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FRIZZLEDS: NEW MEMBERS OF THE SUPERFAMILY OF G-PROTEIN-COUPLED RECEPTORS

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1. ABSTRACT

The superfamily of membrane receptors that signal via heterotrimeric G-proteins includes more than 1500 members, classified into five basic groups, representing about 5-10% of the human genome. These G-protein-coupled receptors operate through a comparatively smaller group of heterotrimeric G-protein family of ~20 members, each displaying an alpha subunit that binds and hydrolyzes GTP in combination with the beta-/gamma-subunit complex that is largely non-dissociable *in vivo*. Frizzleds represent the cell membrane receptors for a family of secreted glycoprotein ligands termed "Wnts" that play essential roles in development, including cell fate, adhesion, polarity, migration, and proliferation. Based upon a compelling set of experimental observations about the structure and downstream signaling of Wnt-Frizzled signaling, one can only conclude that Frizzleds are true members of the GPCR family and require heterotrimeric G-proteins to propagate signals from the Wnts to well-known effectors, including beta-catenin stabilization, mobilization of intracellular Ca2+, and activation of cyclic GMP phosphodiesterase. Careful study of primary structure of Frizzleds reveal heptihelical, 7-transmembrane segments, characteristic of GPCRs. Chimeric forms of Frizzleds, making use of the cytoplasmic domains of Frizzleds, substituted into the exofacial and transmembrane segments of the prototypic GPCR beta2-adrenergic receptor are functional and display the well-known GTP-shift in receptor affinity. Suppression of specific G-protein subunits suppress the ability of chimeric as well as authentic Frizzled-1 and Frizzled-2 to signal to their canonical pathways upon activation. The involvement of beta-arrestin, an important regulator of GPCR signaling, in Frizzled signaling is, therefore, not unexpected. Recognition of the GPCR character of Frizzled enables a

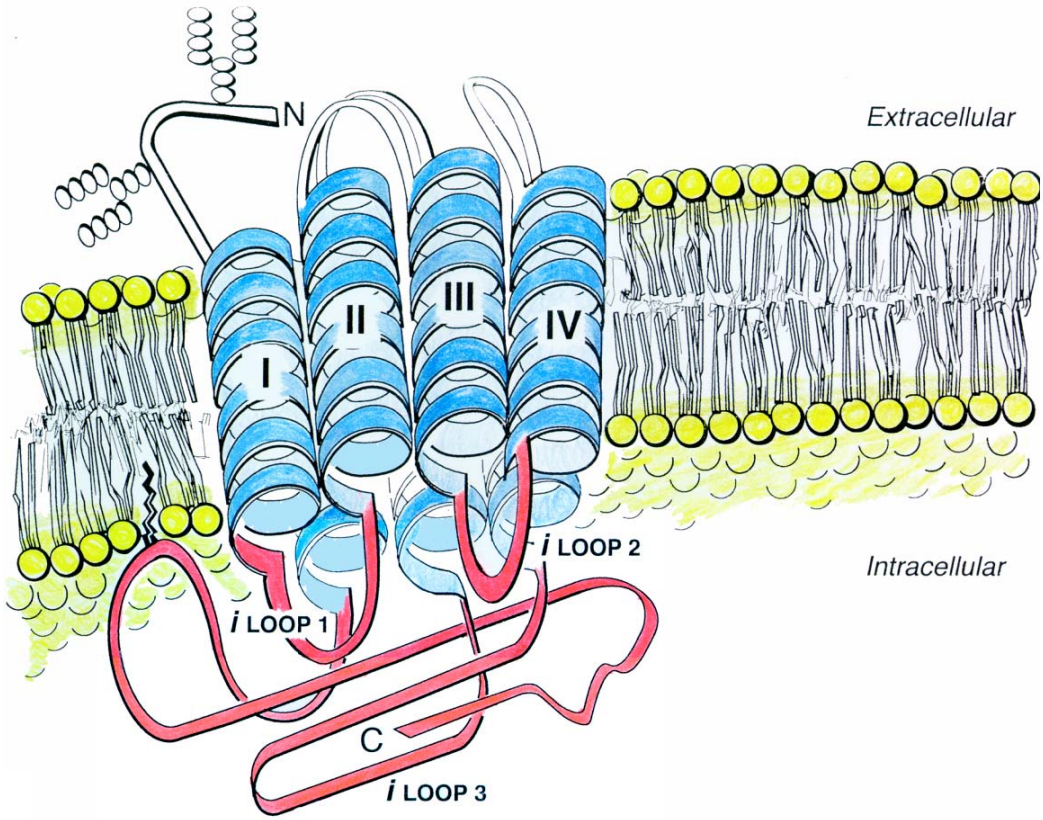

Figure 1. Orientation of N- and C-termini of members of Frizzleds family and other members of the superfamily of G-protein-coupled receptors. The N-terminus of GPCRs is disposed to the extracellular environment accessible to ligands. The C-terminal "tail" and the three intracellular loops (iLOOP1-3) are disposed to the cytoplasmic environment, display canonical sites for phosphorylation by a number of protein kinases, and constitute the regions of the receptor that interface with the heterotrimeric G-proteins.

more broad understanding of these receptors and of their mechanisms of downstream signaling.

2. CRITERIA FOR ESTABLISHING A RECEPTOR AS G-PROTEIN-COUPLED

Since the discovery of heterotrimeric G-proteins and their roles in signal propagation of cell surface receptors more than 20 years ago, evidence has accumulated from study of many members of the GPCR superfamily providing a set of well-established criteria and properties that are shared by all GPCRs (1). Based upon some early insights into the structure and function of Frizzleds (2), the hypothesis was advanced that these distant members of known GPCRs might well be members of the superfamily (3, 4). In an effort to provide a fuller appreciation of the nature of GPCRs, the criteria and properties common to all are briefly discussed below.

2.1. Primary structure with seven transmembrane segments

The hallmark of GPCRs was suspected early by the information deduced by brute-force protein sequencing of the mammalian photopigment rhodopsin that is a GPCR, but one with an interesting twist. Rhodopsin harbors a covalent ligand (11-cis retinal) that captures a photon, isomerizes, and creates the ligand capable of activation this "receptor" which responds to light. The protein sequence includes seven highly hydrophobic regions in the structure, which resembled a ion channel isolated from bacteria*, i.e.,* bacteriorhodopsin. The structure for bacteriorhodopsin elucidated by X-ray crystallography indeed included 7 hydrophobic reaches of sufficient length to traverse the cell membrane (5). Successful cloning of the first true ligand-sensitive GPCRs revealed what might have been suspected from the work on rhodospsin and bacteriorhodopsin, that GPCRs display 7-transmembrane segments (7TMS) or heptihelical hydropathy plots with seven, alpha-helical segments of sufficient length to traverse the lipid bilayer (6, 7). To date, all GPCRs that have been subjected to molecular cloning have been shown to display 7TMS (8).

2.2. N- and C-terminal characteristics of the receptor structure

The N-terminal and C-terminal regions of GPCRs also display some characteristics that are common to GPCRs. The N-terminus is disposed to the extracellular space (*i.e.*, the exofacial side of the lipid bilayer) and is multiply N-glycosylated with complex carbohydrate in most instances (figure 1). The C-terminus, in contrast, is

localized to the cytoplasmic compartment of cells; where in combination with the three intracellular loops (iLoop1, iLoop2, and iLoop3) constitute the domains with which heterotrimeric G-proteins interact (1). The C-teminus and cytoplasmic loops 1-3 of GPCRs also harbor canonical sites for protein phosphorylation by serine/threonine- as well as tyrosine-specific protein kinases. Canonical sites for protein phosphorylation by protein kinase A (PKA), protein kinase C (PKC), casein kinases, G-protein receptor kinases (GRKs), and receptor tyrosine kinases (*e.g.,* insulin and IGF-I) and non-receptor tyrosine kinase (*e.g.*, Src family kinases) abound in these GPCR cytoplasmic domains (9).

2.3. Bacterial toxin sensitivity of the receptor-mediated pathway

Early in the analysis of G-protein-coupled pathways, bacterial toxins such as cholera toxin and pertussis toxin became indispensable tools. Many bacterial toxins display an ADP-ribosyltransferase activity directed to key signaling molecules, many of them G-proteins(10). Cholera toxin ADP-ribosylates the alpha-subunit of Gs, inhibits its intrinsic GTPase activity, and activates the Gs alpha subunit irreversibly. Pertussis toxin, in contrast, makes use of all of the members of the Gi family of G-proteins (which includes Gi1, Gi2, Gi3, Goa, Gob, Gt1, and Gt2) for ADP-ribosylation of their alpha-subunits, but in this case inactivating the subunit and its downstream signaling. The very first indication that signaling pathways were mediated by G-proteins was deduced simply on the ability of bacterial toxins to alter the signaling pathway (11).

2.4. Regulation by introduction of GTP analogues and/or G-protein subunits

One of the most direct approaches to interrogating if a pathway is G-protein–mediated is to introduce GTP analogues (by microinjection) and/or G-protein subunits (via microinjection or transfection) into target cells and measure signaling read-outs. This approach was central to the elucidation of many G-protein-coupled pathways, including phototransduction (12) and early development (13). Introduction of GTP (or the poorly-hydrolyzable analogue GTPgammaS) promotes activation or potentiation of G-protein-mediated pathways. Introduction of the inactive GDP (or its poorly-hydrolyzable analogue GDPbetaS) acts by mass action to attenuate or to block G-protein-mediated pathways. Purified G-protein subunits can be injected into cells or cells can be transfected with an expression vector harboring a G-protein subunit of interest in an attempt to influence the pathway under scrutiny. Introduction or expression of the constitutively-activate mutant versions of G-protein alpha subunits (see below), lacking the intrinsic GTPase activity of the alpha-subunit, provides for a constitutive activation of the pathway, if the G-protein introduced is coupled to the downstream signaling elements. Thus, it is possible to ask if a specific G-protein can turn on the pathway and mimic the action of agonist ligand on a suspected GPCR. Microinjection of a guanine nucleotide provides a second, less specific but equally telltale means to test if the pathway includes a G-protein mediator (14)

2.5. Attenuation of receptor signaling by suppression of specific G-protein subunits

A powerful tool in the analysis of 7-TMS receptors in probing a possible G-protein signaling role is the suppression of specific G-proteins of interest. A variety of techniques have been used to suppress the expression, including microinjection (15) or cell loading with antisense oligodeoxynucleotides (ODN, 16), expression of antisense RNA *in vivo* (17), and gene disruption (18). Much like the inactivation of members of the Gi family of heterotrimeric G-proteins by pertussis toxin blocks the signaling of GPCRs that couple to members of that family, suppression of the expression of specific G-proteins should provide a means to not only demonstrate that a 7TMS receptor is a GPCR, but also to identify precisely those G-proteins to which the receptor couples. It was the application of this "knock-out" strategy that provided insights into not only the receptors, but also the pathways that they regulate. Identification of G-proteins as mediators of such complex processes as differentiation (16), development (19), oncogenesis (20), cell-cycle (21), and life span in *Drosophila* (22) benefited by use of one or more of these techniques.

2.6. Mimicry of GPRC signaling by introduction of activated mutant versions of G-protein alpha-subunits

Although typically used to test the assignment of a specific G-protein as mediating the action of a putative GPCR, expression of the constitutively active mutants of G-protein alpha subunits engineered with diminished endogenous GTPase activity provides a receptor by-pass and answers the question, can the activation of the G-protein stimulate the downstream signaling elements as does the activated GPCR? Although the results of pertussis toxin treatment, suppression by antisense ODNs, and presence of a GTP-induced agonist-specific shifts (see below) have identified a novel role of Galphai2 in differentiation, the ability to mimic the effect of differentiation with expression of the Q205L Galphai2 subunit acted to "close the loop" on the question of whether this G-protein could and did mediate this complex response in mouse F9 teratocarcinoma cells (16). An extension of the strategy of introducing via microinjection GTP analogues as activators (GTPgammaS) or inhibitors (GDPbeta S), investigators have made use of microinjection of purified expressed G-protein subunits (and their constitutively activated mutant forms) to probe complex responses regulated by GPCRs (23, 24).

2.7. Agonist-specific, GTP-dependent shift in receptor affinity

One of the hallmarks of 7TMS that demonstrate them to be GPCRs is the agonist-specific shift in receptor affinity induced by treatment with GTP (or GTPgammaS, 25). Based upon results from reconstitution experiments with purified 7TMS and G-proteins, it has been shown that a GPCR, when coupled to its cognate G-protein partner(s) displays an affinity for agonist ligand that is markedly greater in the absence than in the presence of GTP. In the absence of added GTP the G-protein is GDP-liganded and in a complex with the receptor. Addition of GTP (or GTPgammaS) stimulates exchange of GTP for GDP on the

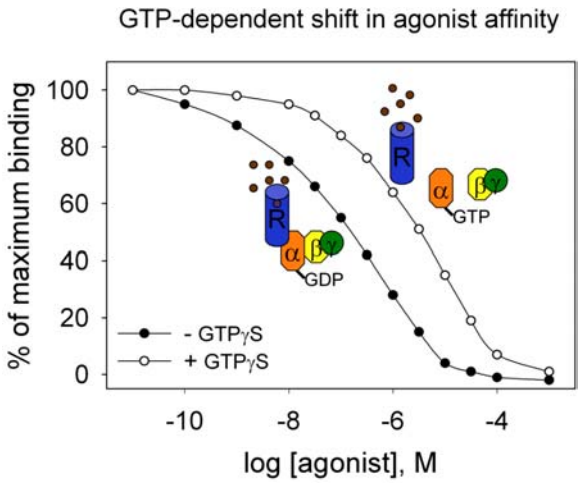

Figure 2. GTP-induced agonist-specific shift in affinity of members of the superfamily of G-protein-coupled receptors, including the Rfz1 chimera. A cardinal property of GPCRs is the ability of GTP (or analogues) to stimulate a reduction in the apparent affinity of the receptor for agonist, but not antagonist, ligands. In the absence of added GTP, the inactive heterotrimeric G-protein is loaded with GDP and forms a complex with the GPCR. In the G-protein-GDP/receptor complex, the receptor displays high affinity for agonists. Introduction of GTP leads to a receptor-mediated guanine nucleotide exchange of the G-protein alpha subunit, its activation and dissociation from both the receptor as well as the Gbeta/gamma complex. The receptor, in the absence of its cognate G-protein, now displays a lower affinity for agonist, but not antagonist, ligands. The inability of the antagonist ligands to activate the receptor and guanine nucleotide exchange of the G-protein is the basis for the agonist-specific nature of the GTP-induced shift.

G-protein, dissociation of the receptor from the activated, GTP-occupied Galpha subunit, and a reduction (rightward shift) in the affinity of the receptor for agonist (figure 2). In the presence of GTP, all GPCRs uniformly display markedly lower affinity for agonist ligands, so it is the higher affinity state of the receptor for agonist in the absence of GTP that reflects its direct, molecular interactions with heterotrimeric G-proteins. All 7TMS for which these types of experiments can be performed display an agonist-specific, GTP-dependent shift in receptor affinity, demonstrating their nature as GPCRs. This effect of GTP on receptor affinity is specific for agonist binding only. The affinity of the receptors for antagonist ligands are unaffected by the absence/presence of GTP. Furthermore, a receptor displaying an agonist-specific, GTP-induced shift in receptor affinity demonstrates a true direct molecular coupling of the receptor with its cognate heterotrimeric G-protein. For this assay, one would predict that addition of pertussis toxin should block the ability to observe the GTP-shift of a GPCR, since the toxin-induced ADP-ribosylation is known to occur on a G-protein residue in the C-terminus of Galphai family members that is critical for receptor/G-protein interaction. Likewise suppression of the specific G-protein(s) to which a 7TMS couples should abolish the ability of the GTP to shift the agonist affinity of the receptor.

2.8. Functional reconstitution of receptors, heterotrimeric G-proteins and effectors

The approach that provided support for the now nearly universal paradigm of GPCRs was defined reconstitution of purified receptors, specific G-proteins and G-protein-regulated effectors. Biochemical reconstitution of these elements into defined, unilammelar vesicles is a heroic effort (26), since the purification of each of these low-abundance molecules from native cells/tissues was in itself a Herculean task. Reconstitution of receptors/G-proteins or of the complete triad of receptor/G-protein/effector is fraught with technical obstacles that challenge investigators. In spite of the formidable challenge, in a few cases the successful reconstitution of a ligand-activated triad of receptor/G-protein/effector has been realized. More common was the reconstitution of a suspected GPCR with a specific G-protein with the read-out being the ligand (agonist) activation of the endogenous GTPase of the alpha subunit of the G-protein or the activation by ligand of the guanine nucleotide exchange reaction for endogenous GDP for radiolabeled GTP (27). The advent of molecular cloning of the G-proteins, many effectors, and a large number of potential GPCRs has provided an alternative approach, *i.e.,* targeted overexpression of members of the triad in various cells (such as insect Sf9 cells) and the use of the same read-outs. The stoichiometries of the overexpression can be so great (1×10^6 copies/cell) that the read-out from endogenous competing G-protein-coupled sources is minor (28, 29) and robust response can be measured. But, as should be obvious from the brief elaboration of the criteria that establish 7TMS receptors as GPCRs, it is not necessary to achieve successful reconstitution of the triad in vitro to establish a 7TMS receptor as a GPCR. From careful testing a few key criteria, it is possible to identify members of the superfamily of GPCRs. Investigators from many laboratories have focused upon a single question posed in early 1995, are Frizzleds members of the superfamily of 7TMS coupled via heterotrimeric G-proteins? The answer to this question is essential to our establishing the downstream signaling events and understanding the mechanisms of action of Frizzleds.

3. FRIZZLEDS AS G-PROTEIN-COUPLED RECEPTORS

Based upon the information outlined above for the characterization of 7TMS receptors as GPCRs, one might consider the testing of the hypothesis that Frizzleds are GPCRs to be a relatively straight forward effort based upon similar effort that have identified several thousand GPCRs to date. The major obstacle encountered to the analysis of Frizzleds actions for more than 20 years was the inability to purify the Frizzled ligands, the Wnts, with the retention of biological activity (30, 31). The lack of purified, active Wnts limited most studies to that which could be gleaned from genetic approaches, which have been invaluable in elucidating many of the features of downstream signaling of Frizzleds. When cell-based

experiments were attempted, investigators had but one option, the use of conditioned media as a source of expressed Wnts. The limitations of conditioned media are well-known in the signaling field. For Wnt-Frizzled studies it was even more challenging, as the identity of all of the Wnts present and their relative abundance were difficult to establish. The presence of other growth factors and potentially confounding agents in conditioned media is also well known. So, it is no surprise that the analysis of the Wnt-Frizzled pathways was impaired for those two decades. The creation of Frizzled chimera (32) that by-pass the need for purified, active Wnts enabled direct analysis of many features of Wnt-Frizzled signaling that were not addressable by traditional approaches.

The solution to the challenge was provided by adapting a tried and tested approach developed for teasing out the biochemical signaling pathways for well-known GPCRs. This strategy was an attempt to create chimeric receptors between Frizzled and prototypic members of the GPCRs (33). For more than a decade it was shown, for a variety of GPCRs, that the exofacial and transmembrane domains of the most populous type I receptors can provide membrane-embedded ligand-binding domains capable of accepting cytoplasmic loops (intracellular loops 1-3) and the cytoplasmic C-terminus of other GPCRs to form functional chimeras that display the functional output of the receptor donating the cytoplasmic domains of the chimera (33). Thus one can substitute the cytoplasmic domains of an alpha1-adrenergic receptor (a receptor that signals through the activation of phospholipase C and phosphatidylinositol phosphate cleavage) to the exofacial and TMS of a beta-adrenergic receptor (a receptor that signals via activation of adenylylcyclase and cyclic AMP) to yield a chimera that binds and is activated by a beta-adrenergic agonist, but now signals via phospholipase C rather than adenylylcyclase. Although the chimeras constructed early on were functional, all were created from closely related members of the superfamily of GPCRs. The task to adapt this strategy to test if the Frizzleds are GPCRs would be the first attempt to create a functional chimera from the most distantly-related members of the 7TMS. The beta2-adrenergic receptor, selected as the 7TMS receptor donating the exofacial and transmembrane segments typically binds beta-agonists such as epinephrine, norepinephrine, or the synthetic agonist isoproterenol at a core binding domain localized to the 7TMS domains within the lipid bilayer (7). The cytoplasmic domains donated to the chimera would be provided by either Frizzled-1 or Frizzled-2. These 7TMS receptors typically bind secreted, glycoprotein ligands Wnts that selectively activate various Frizzleds. Wnt-8 binds and activates Frizzled-1, whereas Wnt-5a binds and activates Frizzled-2. Both native Frizzleds rely upon their relatively large, complex N-terminal binding domains unique for specific Wnts. Each of these N-termini displays a motif of 10 invariant cysteinyl residues, termed a cysteine-rich-domains (CRD). Wnt-8 neither binds to nor activates Frizzled-2; Wnt-5a neither binds nor activates Frizzled-1 (31).

Creating functional chimera between the prototypic GPCR, the beta2-adrenergic receptor (1), and its most distant relative in the 7TMS superfamily would itself constitute a compelling demonstration that Frizzleds are GPCRs and would enable a search for the G-proteins to which they couple. Additionally, both the beta2-adrenergic receptor and the Frizzleds display some interesting aspects about the size of their domains that would be optimized in creating a chimera from a receptor with the shortest length of the exofacial domains (beta2-adrenergic receptor) with receptors that display the shortest cytoplasmic domains (Frizzleds). The 7TMS of all members of the GPCR superfamily, in contrast, are nearly invariant in length, *i.e.*, each segment equivalent to a stretch of alpha helix of sufficient length to span the lipid bilayer. After thoughtful and empirical determination of the optimal junction points, two Frizzled chimera were prepared (32, 34). The chimera in which the cytoplasmic domains of the rat Frizzled-1 were substituted into the corresponding regions of the human beta2-adrenergic receptor was termed the Rfz1 chimera(34), whereas the chimera in which the cytoplasmic domains of the rat Frizzled-2 were substituted in the corresponding regions of the beta2-adrenergic receptor was termed the Rfz2 chimera(32). The chimera were expressed in the mouse totipotent F9 teratocarcinoma cells in which the stimulation of formation of primitive endoderm (PE) by Wnt-Frizzled action can be ascertained by assay of the expression of two hallmark proteins of PE-phenotype, cytokeratin endo-A (*i.e.*, the TROMA antigen) and tissue plamsinogen activator. Below is a brief assessment of the ability of the Frizzleds and Frizzled chimeras to meet the criteria established for characterization of a GPCR.

3.1. Frizzled-2, the regulator of Wnt-Ca^{++} and cyclic GMP signaling

3.1.1. Primary sequence of Frizzled-2

The rat Frizzled-2 gene product, like all GPCRs, displays a hydropathy plot with seven discrete hydrophobic domains(35). Each of these domains is predicted to form an alpha-helix and to be of sufficient length to span the lipid bilayer. Each of the 7TMS receptors examined in depth to date have been demonstrated to signal via heterotrimeric G-proteins, members of the GPCR superfamily of some >1500 gene products(36).

3.1.2. N- and C-temini sequences of Frizzled-2

The rat Frizzled-2 displays a large, complex N-terminus possessing multiple CRDs that reflect the primary binding domain of the Frizzled for Wnt ligand. Although not at all like the N-terminal sequence of the prototypic GPCR beta2-adrenergic receptor, the Frizzled-2 N-terminal sequence displays similarity to the N-terminus of other well known GPCRs, such as the receptors for the gonadotropins leutinizing hormone (LH) and follicle-stimulating hormone (FSH), which display CRDs and also bind glycoprotein agonists. The gonadotropin receptors, which share many of these features with Frizzleds, are well-known to signal via heterotrimeric G-proteins, providing a paradigm for Frizzled signaling deserving of serious consideration. The C-terminal tail and three intracellular loops (iLoop1-3) of Frizzleds have been shown to harbor canonical sites for protein phosphorylation by a number of well-known serine/threonine protein kinases, including protein kinase A and protein kinase C. The C-terminal structures may well

also be substrates for phosphorylation by GPCR receptor kinases (GRKs) through which interactions with beta-arrestins might occur, beta-arrestins acting as adapter proteins for a large number of GPCRs. Supporting these notions are recent experiments implicating beta-arrestins in Frizzled signaling(37), although this is not universally observed (38). Thus, Frizzleds, as analyzed by *in silico* techniques, appear to display several cardinal features of GPCRs. Although the existence of these similarities does not by itself demonstrate that Frizzleds signal via G-proteins, it does provide a compelling argument for the further testing of the hypothesis through direct means.

3.1.3. Effects of bacterial toxins on Frizzled-2 action

Although the bacterial toxin of *Vibrio cholera*, which specifically ADP-ribosylates and activates the heterotrimeric G-protein Gsalpha, clearly elevates intracellular cyclic AMP concentrations, it has not been shown to modulate the ability of Frizzleds to signal in any functional read-out. To quite the contrary, the pertussis toxin elaborated by *Bordetella pertussis* has been shown to attenuate or block the ability of Frizzled-2 to signal to several well-known downstream elements and functional read-outs (39, 40). The Wnt-Ca^{++} response observed in a variety of model systems of development provides the best example of a pertussis toxin-sensitive pathway of Frizzled signaling. The ability of Wnt-5a to mobilize intracellular Ca^{++}, activate calcium calmodulin-sensitive enzymes (such as CaM kinase), stimulate the Ca^{++}-sensitive protein phosphatase calcineurin (also known as PP2B), and regulate the activity of transcription factors such as NF-AT, are all sensitive to blockade by pertussis toxin treatment (41). In mouse F9 embryonic cells, pertussis toxin attenuates the ability of the Rfz2 chimera to stimulate the formation of primitive endoderm (32). In addition, the ability of pertussis toxin to block Frizzled-2 signaling was tested in F9 cells expressing the authentic Rfz2 receptor (not the chimera) and challenged with conditioned media from Wnt-expressing clones. Wnt-5a, but not Wnt-8, stimulated primitive endoderm formation in the F9 clones expressing Rfz2 and this response was attenuated by prior treatment of the cells with pertussis toxin, confirming the more detailed experiments performed with the Rfz2 chimera stimulated with isoproterenol (32). Thus, Wnt-Frizzled-2 signaling to downstream signals demonstrates sensitivity to the effects of pertussis toxin, a toxin that blocks signaling by the Gialpha family of G-proteins. Pertussis toxin sensitivity is a cardinal feature of all GPCRs that signal via this family of G-proteins that includes Gi1, Gi2, Gi3, Goa, Gob, Gt1, Gt2 (11).

3.1.4. Injection of GTP and/or G-protein subunits on Frizzled-2 signaling

The ability of GTP (or its analogues) to mimic and GDP (or its analogues) to attenuate signaling pathways that are mediated by heterotrimeric G-proteins is another cardinal feature of the pathways regulated by GPCRs. The application of this strategy to cell signaling is limited to those cells suitable for microinject of solutions of guanine nucleotides (e.g., GTP) and/or purified G-protein subunits. At least in the *Xenopus* animal cap system, it was possible to demonstrate that introduction of GTPgammaS mimicked the effects of stimulating Frizzled-2 on Ca^{++} mobilization (40). Injection of GDPbetaS, in contrast, blocked the ability of the Frizzled-2 to stimulate the Ca^{++} mobilization. Expression of excess levels of Galphat1 to sequester Gbeta/gamma subunits, forcing the equilibrium by mass action to the heterotrimeric form and reducing the amount of "free" Gbeta/gamma complex that activates phospholipase Cbeta isoforms, also attenuated this Wnt/Ca^{++} pathway (40). These effects of the GTP versus GDP in the Frizzled-2 Ca^{++} pathway conform to the pattern of many GPCR-mediated signaling pathways.

3.1.5. Suppression of G-protein subunits and Wnt-Frizzled-2 signaling

Perhaps the single most definitive proof of the role of G-proteins in Frizzled signaling was obtained by through screening the effects of a systematic elimination of G-protein subunits that constitute the greater family of heterotrimeric G-proteins. For more than a decade oligodeoxynucleotides (ODN) antisense to G-protein subunits have provided a powerful and targeted strategy for identifying GPCRs and the pathways that they regulate. Through careful design of the ODNs, empirical determination of the most efficient means to deliver ODNs to cells in culture, and attention to performing proper missense and sense controls, many laboratories have succeeded in taking advantage of this approach (42). The F9 cells have proven to be a valuable system for studies involving use of ODNs, since they demonstrate little toxicity to ODNs at the levels typically employed and show efficient suppression of G-protein subunits over the course of 48-96 hr treatment required to fully suppress most of the subunits (16). Using this approach it was possible to systematically eliminate all suspected alpha and beta-subunits of heterotrimeric G-proteins in F9 cells and ascertain what effects, if any, does the elimination of these subunits have on the ability of Rfz2 chimera to stimulate formation of primitive endoderm. The results were clear, *i.e.*, suppression of either Goalpha or Gt2alpha nearly abolished the ability of Rfz2 chimera to signal to primitive endoderm formation (32). Furthermore, studies using conditioned media from Wnt-expressing clones to treat F9 clones expressing Frizzled-2 provided confirmation, *i.e.*, suppression of Goalpha/Galphat2 subunits blocks the ability of Wnt-5a to signal via authentic rat Frizzled-2 to the formation of primitive endoderm (32). The ability of pertussis toxin treatment to block Rfz2 signaling implicated members of the Gi-family of heterotrimeric G-proteins in Frizzled-2 signaling and the antisense ODN-induced depletion studies identified two members of the G-protein family, Go and Gt2, as critical for Wnt-Frizzled signaling.

3.1.6. Mimicry of Frizzled-2 signaling by expression of constitutively activated Galpha subunits

Once G-proteins have been implicated in a signaling pathway, it is often possible to mimic, to some extent, the downstream signaling events by expression of a constitutively active mutant form of the Galpha subunit in the target cells. This approach is not typically employed to "fish out" potential G-proteins involved in a pathway, but rather to address the question of whether the input from the G-proteins is not only obligate, but sufficient, to stimulate

the pathway. For Frizzled-2 signaling, only one of the downstream effectors has been studied in detail, the activation of cyclic GMP phosphodiesterase. Using F9 cells as a target, it was shown that expression of the proper combination of constitutively activated G-protein alpha subunits (especially Gt2alpha) was able to mimic the cyclic GMP response noted upon activation of Frizzled-2 by Wnt-5a or by the activation of the Rfz2 chimera (43). The magnitude of cyclic GMP response stimulated by the expression of the constitutively activated Galpha subunits was not as great as that produced by activated Frizzled-2 or its Rfz2 chimera, but this simply may reflect differences in the stoichiometries of the G-proteins expressed endogenously compared to exogenously. More work will be required to determine if the stoichiometry is truly an issue, or whether the activation of the Frizzled-2 yields some additional signal, perhaps through activation of Galphao and/or the phosphoprotein Dishevelled, that is required for full expression of the Frizzled control on the cyclic GMP phosphodiesterase.

3.1.7. GTP-dependent, agonist-specific shift in Frizzled-2 affinity

Involvement of a G-protein in the signaling of a 7TMS receptor must include the demonstration that the receptor and G-protein display protein-protein interaction, if the hypothesis that the receptor is a GPCR is to be exhaustively interrogated. For many of the several thousand GPCRs that have suitably radiolabeled antagonist ligands and unlabeled agonist ligands the demonstration of the protein-protein interaction is manifest in the GTP-dependent, agonist-specific shift of GPCR affinity (figure 2). The GTP-induced shift in affinity is only observed for agonist ligands, detected best by traditional radioligand binding studies using competition studies of radiolabeled antagonists with unlabeled agonist. In the presence of GTP, the receptor-G-protein complex that displays high affinity for agonist ligand is lost, with the dissociation of the GTP-bound Galpha subunit from the receptor as well as from the Gbeta/gamma dimer. The uncomplexed receptor displays a rightward shift in the affinity for agonist; this is a hallmark for GPCRs. Considering the historical problems with the Wnt agonist alone, it becomes clear that this approach is not tenable for study of Frizzleds. The Frizzled chimera, in sharp contrast, can make use of a well-characterized ligand binding domain donated by the beta2-receptor to report on the interactions of the cytoplasmic domains of the Frizzleds with cognate G-proteins, if present. The Rfz2 chimera which makes use of the cytoplasmic domains of the Frizzled-2 does indeed demonstrate a GTP-dependent shift in the affinity of the chimera for agonist (43). Furthermore, either treatment with pertussis toxin or with ODNs antisense to Goalpha/Gt2alpha results in a collapse of the agonist competition curve to the position occupied by the receptor in the presence of GTP (43). Thus, at a molecular level, it has been demonstrated that Frizzled-2 cytoplasmic domains couple functionally to heterotrimeric G-protein necessary for their signaling to downstream effectors. A GTP-shift in agonist affinity has been shown for the gonadotropin receptors that like the Frizzleds also bind secreted glycoprotein ligands. With more detailed understanding of the Wnt-Frizzled interaction and the availability of radiolabeled Wnt antagonists and purified Wnts, it may be possible to test this question more directly making use of the authentic Frizzleds for study of the GTP-dependent shift in receptor affinity.

3.1.8. Functional reconstitution of the Wnt-Frizzled triad of Frizzled-2/G-protein/effector

From a biochemical perspective, the best way to study and to understand cell signaling devices of this nature is to express, purify, and reconstitute the triad Frizzled/G-protein/effector into defined liposomes to see whether and how they operate. As powerful and appealing this approach may seem, in sparingly few cases has the effort been attempted, let alone succeeded. The major issues are retaining the function of each of the triad constituents through-out long, arduous purification schemes and then defining the precise conditions to retain the activities of the receptor, G-protein, and effector while retaining the proper spatial arrangement in a phospholipid, unilammelar vesicle. This convergence of top quality biochemistry and some good fortune has been achieved for the phototransduction pathway and for the stimulatory adenylylcyclase pathway. With the ability to express these interesting molecules at very high levels in insect Sf9 cells infected with a baculovirus construct in which the P-protein promoter directs their expression (29), a second option, but one far less appealing than a true reconstitution, may be available for study of the many other Frizzled/G-protein/effector interactions likely to be operating in Wnt signaling.

3.1.9. Conclusion

Based upon the fulfillment of nearly all of the criteria outlined for establishing a 7TMS receptor as a GPCR, Frizzled-2 can be considered one of the newest members to the superfamily of G-protein-coupled receptors. The data are compelling and at least at some level would seem expected based upon the large body of literature demonstrating 7TMS receptors as GPCRs. However, through careful analysis at many levels, making optimal use of many approaches including the confirmation of key observations with conditioned media containing Wnts and expression of authentic Frizzled-2, it is clear that developing a further understanding of the downstream signaling of Wnts will require greater effort devoted to interrogating the many leads that have been established for many GPCRs and the G-proteins to which they couple.

3.2. Frizzled-1, regulator of the Wnt/beta-catenin pathway

Primary sequence- The primary sequence deduced first for the rat Frizzled-1 displayed a hydropathy plot with 7 prominent, hydrophobic, suspected alpha-helical segments of sufficient length to span the lipid bilayer, *i.e.*, 7TMS. The disposition of the 7TMS in the sequence is a characteristic of all GPCRs subjected to molecular cloning to date (35).

3.2.1. N- and C-termini of Frizzled-1

The N-terminus of Frizzled-1 is localized to the exofacial, extracellular space and includes the characteristic large, complex Wnt-binding domain displaying multiple CRDs, as described for Frizzled-2 above. This organization

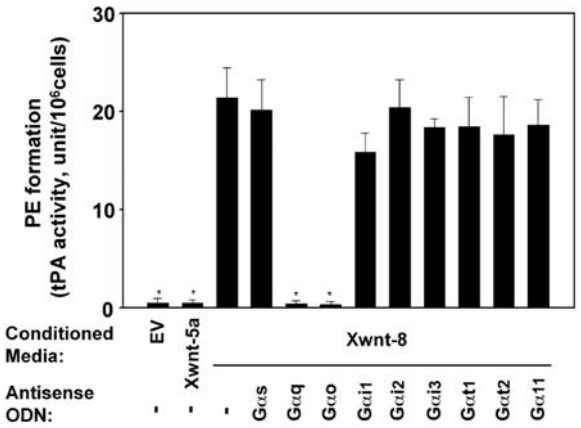

Figure 3. Suppression of Go and Gq heterotrimeric G-protein alpha subunits attenuates Frizzled-1 signaling. Mouse totipotent F9 teratocarcinoma cells were stably transfected with authentic rat Frizzled-1. The Frizzled-1 expressing clones were first treated with oligodeoxynucleotides (ODN) antisense to a specific G-protein alpha subunit, to suppress the expression of each of these G-proteins. The ODN-treated Frizzled-1 expressing clones were then treated with conditioned media from one of three sources, F9 clones stably transfected with an empty vector (EV) or vector harboring either *Xenopus* Wnt-5a (Xwnt-5a) or *Xenopus* Wnt-8 (Xwnt-8). Four days later, the clones were assayed for formation of primitive endoderm (PE), by assay of tissue plasminogen activator (tPA). tPA expression is the hallmark of PE formation. The clones treated with conditioned media from F9 cells expressing Xwnt-8, but not Xwnt-5a or the EV control, displayed robust PE formation. Treating the Xnt-8 induced Frizzled-1 clones with ODNs antisense to the alpha subunit of Gq and Go, but to none of the other Galpha subunits, specifically blocked the Xwnt-8-induced formation of PE.

is similar to that of the GPCRs that like Frizzleds bind glycoprotein ligands, such as LH, FSH, and other gonadotropins. The C-terminus of Frizzled-1 is disposed to the cytoplasmic space, displays one of the shortest lengths, and in combination with the intracellular loops (iLoop1-3) display canonical phosphorylation sites for protein kinase A, protein kinase C, and other protein kinases.

3.2.2. Effects of bacterial toxins on Frizzled-1 signaling

As was noted for Frizzled-2, the treatment of cells with cholera toxin to constitutively activate Gs and adenylylcyclase appears to have little influence on Frizzled-1 signaling. Pertussis toxin, on the other hand, has been shown to attenuate the ability of Frizzled-1 to signal in a variety of systems, including Frizzled-1 promotion of primitive endoderm in mouse F9 embryonic cells and Frizzled-1 activation of hallmark genes of early development in *Xenopus* embryos (34). Careful review of reports that are unable to observe pertussis toxin-induced attenuation of Frizzled actions reveals the apparent discord in the data, since for the studies in which pertussis toxin had no apparent effect the holotoxin if often introduced to the cells, whereas in the studies in which the RNA encoding the A-subunit (the ADP-ribosyltransferase) is microinjected, the pertussis toxin blocks frizzled-1 signaling. Studies from more than 20 years ago documented that addition of holotoxins, like that for pertussis toxin, requires the availability of a cell surface receptor for the toxin B-protomer in order for the complex process of transmembrane transport and protease processing to release the active A-protomer to the intracellular compartment. Absence of the cellular receptor for the toxin (or of any of the cellular machinery necessary for insertion and protease activation) renders a cell insensitive to pertussis toxin. We can only speculate that some systems, such as the *Xenopus* embryo, are deficient in one or more of these factors and that this limitation can be obviated by injection of the RNA encoding the active A-protomer. The ability of pertussis toxin to block Frizzled-1 signaling extends from the read-outs of stabilization of beta-catenin, to activation of the Lef/Tcf transcriptional elements, gene expression, and other feature of early development (34). Thus, pertussis toxin acts at a point distal to the Frizzled and proximal to downstream signaling elements such as Dishevelled. These data and those obtained with the analogous Frizzled-2 provided the basis for the hypothesis that Frizzled-1 and its control over the Wnt-beta-catenin "canonical" pathway needed to be evaluated as a G-protein-mediated pathway.

3.2.3. Effects of antisense ODNs to suppress G-protein subunits on Frizzled-1 action

Making use of antisense technology to suppress the expression of G-protein subunits, it was possible to screen the major alpha and beta subunits for their role in Frizzled-1 activation of a luciferase reporter gene (Topflash) possessing the Lef/Tcf –sensitive promoter that is activated by beta-catenin (34). The initial screens were performed in mouse F9 embryonic cells making use of a rat Frizzled-1 (Rfz1) chimera composed of the exofacial and 7TMS of the beta2-adrenergic receptor to which the cytoplasmic domains (intracellular loops 1-3 and the C-terminal tail) of the rat Frizzled-1 were substituted. Activation of this Rfz1 chimera with isoproterenol was shown to activate Topflash activity and to promote the formation of primitive endoderm in F9 cells. Using both Topflash activation and primitive endoderm formation as read-outs, each of the candidate alpha and beta subunits were individually suppressed with antisense ODNs. The results of these screens identified two alpha subunits, Goalpha and Gqalpha, as essential to activation of Topflash and primitive endoderm formation in response to the activation of Rfz1 chimera by isoproterenol. These data obtained with the chimera were tested further through the use of conditioned media containing Wnt-8 to stimulate activation of authentic rat Frizzled-1 in the F9 cells (figure 3). The conditioned media containing Wnt-8, but not that containing Wnt-5a, stimulated activation of Topflash and formation of primitive endoderm. The results of the screens of the effects of suppression of individual G-protein subunits on Wnt-8 activation of Frizzled-1 signaling was the same as that observed using the Rfz1 chimera, *i.e.*, both Goalpha and Gqalpha are required for Frizzled-1 action.

3.2.4. Mimicry of Frizzled-1 action by expression of constitutively activated mutants of G-proteins

Studies complementary to the antisense ODN-based suppression strategy were performed, targeting

analysis of the effect of expressing the constitutively activated mutant versions of Goalpha and Gqalpha on the signaling elements downstream of Frizzled-1. The results of the experiments demonstrated in mouse F9 cells that the expression of activated mutant form of Goalpha provided significant Frizzled-1-like downstream responses (34). Co-expression of both activated Go and Gq alpha subunits resulted in a substantial activation of Topflash and formation of primitive endoderm, although not as large in magnitude as that obtained by stimulation of Frizzled-1 of the Rfz1 chimera (34). These data can be interpreted in two ways, the co-expression may be not be stoichiometric for both subunits, or the full activation of the pathway requires some additional signal provided by Frizzled-1 activation that is not mediated by the G-proteins directly, such as activation of Dishevelled. In any case, these experiments confirm and extend the antisense suppress data, demonstrating that introducing activated forms of the implicated G-proteins does in fact lead to activation of the Frizzled-1 downstream signaling.

3.2.5. GTP-dependent, agonist-specific shift in Frizzled-1 affinity

The Rfz1 chimera possessing the cytoplasmic domains of the rat Frizzled-1 fused to the exofacial and 7TMS regions of the beta2-adrenergic receptor is properly configured to allow determination if a GTP-dependent, agonist-specific shift occurs for the Frizlled-1 receptor domains that are known to interact with G-proteins. The results of exhaustive radioligand binding experiments of GTP-induced shifts demonstrated that indeed addition of GTP to membranes prepared from F9 cells expressing Rfz1 chimera induced a rightward shift in receptor affinity for agonists, but not antagonists, a hallmark of GPCRs (34, 38). Furthermore, suppression of the G-protein alpha subunits essential for Rfz1 chimera and Rfz1 signaling to activation of Topflash and formation of primitive endoderm resulted in a collapse of the agonist competition curves in the absence of GTP to the same position as observed in the presence of GTP. These data demonstrate not only that the Frizzled-1 cytoplasmic domains interact directly with their cognate G-proteins, but also that in the absence of these key G-protein alpha subunits the high affinity state of the receptor for agonist is not manifest.

3.2.6. Functional reconstitution studies of Frizzled-1 signaling

In view of the fact that the effectors for Frizzled-1 signaling beyond the level of G-proteins and Dishevelled are not known with precision, reconstitution experiments are not possible. It should be highlighted that for at least the Goalpha subunit, no well-established effector has been identified.

3.3. Other Frizzleds as GPCRs

Other members of the Frizzled family are being interrogated by the creating of Frizzled chimeras very similar in design as those first prepared for Frizzled-1 and Frizzled-2. The extent to which these constructs prove to be functional chimeras and employed in studies similar in nature to those outlined above will require additional studies.

4. CONCLUDING REMARKS

Analysis of the protein-protein signal linkage map for Frizzleds remains an important target for study. There is no doubt that Frizzleds are 7TMS receptors that couple to downstream signaling elements by heterotrimeric G-proteins. Several major unresolved tasks remain for those developmental biologists and cell signaling experts alike. For Frizzled-1 and Frizzled-2, Go plays a key role. Effectors for this abundant G-protein that was discovered almost 20 years ago remain, however, enigmatic. For Frizzled-1, and perhaps Frizzled-2, the role of the phosphoprotein Dishevelled in downstream signaling is unambiguous, but how Dishevelled and G-proteins coordinate their signals remains a mystery worthy of intensive study. Gene profiling of Frizzled-1 and Frizzled-2, post-translational regulation of Frizzleds in general, and the unique spatial constraints on Wnts and their interactions with Frizzleds are all major issues to be addressed. Perhaps to some extent experts in GPCR-based signaling would only be moderately surprised by the demonstration that Frizzled-1 and Frizzled-2 are members of the this superfamily of 7TMS that coupled to heterotrimeric G-proteins. For developmental biologists the discovery of G-proteins mediating aspects of signaling was unexpected, although new members of these complex networks are still being discovered. The application of proteomics, advanced multi-photon microscopy, and other new strategies will undoubtedly yield clues to the unresolved mysteries of the Frizzleds and other potential members of its family of 7TMS, such as the Smootheneds.

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