Activation of Rat Frizzled-1 Promotes Wnt Signaling and Differentiation of Mouse F9 Teratocarcinoma Cells via Pathways That Require Gαq and Go Function*

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The frizzled gene family of putative Wnt receptors encodes proteins that have a seven transmembrane-spanning motif characteristic of G-protein-linked receptors, although no loss-of-function studies have demonstrated a requirement for G-proteins for Wnt signaling by the gene product of frizzled-1. Medium conditioned by mouse F9 teratocarcinoma stem cells stably transfected to express either Xenopus Wnt-5a or Wnt-8 was used to test primitive endoderm formation of F9 stem cells. F9 stem cells expressing the rat Frizzled-1 receptor demonstrated endoderm formation in response to conditioned medium containing Wnt-8 but not to medium containing Wnt-5a. Primitive endoderm formation stimulated by Wnt-8 acting on the rat Frizzled-1 receptor was blocked by treatment with pertussis toxin by depletion of either Goq or Go, via antisense oligodeoxynucleotides, as well as by inhibitors of protein kinase C (bisindoylmaleimide) and of mitogen-activated protein kinase kinase (PD98059). Our results demonstrate the requirement for G-protein subunits Goq (a pertussis toxin substrate) and Go, for signaling by Frizzled-1, and an obligate role for the protein kinase C (likely mediated through stimulation of Goq) and mitogen-activated protein kinase network at the level of mitogen-activated protein kinase network.

Wnts are a class of vertebrate genes encoding secreted signaling proteins, which appear to modulate diverse processes in developing vertebrate embryos and in some adult tissues (1–4). The actions of Wnts are thought to be mediated by the function of members of the frizzled gene family of prospective heptihelical receptors (5–10). In the absence of a Wnt signal, active glycogen-synthase kinase 3 (zeste white 3/shaggy in Drosophila) phosphorylates β-catenin at an amino-terminal site (11), targeting it for ubiquitination and degradation through a proteasome pathway that also involves axin and the product of the adenomatous polyposis coli gene (12–21). Signaling by Wnt-1 via Frizzled homologues activates the function of Dishevelled, which represses the activity of glycogen-synthase kinase-3 (3, 22), promoting elevation of intracellular β-catenin levels and accumulation of β-catenin in nuclei (11, 23). Nuclear β-catenin interacts with members of the lymphoid-enhancer factor/T-cell factor (LEF/TCF) classes of architectural high mobility group (HMG) box transcription factors (24–27) to regulate expression of genes involved in vertebrate development (24, 28–32).

Some Wnts can work through a pathway distinct from the glycogen-synthase kinase 3-mediated one described above (2, 33–35), depending upon what receptors are present. Xwnt-5a, unlike Wnt-1 and Xwnt-8, does not induce a duplication of the axis in Xenopus when ectopically expressed, but instead causes morphogenetic defects (33, 34). Whereas Xwnt-1, -8, -8b, and -3a are functionally equivalent in axis induction assays, Xwnt-5a, -4, and -11 are functionally equivalent in this second, distinct Wnt signaling activity (36, 37). This classification of Wnt ligand resembles the classification by the McMahon laboratory (38), based on the ability of Wnts to transform mouse mammary epithelial cells (for review, see Ref. 2). Wnt7a regulates dorsoventral polarity in the chick limb in a manner distinct from the function of β-catenin (35), further suggesting that not all Wnts work through the β-catenin pathway.

Several lines of evidence suggest that one frizzled gene product (Frizzled-2) is a member of the superfamily of G-protein-linked receptors, including: a proposed heptihelical structural motif typical of G-protein-linked receptors (39); sensitivity to the inhibitory action of pertussis toxin, a cardinal property of G-protein-coupled receptors; signal; a cardinal property of G-protein-coupled receptors; and coupling to effectors often associated with G-protein mediation (40). Although Frizzled-1 members display the same tentative heptihelical motif, far less is known about how Frizzled-1 receptors signal. In this work, we express Xenopus Wnt-5a and -8 in mouse F9 teratocarcinoma stem cells to generate conditioned medium with which to supplement clones expressing the rat frizzled 1 (Rfz-1)1 receptor. Formation of primitive endoderm in response to Wnt stimulation is used as the read-out for activation of the Rfz-1 receptor. Xwnt-8, but not Xwnt-5a, induced formation of primitive endoderm in F9 stem cells, and this response can be blocked by treatment with pertussis toxin and by depletion of G-proteins Goq and Go.

EXPERIMENTAL PROCEDURES

F9 Cell Culture and Transfection Studies—Mouse F9 teratocarcinoma cells were obtained from the ATCC collection, propagated, and stably transfected using LipofectAMINE (Life Technologies, Inc.) and

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1 The abbreviations used are: Rfz-1, rat frizzled 1; ODN, oligodeoxynucleotide; tPA, tissue plasminogen activator; PE, primitive endoderm; GFP, green fluorescent protein; EV, empty vector; PCR, polymerase chain reaction; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase DOTAP, N,N,N′,N′-tetramethylammonium methylsulfate.
FIG. 1. Reverse transcription-PCR identification of mouse F9 teratocarcinoma clones stably expressing mRNA for Rfz-1, Xwnt-5a, or Xwnt-8. The RNA of F9 clones harboring either the empty expression vector (EV) or the vector expressing Rfz-1, Xwnt-5a, or Xwnt-8 were reverse-transcribed and amplified. The molecular markers (MK) indicate the relative size in base pairs (bp) of the amplified products. The size of the amplified products from reverse transcription-PCR of the target mRNAs are as follows: 270 bp base pair for Rfz-1; 410 base pairs for Xwnt-5a; and 504 base pairs for Xwnt-8. The results are shown from representative independent clones selected for maximal expression of the target mRNAs. Ten to twenty G418-resistant clones of each transfection were screened for high expression of the vector, Rfz-1, Xwnt-5a, of Xwnt-8 and two or three of the highest expresser clones propagated for use in these studies.

FIG. 2. Conditioned medium from cells expressing Xwnt-8, but not Xwnt-5a, stimulates differentiation of F9 cell expressing Rfz-1 to PE, as established by the expression of the PE marker tPA. Conditioned medium was collected from F9 clones stably transfected with Xwnt-5a, Xwnt-8, or the EV and used to supplement 1:9 (ratio of conditioned medium of target Rfz-1-expressing clones to that of the Wnt-expressing clones) the medium of the F9 cells stably expressing either Rfz-1 or the empty vector, shown in the left panel. Nil denotes the clones to which no conditioned medium was added. The ratio of conditioned medium of the target Rfz-1-expressing clones to that of the Wnt-expressing clones was varied from 9:1 to 1:9, and the PE formation followed by tPA activity, shown in the right panel. The tPA activity is calculated as described (52). The results are presented as the mean values ± S.E. of at least four separate experiments. *, denotes p < 0.05 for difference from the mean. **, denotes p < 0.01 for the difference from the mean.

RESULTS AND DISCUSSION

Selection of F9 Clones Expressing Either Xwnt-5a, Xwnt-8, or Rat Frizzled-1—F9 teratocarcinoma cells are proven as a cultured cell system amenable to depletion of specific G-proteins followed by analysis of differentiation phenotypes (41, 42, 46–48). We first ascertained whether or not the F9 cells were a suitable system for analysis of Wnt signaling by Frizzled homologues. F9 teratocarcinoma cells were stably transfected with rat Frizzled-1 (Rfz-1), Xenopus Wnt5a (Xwnt-5a), Xenopus Wnt8 (Xwnt-8), or empty vector (EV) and clones selected. In the absence of antibodies specific for these individual gene products, clones were selected based upon their relative level of expression of target mRNA, using reverse-transcription PCR as the read-out (Fig. 1). Reverse transcription and amplification by PCR reveals from a large number of individually tested clones, clones expressing the highest levels of Rfz-1, Xwnt-8, or Xwnt-5a. Several independent stably transfected clones displaying the highest levels of reverse-transcription PCR product as an index of expression of Xwnt-5a, Xwnt-8, or Rfz-1 receptor were selected and propagated for use in these studies.

Conditioned Medium Containing Xwnt-8 but Not Xwnt-5a Activates the Rat Frizzled-1 Receptor—Clones expressing either Rfz-1 or empty vector were incubated with medium conditioned by the stable transfecant F9 clones expressing either Xwnt-5a, Xwnt-8, or EV. Application of the Xwnt-8-containing medium to the Rfz-1-expressing cells induced differentiation of F9 cells to PE, as indicated by the secretion of the PE-specific marker tPA (Fig. 2). In contrast, challenge of the F9 clones transfected

specific marker antigen cytokeratin endo A by the monoclonal antibody TROMA-1 (University of Iowa Developmental Studies Hybrida Bank, Iowa City, IA). The TROMA-stained co-cultures were stained using a Texas Red-labeled, goat anti-mouse IgG second antibody to identify those cells that were stimulated to PE in response to co-culture with the Wnt-producing cells. The original intent was to stain for PE-marker with Texas Red-labeled secondary antibody and to contrast the Texas Red signal of PE formation with the autofluorescent signal from the GFP expressed by the Wnt-producing cells. The conditions required for permeabilization and TROMA staining were found to be incompatible, however, with the retention of the autofluorescent signal of the GFP, precluding simultaneous monitoring of the signals.

the pCDNA3 expression vector (Invitrogen) alone (empty vector), or pCDNA3 vector engineered by standard techniques to express Xwnt-5a (2), Xwnt-8 (2), or rat Frizzled-1 (6) under the control of the cytomegalovirus promoter (41, 42). The pCDNA3 vector harbors a copy of the neomycin resistance gene and clones were selected in medium containing the neomycin analogue G418 (Life Technologies, Inc., 0.4 mg/ml). Ten to twenty independent clones resistant to the G418 were propagated in the transfections for each construct. The level of expression of the mRNA for each of the target proteins was measured indirectly via reverse-transcription, polymerase chain reaction amplification. The clones displaying the highest level of expression of mRNA for Xwnt-5a, for Xwnt-8, or for Rfz-1 receptor were then used for the studies reported herein.

Antisense Oligodeoxynucleotides Treatment—The F9 clones expressing the Rfz-1 receptor were propagated on 96-well plates (~800 cells/well) and allowed to attach overnight. The clones were treated with phosphorothioate oligodeoxynucleotides (ODNs; cell culture-grade, HPLC-purified, from Operon Technologies, Inc.) antisense to specific G-protein subunits at least 48-h in advance of challenge with Xwnt-5a or Xwnt-8 (41, 43). Antisense ODNs (28-nucleotide) were complexed with the DOTAP liposomal carrier (5 µg oligomers complexed with 1 µl of DOTAP carrier) and used at final concentrations of ~1 µM. Antisense oligomers were designed against the 5’-untranslated regions of the G-protein subunits and include the ATG start codon.

Immunoblotting—Aliquots of crude membrane fractions (0.1 mg of protein/lane) from each F9 clone were subjected to SDS-polyacrylamide gel electrophoresis, the separated proteins transferred to nitrocellulose, and blots were stained with a rabbit polyclonal, anti-peptide antibodies to the indicated G-protein subunits (Signal Transduction Labs). The immune complexes were made visible by staining with a second antibody (goat anti-rabbit IgG) coupled to calf alkaline phosphatase (44).
with the empty vector alone with medium conditioned by cells expressing Xwnt-8 failed to provoke PE formation. Rfz-1-expressing cells formed PE in response to conditioned medium from cells transfected with Xwnt-8, but not from the cells expressing Xwnt-5a or transfected with the empty vector. Rfz-2 has been shown to respond to stimulation by Xwnt-5a, but not Xwnt-8, to induce a pertussis toxin-sensitive release of intracellular calcium (40). Rfz-1, in contrast, is stimulated by Xwnt-8 but not Xwnt-5a. For Rfz-1 receptor-expressing but not empty vector clones, the induction of PE by conditioned medium from clones secreting Xwnt-8 was dose-dependent (Fig. 2), increasing the amount of conditioned medium from the Xwnt-8 as compared with medium from EV clones increased the formation of PE. These data establish the F9 cells as a viable read-out for signaling via Rfz-1, measured by the ability of various Wnts to stimulate formation of PE.

A second read-out of the formation of primitive endoderm is the expression of the endoderm-specific marker antigen cytokeratin endo A detected by the monoclonal antibody TROMA. To test endo A expression (i.e., positive TROMA staining), a co-culture system was used (Fig. 3). Clones expressing a Xwnt in combination with GFP or expressing Rfz-1 receptor alone were seeded onto a Petri dish in which coverslips were present. After initial cell growth, the coverslips were removed from each plate and transferred to another plate so that the coverslip on which cells expressing Rfz-1 occupied a vacant space among the cells expressing either Xwnt-5a or Xwnt-8. Phase-contrast images reveal the edge of the coverslip and display the cells expressing the Xwnts (left side of panel), the edge of the coverslip, and the cells expressing the Rfz-1 (right side of panel). Epifluorescence images identify the clones on the left side as the cells expressing Xwnt and the autofluorescent GFP. The Rfz-1 cells do not express GFP and are not detected by epifluorescence microscopy. After 4 days of incubation, the coverslips for the cells expressing Xwnt-5a and Xwnt-8 are fixed, permeabilized, and stained for the PE-marker antigen using the TROMA monoclonal antibody and a secondary goat-anti mouse IgG coupled to Texas Red. The cells expressing Wnts stain negative for TROMA. The Rfz-1-expressing clones grown in close proximity to cells secreting Xwnt-8, but not Xwnt-5a, stained positive for TROMA antigen. The results displayed are representative of more than four separate trials of this design.

The coverslips on which F9 clones expressing Xwnt-5a alone, Xwnt-8 alone, or Rfz-1 receptor had been seeded were transferred to and then incubated for 4 days in a Petri dish in which clones expressing a specific Xwnt had been seeded. On the fourth day, the coverslips were examined by epifluorescence microscopy, and the Rfz-1-expressing cells then fixed, permeabilized, and stained with the PE-specific monoclonal antibody TROMA. The indirect immunofluorescence images reveal positive TROMA staining only for the F9 clones expressing Rfz-1 receptor that had been co-cultured with the clones secreting Xwnt-8 but not Xwnt-5a. The results from the TROMA staining (Fig. 3) agree well with the data obtained using the PE-marker tPA secretion as a read-out (Fig. 2). Both read-outs demonstrate that the F9 cells expressing the Rfz-1 receptor respond to...
stimulation by Xwnt-8, but not Xwnt-5a, by formation of primitive endoderm.

Pertussis Toxin Blocks Xwnt-8 Activation of Rat Frizzled-1 Receptor—We investigated whether inhibitors of G-protein signaling would block formation of PE in F9 cells in response to activating Rfz-1 signaling with Xwnts (Fig. 4). Pertussis toxin and inhibitors of 1-phosphatidylinositol 3-kinase, protein kinase C (PKC), and cyclic nucleotide phosphodiesterases were tested in F9 clones expressing Rfz-1 receptor before stimulation by either Xwnt-5a- or Xwnt-8-conditioned medium. Pertussis intoxication abolished the Xwnt-8-stimulated formation of PE (Fig. 4). Pertussis toxin has been shown to block the stimulation of calcium transients mediated by Rfz-2 receptor in response to Xwnt-5a, but not Xwnt-8, in Zebrafish embryos (40). The pertussis toxin sensitivity of the Rfz-1 receptor-mediated formation of PE in response to Xwnt-8 stimulation implicates heterotrimeric G-proteins of the $G_\alpha$, $G_\beta$ and/or $G_\delta$ family in responding to Frizzled-1. This G-protein requirement could reflect either a direct role for G-proteins in Frizzled signaling or an indirect role obligate later for the cellular response to Frizzled-1 activation. However, our data showing that some Frizzled homologues, but not Frizzled-1, F9 clones stably expressing the Rfz-1 receptor responded to the signaling. As there previously has not been any implication of pertussis toxin-sensitive G-proteins in the $\beta$-catenin pathway activated by Frizzled-1, precise determination of when pertussis toxin-sensitive G-proteins are required for PE formation will be important. Taken together with the heptihelical nature of the predicted structures of the frizzled gene products, pertussis toxin sensitivity of Rfz-1 receptor-mediated responses, if direct, does provide additional support that the frizzled-1 gene product is a member of the superfamily of G-protein linked receptor.

Effects of Inhibitors of 1-Phosphatidylinositol 3-Kinase, PKC, MEK, and Cyclic GMP Phosphodiesterase on Rat Frizzled-1 Signaling—Inhibition of 1-phosphatidylinositol 3-kinase activity with the LY294002 inhibitor (20 $\mu M$) or inhibition of cyclic nucleotide phosphodiesterases with 3-methylisobutylxanthine (0.5 mM) did not block the ability of Xwnt-8 to stimulate formation of PE by the F9 cells expressing the Rfz-1 receptor (Fig. 5). Inhibition of MEK signaling by the PD98059 inhibitor (4 $\mu M$), in sharp contrast, effectively blocked the ability of conditioned medium from the Xwnt-8-expressing cells from stimulating PE formation in the F9 cells expressing the Rfz-1 receptor. Depletion of ERK1,2, the substrates for MEK, blocks the ability of the morphogen retinoic acid from inducing PE formation in F9 stem cells (41), as does inhibition of MEK with the PD98059 compound (not shown). Treating Rfz-1-expressing F9 clones with bisindoylmaleimide (1 $\mu M$), a selective protein kinase C inhibitor, blocked Xwnt-8-induced formation of PE. It has been shown that either depletion of PKC by antisense oligodeoxynucleotides or inhibition of PKC with bisindoylmal- eimide blocks the ability of retinoic acid to promote PE formation by wild-type F9 stem cells (41, 42). These data implicate both activation of PKC and MEK (and ERK1,2 activation) in mediating PE formation to Rfz-1, as is true for their roles in retinoic acid-induced PE formation. The data cannot discriminate between a direct involvement of PKC and MEK activation in Frizzled signaling or an indirect role obligate later for the cellular response to Frizzled-1 activation. However, our data showing that some Frizzled homologues, but not Frizzled-1, lead to elevation of PKC activity suggests that in mediating PE formation PKC may be required for the cellular response to Frizzled-1, rather than directly induced in response to Frizzled-1 signaling.

Suppression of $G_\alpha_q$ and of $G_\alpha_o$ Abolishes Rat Frizzled-1 Signaling—To ascertain the role of specific subunits of the heterotrimeric G-proteins in mediating the cellular responses to Frizzled-1, F9 clones stably expressing the Rfz-1 receptor were treated with phosphorothioate ODNs antisense to specific G-protein subunits, as previously used to suppress expression of these subunits (41, 43, 47, 48, 50). Depletion of either $G_\alpha_q$ or $G_\alpha_o$ by antisense ODNs selectively blocked the ability of Xwnt-8 to promote PE formation in cells expressing Rfz-1 receptor (Fig. 6). Clones expressing Rfz-1 receptor and treated with ODNs antisense to $G_\alpha_q$, $G_\alpha_o$, $G_\alpha_{11}$, or $G_\alpha_{12}$, and $G_\alpha_{13}$, in sharp contrast, displayed normal PE formation in response to conditioned medium of Xwnt-8-expressing cells (Fig. 6). Treatment with ODNs antisense to $G_\beta_2$, $G_\delta_2$, and $G_\delta_3$ also failed to influence the ability of the clones expressing the Rfz-1 receptor to differentiate to PE in response to Xwnt-8. In all
cases where the antisense oligodeoxynucleotide blocks Xwnt-8-induced formation of PE in cells expressing Rfz-1, analysis of the protein reveals that the expression of the targeted G-protein subunit is reduced (Fig. 6B). G-protein subunit expression was suppressed >84% by ODN treatment, except for Gαq. Suppression of Gαo was >75% for both molecular species. Elevation of intracellular cyclic AMP (by cholera toxin treatment or by addition of 5 mM dibutyryl cyclic AMP), depression of intracellular cyclic AMP (with 50 μM 2, 5'-dideoxyadenosine), and inhibition of protein kinase A (with 1 mM KT5720) do not alter F9 stem cell differentiation to primitive endoderm (41, 42, 51). Taken together, these data suggest no role for cyclic AMP in the differentiation response to activation by Rfz-1 receptor.

Pertussis toxin effectively blocks Xwnt-8-stimulated formation of PE by the F9 teratocarcinoma cells expressing Rfz-1 receptor, implicating substrate G-protein α-subunits for toxin-catalyzed ADP-ribosylation and inactivation of the signals that they transduce. We extended analysis of G-protein involvement implicated by pertussis toxin action via specific depletion of G-protein subunits by antisense ODNs. The results of these studies demonstrate a requirement for both Gαo and Gαq in signaling from the activation of Rfz-1 receptor by Xwnt-8 to PE formation, suggesting that a network of G-protein signaling may be required for signaling to PE formation. Gαo is a substrate for pertussis toxin-catalyzed ADP ribosylation and inactivation, hence the results of the pertussis toxin and subunit depletion studies agree well. Although the effectors for Gαo remain unknown, phospholipase Cβ is a well known effector for Gαq. Activation of phospholipase Cβ generates inositol phosphates as well as diacylglycerol, an intracellular activator of PKC. The ability of Gαo depletion and of the bisindolylmaleimide PKC inhibitor to block PE formation of F9 clones expressing the Rfz-1 receptor in response to Xwnt-8 stimulation implicates Gαq, phospholipase Cβ, and ultimately PKC as obligate components in cellular responses to Rfz-1 in this system. The ability of specific inhibition of MEK by PD98059 to block signaling from Rfz-1 receptor to PE formation in this model is of note and mimics the action of ERK1.2 depletion on PE formation in F9 cells (41). The results of this work are the first indication of a requirement for these specific signaling molecules in Fz signaling.
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