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PERSPECTIVE

Bring Your Own G Protein[S]

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ABSTRACT

G protein-coupled receptor (GPCR)-Gα fusion proteins were first characterized more than 10 years ago as a strategy for studying receptor-G protein signaling. A large number of studies have used this approach to characterize receptor coupling to members of the G_s, G_i, and G_q families of Gα subunits, but this strategy has not been widely used to study Gα_{12} and Gα_{13}. As described in the article by Zhang et al. in this issue of *Molecular Pharmacology* (p. 1433) characterization of the signaling properties of thromboxane A_2 receptor (TPα) -Gα_{12} and -Gα_{13} fusion constructs demonstrates the applicability of this strategy to members of this unique family of Gα subunits, and how this strategy can be used to resolve otherwise difficult problems of receptor pharmacology associated with these proteins. The general strategy of making receptor-Gα fusion constructs has wide applicability to a number of research problems, but there are perhaps also "hidden messages" in how different receptor-Gα subunit fusion pairs behave.

Gα_{12} and Gα_{13} are the least understood of the larger family of heterotrimeric G proteins that mediate the effects of a multitude of endogenous and exogenous regulators of cellular function (Riobo and Manning, 2005). From the beginning, Gα_{12} and Gα_{13} were pursued by a different tack than their better-characterized cousins that are members of the G_s, G_i, and G_q subfamilies of Gα subunits. These latter three families were initially described by following the biology of signaling pathways—for example, by looking for the transducers of the regulation of cAMP or phosphatidylinositol turnover. In contrast, Gα_{12} and Gα_{13} were "discovered" by cloning studies designed to look for homologs of already-identified proteins (Strathmann et al., 1989). Hence, they were accorded numbers instead of the earlier names that were used to denote primary downstream signaling targets, such as "s" for stimulation of adenylyl cyclase, "i" for inhibition of adenylyl cyclase and, whimsically, "q" for stimulation of phospholipase C (p having already been claimed). The Gα_{12}/Gα_{13} proteins, however, segregate into a distinct arm of the G protein α subunit family (Strathmann and Simon, 1991) and were, from the beginning, orphan proteins in search of an intracellular function. One such function, at least for Gα_{13}, turned out to be regulation of a Rho-GEF (Hart et al., 1998) (i.e., a guanine nucleotide exchange factor for a member of the small G protein family of GTP binding proteins). Numerous variants of this protein have been identified as Gα_{12}/Gα_{13} targets, as have several other interacting proteins (Riobo and Manning, 2005).

Several features of the biology of Gα_{12} and Gα_{13} have made them difficult to study. They have fairly slow nucleotide exchange rates and are hard to express (Singer et al., 1994; Kozasa and Gilman, 1995); they regulate cellular processes that have coincident regulation through multiple other G protein-related processes—as by Gα_q and G$\beta\gamma$; and they do not seem to cause generation of a specific small molecule mediator, such as cAMP or inositol 1,4,5-trisphosphate, that would lead to easily assayed downstream effects (Riobo and Manning, 2005). Consequently, it has not been easy to evaluate whether a receptor signals through this family of proteins or to study the pharmacology of their interactions with receptors. To circumvent these limitations, Zhang et al.

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ABBREVIATIONS: GEF, guanine nucleotide exchange factor; TPα, thromboxane A_2 receptor α; PTA_2, pinane thromboxane A_2; U46619, 9*a*,11*a*-methanoepoxy-15-hydroxyprosta-5,13-dienoic acid; U44069, (5*Z*)-7-((1*S*,4*R*,5*S*,6*R*)-5-((1*E*,3*S*)-3-hydroxy-1-octenyl)-2-oxabicyclo(2.2.1)hept-6-yl)-5-heptenoic acid; SQ29548, 7-[3-[[2-[(phenylamino)carbonyl] hydrazino]methyl]7-oxabicyclo[2.2.1]hept-2-yl]-,[1*S*-]1α,2α(*Z*),3α,4α]]-]5-heptenoic acid; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$.

(2006) report in this issue the characterization of fusion proteins between a thromboxane A_2 receptor (TPα) and Gα_{12} or Gα_{13}, characterizing direct responses of these constructs expressed in Sf9 cells by [^{35}S]GTPγS binding in response to known and suspected TPα receptor agonists and antagonists. Using this assay, the authors show that isoprostanes related to 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) target Gα_{12} and Gα_{13} through TPα receptor activation. These compounds are generated nonenzymatically from arachidonic acid in response to oxidative stress and may play a role in multiple human diseases (Montuschi et al., 2004). Previous studies have ambiguously associated these compounds with multiple receptors, and uncertainly with TPα (for review, see Zhang et al., 2006). Zhang et al. (2006) also characterize the response of TPα-Gα_{12} and TPα-Gα_{13} to other agonists (U46619 and U44069) and antagonists [pinane thromboxane A_2 (PTA$_2$) and SQ29548] of TPα. Their studies indicate that all ligands tested, except SQ29548, have agonist activity for TPα-Gα_{13}, including the purported antagonist PTA$_2$, and that SQ29548 decreases activity of TPα-Gα_{13}, compatible with the idea (but, as admitted by the authors, not definitive proof) that it is an inverse agonist. In contrast to TPα-Gα_{13}, TPα-Gα_{12} did not respond to PTA$_2$ and had a substantially decreased potency for 8-iso-PGF$_{2\alpha}$ that precluded evaluation of its efficacy. To validate the conclusions from the fusion constructs, Zhang et al. (2006) showed that PTA$_2$ and 8-iso-PGF$_{2\alpha}$ were also agonists for Gα_{13} in human embryonic kidney 293 cells through both expressed and endogenous TPα receptors that are not fusion constructs. These cells do not express Gα_{12}, which precluded validation of those results.

The report by Zhang et al. (2006) is the latest to use receptor G protein fusion constructs to study the biology and pharmacology of signaling through specific receptor/G protein interactions (Seifert et al., 1999; Milligan, 2000; Wurch and Pauwels, 2001; Milligan et al., 2004). These constructs express Gα subunits as a C-terminal extension of the receptor protein so that their expression is linked in a 1:1 stoichiometry, with the expressed proteins in obligatory close proximity. This strategy was first used more than 10 years ago to characterize a β2-adrenergic receptor-Gα_s fusion construct (β2-AR-Gα_s), showing, first and foremost, that it was expressed as a functional protein and that it had increased sensitivity to agonists (Bertin et al., 1994). Receptor-G protein fusion proteins have now been used extensively to characterize a large number of receptors, notably those coupled to members of the G_s, G_i, and G_q family of proteins (Table 1). (An extended version of Table 1 is available as an online supplement.) Only one previous report has characterized a receptor construct fused to a member of the Gα_{12}/Gα_{13} subunit family (Sugimoto et al., 2003). That study used a similar fusion construct to show that sphingosine-1-phosphate activation of SIP$_2$/Edg5 receptors can use either Gα_{12} or Gα_{13} to stimulate Rho and inhibit Rac and cell motility, but the authors of that study did not evaluate the utility of the constructs for studies of receptor pharmacology. A primary goal of the work reported here was to establish the Gα_{12}/Gα_{13} family of proteins as targets of this strategy for studying receptor-G protein signaling (Zhang et al., 2006). Thus, this work opens up the possibility of using such constructs both for characterizing responses to additional receptors and, perhaps, for studying the unique signaling properties associated with this particular family of Gα proteins.

The future utility of the constructs characterized by Zhang et al. relates in part to the utility of these receptor-fusion constructs in general. The advantages of the 1:1 receptor/G protein stoichiometry of these constructs has led to a large number of studies evaluating specific receptor-G protein pairs (Table 1); there are theoretical reasons for using such constructs to study (GPCR) receptor theory (Colquhoun, 1998). These constructs have been widely used for characterizing mutations and modifications of receptors (Loisel et al., 1999; Ward and Milligan, 1999; Pauwels and Colpaert, 2000; Moon et al., 2001; Stevens et al., 2001; McLean et al., 2002; Ward and Milligan, 2002; Barclay et al., 2005) and G proteins (Wise and Milligan, 1997; Dupuis et al., 1999; Kellett et al., 1999; Loisel et al., 1999; Wang et al., 1999; Moon et al., 2001;

TABLE 1

Receptors expressed as fusion proteins with C-terminal G protein α subunits

Summary of receptors that have been reported on in the literature as fusion constructs with different G protein α subunits. The table is arranged according to Gα subunit family, and the specific isoforms of each family for which constructs have been made. A complete list of references for these constructs is available as an online supplement to this article.

G_s Family			G_i Family						G_q Family				G_{12} Family	
$G_{s\alpha L}$	$G_{s\alpha S}$	$G_{olf\alpha}$	$G_{i1\alpha}$	$G_{i2\alpha}$	$G_{i3\alpha}$	$G_{o1\alpha}$	$G_{z\alpha}$	$G_{q/i1\alpha}$ Chimera	$G_{q\alpha}$	$G_{11\alpha}$	$G_{15\alpha}$	$G_{16\alpha}$	$G_{12\alpha}$	$G_{13\alpha}$
β1-AR	β1-AR	β2-AR	α2A-AR	α2A-AR	α2A-AR	α2A-AR	m2	α2A-AR	β2-AR	α1B-AR	α2A-AR	β2-AR	Edg5	Edg5
β2-AR	β2-AR		A1	A1	A1	A1		α2B-AR	Edg5			CX3C		
D1	GR		5HT1A			5HT1A		α2C-AR	NK1			M1		
GR	H2		β2-AR	β2-AR	β2-AR				NTS-1			M2		
H2			M2	CXCR1	FPR	D2short						UR-II		
IP			δOR	FPR		δOR								
NK1			Edg2	μOR		μOR								
δOR			FPR	NR		NR								
V2			IP											
			MOR											
			NTS-1											
			TG1019											
			V2											

5HT1A, 5-hydroxytryptamie-1A receptor; A1, adenosine A1 receptor; α1B-AR, α1B-adrenergic receptor; α2A-AR, α2A-adrenergic receptor; α2B-AR, α2B-adrenergic receptor; α2C-AR, α2C-adrenergic receptor; β1-AR, β1-adrenergic receptor; β2-AR, β2-adrenergic receptor; CX3C, CX3C chemokine receptor 1; CXCR1, CXCR1 chemokine receptor; D1, dopamine D1 receptor; D2short, dopamine D2 short receptor; δOR, Delat opioid receptor; Edg2, Edg2 receptor; Edg5, Edg5/S1P2 receptor; FPR, formyl peptide receptor; GR, glucagon receptor; H2, histamine H2 receptor; IP, IP prostanoid receptor; m1, muscarinic m1 receptor; m2, muscarinic m2 receptor; μOR, μ opioid receptor; NK1, tachykinin NK1 receptor; NR, nociceptin receptor; NTS-1, NTS-1 (neurotensin receptor); TG1019, orphan (eicosanoid) receptor; UR-II, urotensin II receptor; V2, Vasopressin V2 receptor.

Stevens et al., 2001; Liu et al., 2002; Ugur et al., 2003; Barclay et al., 2005). Such constructs are similarly useful for characterizing receptor and G protein polymorphisms (essentially mutant constructs) (Milligan, 2002). Receptor-G protein fusion constructs have also been implemented as a successful means for characterizing orphan receptors, so as to identify exogenous (Takeda et al., 2003), as well as endogenous (Hosoi et al., 2002), regulators of pharmacological significance. They have even been targeted for developing gene therapy reagents (Small et al., 2001). The work of Zhang et al. (2006) indicates the likelihood that members of the $G\alpha_{12}$ family, and possibly all G proteins, will be amenable targets for this research strategy.

Some of the interesting results reported by Zhang et al. (2006) are differences in the responses of the $G\alpha_{12}$ and $G\alpha_{13}$ constructs. TPα-$G\alpha_{13}$ responded to PTA_2 as a partial agonist with relatively high potency, whereas TPα-$G\alpha_{12}$ did not respond to PTA_2 and this compound functioned as an antagonist. Nevertheless, both constructs responded to the full agonist U46619 with similar potency. This may indicate ligand-dependent conformations of TPα that differentially interact with G proteins (i.e., some form of agonist-directed trafficking) (Leff et al., 1997; Kenakin, 2003; Perez and Karnik, 2005). There was, however, also a more subtle difference between the two constructs that may or may not relate to the same phenomenon. Zhang et al. (2006) measured activation by agonist-induced GTPγS binding. Whereas TPα-$G\alpha_{12}$ responded to agonists with slow GTPγS binding, the TPα-$G\alpha_{13}$ response was rapid and had to be assayed at very short time points to obtain valid estimates of potency. Previous studies of purified proteins do not suggest differences in these two G proteins for GDP/GTPγS binding kinetics, and both of them have slow binding kinetics relative to other Gα proteins (Singer et al., 1994; Kozasa and Gilman, 1995). Thus, results with the receptor fusion contrasts could be due to receptor-Gα specific interactions indicative of important biological properties or perhaps to differences in the constructs. It is interesting that the recent report of crystals of $G\alpha_{12}$ and $G\alpha_{13}$ as chimeric proteins containing the N-terminal helix of $G\alpha_{i1}$ found that they crystallized in opposite (active versus inactive, respectively) conformations under otherwise similar conditions in the presence of aluminum fluoride and GDP (Kreutz et al., 2006). Such results argue for Gα-specific preferences of these proteins that have inversely related interactions with guanine nucleotides, on the one hand, as in the crystallization studies, and with receptors, on the other hand, as in fusion constructs.

In the most general sense, the report of Zhang et al. (2006) focuses attention on the utility of receptor-Gα fusion constructs for studying GPCR signaling mechanisms. These constructs have been very successful for a number of applications, but as artificial constructs, they have both hidden caveats and potential important surprises for otherwise unapparent biological processes. In general, fusion constructs are perceived to have increased sensitivity to activation, which can include increased constitutive activity. Such observations make sense based upon the proximity and theoretical interactions of the two components of the fusion protein (Seifert et al., 1999; Milligan, 2000; Wurch and Pauwels, 2001; Milligan et al., 2004). These results are not universal, however, and there are substantial differences in the properties of various constructs formed of different receptor-G protein fusion pairs. For example, some constructs, but not all, activate endogenous G proteins as well as their tethered protégé (Burt et al., 1998; Fong and Milligan, 1999; Vorobiov et al., 2000; Massotte et al., 2002; Molinari et al., 2003). Although some of these results may be due to high level overexpression of the fusion constructs (Carrillo et al., 2003), it is not clear that this accounts for all of them or that such signaling is necessarily seen only at high levels of expression. Colquhoun has analyzed the utility of the use of fusion constructs of defined 1:1 stoichiometry for evaluating receptor signaling mechanisms for systems that are otherwise designed to be catalytic with spare receptor and other phenomenon that circumvent an easy analysis of efficacy and potency (Colquhoun, 1998). The activation of endogenous G proteins by fusion constructs seems to minimize these advantages, along with one of the potential uses of the constructs (i.e., evaluation of mechanisms of receptor theory). But is this really true, or are these proteins and this strategy telling us something (important) by disclosing otherwise unsuspected events?

Another emerging concept in GPCR action is the role of receptor dimerization in their synthesis, trafficking and action (Javitch, 2004; Milligan, 2004; Terrillon and Bouvier, 2004). The functional existence of GPCR dimers took years to establish but is now well accepted, particularly for their role in GPCR biosynthesis and maturation (Bulenger et al., 2005) and for the class C receptors (Javitch, 2004; Milligan, 2004; Terrillon and Bouvier, 2004). The general functional role of receptor dimerization in signaling, particularly for the rhodopsin-related class A GPCRs, is still being established (Javitch, 2004; Milligan, 2004; Terrillon and Bouvier, 2004). Is it possible that signaling by fusion constructs is mediated in part through dimer complexes either with themselves or with endogenous proteins? Dimerization of such fusion proteins has in fact been demonstrated, along with the ability of such dimers to cross-regulate one another (Carrillo et al., 2003). Could the dimerization of fusion constructs play a major role in the divergent phenotypes of different receptor-G protein pairs? For example, might the constructs characterized by Zhang et al. have different properties because of differences in their ability to form dimers? Do associated G proteins play a role in receptor dimerization? According to the concepts of agonist-directed trafficking, agonists select different G proteins by inducing agonist-specific conformations of the receptor compatible with that G protein (Leff et al., 1997; Kenakin, 2003; Perez and Karnik, 2005). According to the principle of microscopic reversibility, if agonist induces a conformation of the receptor specific for a G protein, binding of that G protein should also induce a conformation of the receptor specific for that agonist (i.e., G protein-specific receptor conformations). If this interaction is stable, perhaps indicative of precoupling, then some of these G protein-specific receptor conformations may have a tendency to dimerize, whereas others might not. Might such dimerization also explain other observations about fusion constructs that have remained elusive? For example, these constructs often exhibit high- and low-affinity binding states that are not easily explained theoretically or experimentally (Seifert et al., 1999, 2000; Hoare, 2000). Although the functional role of dimers in the actions of these constructs is speculative, access to the full repertoire of Gα isoforms capable of serving as donors for fusion constructs, in part resulting from the work

reported by Zhang et al., provides a mechanism to address this and other questions regarding the signal transduction mechanisms of these proteins and GPCRs in general.

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