, extension, retraction, or some combination of all three forces likely to play a role in propagating the signal to the cognate G-protein(s) involved.

One of the persistent, nagging questions about GPCRs is that of receptor retention and trafficking. The yeast αMFR provides one extreme for this discussion, a GPCR with little receptor retention or recycling after activation (Hicke et al., 1998). The αMFR undergoes activation and rapid ubiquitination/degradation, providing an example of a “one-pass” receptor. In contrast to the yeast αMFR, most mammalian GPCRs have evolved a complex biology that includes surface retention, sequestration from and re-cycling to the cell membrane, in addition to some ubiquitination and subsequent degradation by the proteosome (Shenoy et al., 2001). Some GPCRs, such as the β2AR, are subject to internalization in response to agonist (i.e., agonist-induced sequestration) (Lefkowitz, 1998), as well as in response to growth factors such as insulin and insulin-like growth factor-1 (i.e., counter-regulation) (Shumay et al., 2002). Our understanding of the receptor domains responsible for conferring spatial and regulated retention of GPCRs is fragmentary at best. Chimeric GPCRs making use of the αMFR as a target for the substitution/insertion of domains from mammalian receptors that are suspected to confer interesting retention properties may provide a powerful approach to this fundamental question. It may be profitable to explore many of the current compelling questions about GPCR retention/trafficking by making autofluorescent fusion proteins of some of the existing 100+ GPCR chimeras available that incorporate unique protein motifs and by using these tagged receptors for cellular imaging. A similar strategy may be profitable that makes use of the extensive list of GPCR chimeras to understand the receptor domains central to receptor oligomerization (Hebert and Bouvier, 1998).

The evidence is compelling that clever chimeric GPCRs can transform one type of binding domain into another (e.g., dopamine D2 to D1), from one ligand to another (e.g., FSH to LH), and from one pathway to another (e.g., AC activation to Lef-Tcf–sensitive transcription). These success stories provoke the possibility that it may be possible to create new chimeric receptors that can translate the presence of one ligand that might provoke a negative outcome (e.g., apoptosis) in its native state, to an alternative downstream pathway that might provoke a preferred outcome (e.g., cell proliferation or terminal differentiation).

Chimeric GPCRs can offer a great deal of insights on ligand binding determinants, G-protein, and effector coupling, and/or landmarks necessary for receptor internalization and trafficking. Since Kobilka et al. (1988) and Kubo et al. (1988) constructed the first chimeric GPCRs, more than several hundred studies have been published on or using chimeric GPCRs. In general, homologous domains are substituted in receptors within the same GPCR family, which probably minimizes disruption of basic receptor structure. Transmembrane spanning segments and intracellular loops are the domains most frequently exchanged, because these regions usually are responsible for determining critical aspects of ligand binding and G-protein coupling, respectively. Multiple chimeras are required to determine a minimal structural basis for ligand binding or effector coupling. With respect to G-protein selectivity and specificity, the third intracellular loop is most commonly exchanged between two GPCRs that have different effects on a downstream signaling (i.e., adenyl cyclase versus phospholipase C activation) or couple different families of G-proteins (i.e., Gi versus Gs). It is remarkable that these exchanged domains examined so far retain most of their function with respect to ligand binding, G-protein coupling, and/or receptor internalization and trafficking. In contrast with standard mutagenesis approaches, in which the endpoint is the loss of function resulting from amino acid deletions or substitutions, the advantage of the chimeric approach is that conclusions can be drawn from qualitative changes in chimeric receptor function, spanning...
from the acquisition of new functions to the ability to respond to new agonists or to signal to new G-protein partners. There are limitations of the chimeric receptor approach, however; some chimeric receptors may not function because of problems in folding and/or targeting or to competing interference of endogenous receptors with which they must compete or oligomerize. Nevertheless, the chimeric approach provides an effective tool to elucidate new insights into structure/function relationships within the superfamily of GPCRs.

Targeted expression of such receptors in a failing heart or atrophic muscle may provide regulated, beneficial outcomes. Likewise, grafting onto a chimer a domain that drives the receptor to re-cycling rather than to ubiquitination/degradation may re-establish a regulatory pathway under assault by the overexpression of some growth factor or hormone that impacts negatively on a G-protein-mediated pathway. Perhaps it is time for us to think of a new generation of chimera, creating a molecular “toolbox” with a mix/match potential for GPCRs to address nagging issues in cell signaling as well as offering opportunities for new therapeutic strategies.

Acknowledgments
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