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Frizzled receptors signal through G proteins

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ABSTRACT

Frizzled receptors have long been thought to couple to G proteins but biochemical evidence supporting such an interaction has been lacking. Here we expressed mammalian Wnt-Frizzled fusion proteins in *Saccharomyces cerevisiae* and tested the receptors' ability to activate the yeast mitogen-activated protein kinase (MAPK) pathway via heterotrimeric G proteins. Our results show that Frizzled receptors can interact with $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ proteins, thus confirming that Frizzled functions as a G protein coupled receptor (GPCR). However, the activity level of Frizzled-mediated G protein signaling was much lower than that of a typical GPCR and, surprisingly, was highest when coupled to $G\alpha_s$. The Frizzled/$G\alpha_s$ interaction was further established in vivo as *Drosophila* expressing a loss-of-function $G\alpha_s$ allele rescued the photoreceptor differentiation phenotype of Frizzled mutant flies. Together, these data point to an important role for Frizzled as a nontraditional GPCR that preferentially couples to $G\alpha_s$ heterotrimeric G proteins.

1. Introduction

Frizzleds are seven transmembrane-spanning receptors for large secreted ligands called Wnts [1–3]. Wnt signaling is essential for many developmental processes, including neural crest induction, cell fate specification and tissue morphogenesis [4–8]. In the canonical Wnt-signaling pathway, Wnt binds to Frizzled and a lipoprotein receptor-related protein (LRP) co-receptor. This results in the stabilization and translocation of β-catenin to the nucleus, where it acts on the Tcf/Lef family of transcription factors [9–12]. Characterized by the features common to all G protein-coupled receptors (GPCRs) – seven transmembrane segments, an extracellular N-terminal domain, and a cytoplasmic C-terminal tail – Frizzleds have long been considered likely GPCRs [2,13]. Until recently, however, studies aimed at showing a direct interaction between Frizzled and G proteins have proven inconclusive.

Typically, ligand binding to a GPCR induces a conformational change that catalyzes guanosine diphosphate (GDP) release and guanosine triphosphate (GTP) capture by the α-subunits of heterotrimeric G proteins. Gα then dissociates from the Gβγ portion of the heterotrimer and both components activate effector proteins. Initial studies of potential G protein signaling interactions demonstrated that rat Frizzled 2 and XWnt5a RNA injection in zebrafish embryos caused calcium mobilization [14]. More recently, studies of mouse embryonic cells stably expressing Frizzled 2 also showed Wnt5a-stimulated calcium mobilization [15]. In both cases, Wnt-stimulated activity could be blocked by pertussis toxin, which ADP-ribosylates both $G\alpha_i$ and $G\alpha_o$ subunits and turns off G protein signaling. Additional studies have demonstrated that depletion of $G\alpha_o$ or $G\alpha_q$ in cultured mammalian cells inhibits Wnt-stimulated stabilization of β-catenin, and, conversely, that direct activation of G proteins by GTPγS leads to β-catenin stabilization in the absence of Wnt [16]. Genetic evidence for possible Frizzled-G protein signaling places *Drosophila* $G\alpha_o$ downstream of Frizzled and upstream of cytoplasmic proteins in the pathway, such as Disheveled and glycogen synthase kinase 3-β (GSK3β) [17]. Studies have also shown that Regulators of G Protein Signaling (RGS) proteins can modulate Wnt signaling and that effector molecules downstream of $G\alpha_s$ are involved in Wnt-regulated gene expression [6,18].

Recent studies have provided the first direct evidence that Frizzleds interact with heterotrimeric G proteins [19,20]. Isolated membranes from N13 cells showed G protein activation by GTPγS binding in response to Wnt5a stimulation; the response was abrogated in the presence of pertussis toxin [20]. Similar work by Koval and Katanaev suggests that GTP binding activity is pertussis toxin sensitive in cells expressing human Frz1, thus also implicating $G\alpha_{o/i}$ proteins [19]. However, since the experiments were performed using intact mouse fibroblasts it is unlikely that the GTP binding activity assayed can be exclusively attributed to heterotrimeric G proteins as small G proteins are present throughout the cell.

Abbreviations: Frz, frizzled; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor.

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Frizzled pharmacology studies are generally complicated by the insolubility of Wnts [21] and the potential interplay between the canonical β-catenin and G protein signaling pathways. Using *Saccharomyces cerevisiae* as a model system to study Frizzled receptor function has several advantages. Mammalian GPCR function has been studied extensively in yeast strains engineered to express chimeric mammalian Gα subunits [22–25]. Also, yeast do not encode proteins related to the canonical Wnt signaling pathway, thus providing a null environment in which to study mammalian receptors.

In this study, we asked whether Frizzled proteins, when expressed with chimeric G proteins, could activate transcription of yeast reporter genes downstream of the yeast MAPK pathway. We found that chimeric Frizzled receptors induced G protein signaling and that chimeric G proteins containing the last five amino acids of mammalian $G\alpha_s$ were preferentially activated over chimeric G proteins containing the last five amino acids of mammalian $G\alpha_i$ or $G\alpha_q$.

2. Materials and methods

2.1. Yeast strains

Yeast strains BY1173 ($G\alpha_i$), BY1172 ($G\alpha_q$), and BY1404 ($G\alpha_s$) have previously been described [23]. BY1173 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2::G418R trp1::GPA1/$G\alpha_{i3}$*. BY1172 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2::G418R trp1::GPA1/$G\alpha_q$*. BY1404 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2ΔG418R lys2Δ Δtrp::GPA1/$G\alpha_s$*. BY1173, BY1172, and BY1404 express a chimeric Gα subunit that is made up of amino acids 1–467 of the yeast Gα protein, Gpa1, followed by the last five amino acids of human $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$, respectively. In all three strains, presence of an activated GPCR leads to signaling through the yeast mitogen-activated protein kinase (MAPK) cascade and expression of the P_{FUS1}-*lacZ* reporter gene (Fig. 1).

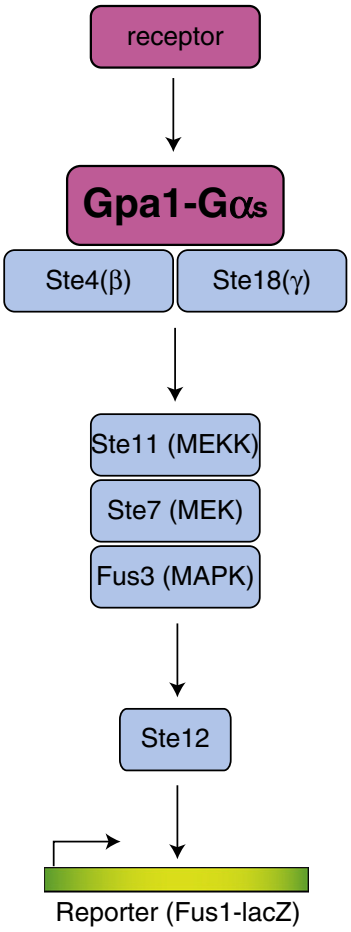

Fig. 1. Schematic outlining the MAPK signaling pathway in BY1404. The single endogenous GPCR, Ste2 is not expressed in the modified *S. cerevisiae* strain. A chimeric yeast Gpa1-mammalian $G\alpha_s$ transplant is expressed in place of endogenous Gpa1. Signaling through the endogenous MAPK pathway initiates transcriptional activation of the Fus1-lacZ reporter gene.

2.2. Construction of plasmids

All constructs were verified by sequencing at the Washington University Protein and Nucleic Acid Chemistry Laboratory.

2.2.1. XWnt8-rFrz1-3xHA (pBN 2245) and XWnt8-rFrz2-3xHA (pBN 2247)

Plasmids encoding *Xenopus* Wnt8 (XWnt8, GenBank Accession NM_001088168), rat Frizzled 1 (Frz1, NM_021266) and rat Frizzled 2 (Frz2, NM_172035) were generously provided by Randall Moon. ΔN-terminal Frz1 and ΔN-terminal Frz2 were amplified with oligos containing homology to a yeast ADE2 plasmid encoding XWnt8. PCR products were recombined with the linearized yeast vector in *S. cerevisiae*, generating plasmids that encode XWnt8-Δ222 Frz1 (pBN 2060) and XWnt8-Δ156 Frz2 (pBN 2062) fusion proteins. The 3xHA-URA3-3xHA sequence was amplified from pBN 687 with homology to the 3′ end of Frz1 or Frz2 and the 3′ UTR. Recombination of the PCR product and linearized pBN 2060 or pBN 2062 generated yeast plasmids encoding two selectable marker genes: ADE2 and URA3. In the presence of 5-fluoroorotic acid (5-FOA), the URA3 gene product becomes toxic to yeast. Thus, yeast subjected to homologous recombination were screened for resistance to 5-FOA, indicating loss of the URA3 gene by recombination and generation of Wnt8-Frz-3xHA fusions.

2.2.2. XWnt8-mFrz6-3xHA (pBN 2353) and XWnt8-mFrz7-3xHA (pBN 2352)

Plasmids encoding mouse Frizzled 6 (Frz6, GenBank Accession NM_008056) and mouse Frizzled 7 (Frz7, NM_008057) were generously provided by Dr. Fanxin Long. Oligos were designed to amplify the Frizzled coding sequence excluding all but approximately 100 bp of the predicted extracellular N-terminal domain. Homologous recombination in *S. cerevisiae* was used to recombine the purified PCR product and linearized pBN 2245, thus generating plasmids encoding XWnt8-Δ113 mFrz6-3xHA (pBN 2353) and XWnt8-Δ161 mFrz7-3xHA (pBN 2352).

2.2.3. A2B-3xHA in ADE2 (pBN 2348)

The human Adenosine 2B (A2B) receptor (GenBank Accession NM_000676) was subcloned into a yeast ADE2 vector (pBN 741) by homologous recombination. Oligos TJB 2425 and 115 were used to amplify the 3xHA tag from pBN 2245. Homologous recombination was utilized to insert the 3xHA in place of the A2B stop codon in linearized pBN 2320. The generated plasmid, pBN 2348, encodes the human A2B-3xHA fusion protein.

2.2.4. LRP6 in URA3 (pBN 2361)

A cDNA clone of mouse LRP6 (GenBank Accession BC060704) was obtained from ATCC. Full length mLRP6 was subcloned into a yeast URA3 vector (pBN 2362) with EcoRI (5′) and NotI (3′).

2.2.5. XWnt8-Frz1 in URA3 (pBN 2369)

In order to co-transform Frz1 and Frz2, the ADE2 selectable marker gene of pBN 2245 was replaced with URA3 by homologous recombination. Briefly, oligos were designed to amplify the URA3 gene from pBN 2265, generating a PCR product with homology to both the 5′