Frizzled-9 Is Activated by Wnt-2 and Functions in Wnt/β-Catenin Signaling*

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Frizzled has been known to function as a Wnt receptor. Although there have been a number of mammalian Frizzled members identified, their binding specificities with Wnt and functions in mammalian cells have been poorly understood. Here, we demonstrate that rat Frizzled-9 (Rfz9) functions in Wnt/β-catenin signaling in 293T cells. Rfz9 overexpression induces the hyperphosphorylation and relocation of mouse Dishevelled-1 (Dvl-1) from the cytoplasm to the cell membrane and the accumulation of cytosolic β-catenin. Transfections of Rfz9 with each of several Wnts show that only Wnt-2 activates Rfz9 in T cell factor (TCF)-dependent transcription. Deletion mutant analysis determines that there is a difference in Rfz9 C-terminal residues required for the modifications of Dvl-1 and those required for the inductions of β-catenin stabilization and TCF transactivation. Deletion of the Wnt-binding domain does not abolish Rfz9 activity completely, although it causes the inactivation of Wnt-2-dependent TCF transcription. Rfz9 also relocates Axin from the cytoplasm to the plasma membrane in the presence of Dvl-1, suggesting that one of the consequences of Dvl-1 relocalization by Rfz9 is to bring Axin to the cell membrane.

Wnt proteins are a family of ligand molecules that play major roles in development, such as embryogenesis, cell polarity generation, and cell fate specification (1, 2). Studies on Drosophila have identified a number of genes that are involved in the Wnt signaling, including Frizzled as a Wnt receptor (3). Recent studies have characterized the molecular mechanisms of the signaling pathway that involves β-catenin. In the absence of the Wnt signaling, phosphorylation by glycogen synthase kinase 3β causes the cytoplasmic β-catenin to be ubiquitinated and degraded through a mechanism that requires adenomatous polyposis coli and Axin (4, 5). Wnt binds and activates the co-receptors Frizzled and the low density lipoprotein-related proteins 5 and 6 (LRP5/6)1 (6). Wnt-dependent activation of LRP5/6 recruits Axin to the membrane, where Axin is destabilized (7). Frizzled relocalizes Dishevelled from the cytoplasm to the cell membrane, and the membrane-associated Dishevelled is hyperphosphorylated (8, 9). Dishevelled binds to a protein complex containing Axin, glycogen synthase kinase 3β, and adenomatous polyposis coli, which leads to suppression of glycogen synthase kinase 3β activity, resulting in stabilization and accumulation of cytoplasmic β-catenin (10–15). In the nucleus, accumulated β-catenin binds to members of the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family (16, 17) and induces transcription of target genes (18–21).

Frizzled family proteins are seven transmembrane-spanning receptors that are activated by Wnt. The N-terminal extracellular cysteine-rich domain (CRD) has been identified as the Wnt-binding domain (22, 23). The C-terminal tail is the most variable part among Frizzled homologs (24). Using assays in Xenopus embryos, rat Frizzled-1 (Rfz1) has been shown to relocalize Xenopus Dishevelled (Xdsh) and induce expression of β-catenin-responsive genes, Xnr-3 and Six5/6 (25). Human Frizzled-5 (Hfz5) can be activated by Wnt-5a and induce the secondary axis formation (26). Studies in mammalian cells have revealed that human Frizzled-1 (Hfz1) is activated by several Wnt homologs in TCF reporter assays in 293T cells (27). Mouse Frizzled-8 (Mfz8) can also be activated by Wnt-1 in a similar assay (28). Direct interactions between Wnt and mammalian Frizzled were confirmed by the demonstrations that Drosophila Wingless (Wg) and Xenopus Wnt-8 (Xwnt-8) can bind to several mouse Frizzled members in 293 and COS cells (3, 22).

Despite the fact that a number of mammalian Wnt and Frizzled members have been identified, there has been little characterization of Wnt-Frizzled binding specificities. In fact, only a few Frizzled members have been shown to function in Wnt/β-catenin signaling in mammalian cells. In the present study, we characterize a recently identified Frizzled family member, Frizzled-9, in Wnt/β-catenin signaling. Frizzled-9 is highly expressed in the brain, and its gene is one of several genes that are deleted in a developmental disorder, Williams syndrome (29, 30). The gene deletion is thought to contribute to the neurological symptoms of Williams syndrome (29, 30). We demonstrate that rat Frizzled-9 (Rfz9) functions in Wnt/β-catenin signaling in mammalian cells and that Rfz9 can act as a receptor for Wnt-2. Importantly, there is a difference in the Rfz9 C-terminal residues required for the inductions of mouse Dishevelled-1 (Dvl-1) modifications and those required for β-catenin accumulation and TCF transactivation. Deletion of a large part of the CRD retains the Rfz9 activity, although it...
causes the inactivation of Wnt-2-dependent TCP transcription. Rfz9 also relocates Axin to the cell membrane in Dvl-1-dependent manner, and the Rfz9 mutants that can translocate Dvl-1 also relocate Axin, suggesting that one of the consequences of Dvl-1 relocalization by Rfz9 is to bring Axin to the cell membrane.

**EXPERIMENTAL PROCEDURES**

**Rfz9 Cloning**—Based on the DNA sequences of human and mouse Frizzled-9 (Hfz9 and Mfz9, respectively), PCR primers were designed to isolate a 800-bp fragment at the 3' end of the Rfz9 coding region. The DNA fragment generated by PCR was used as the probe to screen a rat cortex cDNA library cloned in the Lambda ZAP II vector (Stratagene). After three rounds of selection by hybridization, pBluescript phagemids were excised from the positive clones and sequenced.

**Plasmid Constructions**—Rfz9 wild-type, Rfz9-Myc, Rfz9ΔC5, ΔC15, ΔC26, and ΔC38 expression constructs were generated from the cloned full-length Rfz9 cDNA by PCR amplification and/or restriction digestion and subcloning into pCMV-Tag5B or pCMV-Tag5C (Stratagene). Rfz9ΔN and Rfz9ΔN-Myc were generated by the removal of a smaller fragment and self-ligation of a larger fragment from restriction digestion of the Rfz9 wild-type and Rfz9-Myc constructs, respectively. Green fluorescent protein (GFP)-tagged and V5-tagged Dvl-1 (Dvl-1-GFP and Dvl-1-V5) cDNAs were isolated by PCR amplification of Dvl-1 (a gift from D. J. Sussman) and subcloning into pEGFP-N2 (CLONTECH) and pcDNA3.1/V5/His (Invitrogen), respectively. To remove the His6 epitope from pcDNA3.1/V5/His in the Dvl-1-V5 construct, Dvl-1-V5 was amplified by PCR and subcloned into pEGFP-N2. GFP-tagged Axin (Axin-GFP) was generated by PCR amplification of amino acids 125–956 (form 1) of Axin (a gift from F. Costantini) and subcloned into pEGFP-N2.

**Cell Culture and Transfection**—293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% sodium pyruvate, and 100 units/ml penicillin/streptomycin. Cells were transfected using LipofectAMINE 2000 (Invitrogen) for immunofluorescence experiments and using GeneJammer transfection reagent (Stratagene) for the other transfection experiments. Except for immunofluorescence, all transfected cells were assayed 48 h after transfection.

**Immunofluorescence**—For Dvl-1-GFP localization, transfections were performed in 35-mm dish cultures with Dvl-1-GFP (1.0 μg) together with either an Rfz9 construct or vector control (2.2 μg). For Axin-GFP localization, transfections were performed with Axin-GFP (1.0 μg) together with either Rfz9 or vector (2.2 μg). For Axin-GFP localization in the presence of Dvl-1-V5, transfections were performed with Axin-GFP (0.4 μg), Dvl-1-V5 (0.5 μg), and an Rfz9 construct or vector (2.0 μg). 24 h after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS. After incubation with monoclonal anti-Myc (9E10) or anti-V5 antibody (Invitrogen), immunoreactivity was detected by Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Images were obtained using a confocal laser-scanning fluorescence microscope (Carl-Zeiss).

**Alkaline Phosphatase Treatment**—Cells in 6-well plates were transfected with 0.3 μg of Dvl-1-V5 and 0.7 μg of Rfz9 or vector for each well. The cells were washed twice with ice-cold PBS, scraped, and centrifuged in lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% SDS) containing protease inhibitors at 4 °C for 20 min to remove cell debris. Cell lysates were preclaried with 20 μl of protein A-agarose at 4 °C for 1 h. The sample was spun down to remove the pellet, and 1 μl of anti-V5 antibody and 20 μl of protein A-agarose were added and incubated at 4 °C overnight. The mixture was washed with 500 μl of lysis buffer twice, 90 μl of phosphatase buffer and 10 μl (10 units) of alkaline phosphatase (Boehringer Molecular Biochemicals) were added, and the reaction mixture was incubated at 37 °C for 2 h. The sample was washed with lysis buffer twice and resuspended in 30 μl of SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot.

**Cell Fractionation and Immunoblot**—Cell fractionation was performed as described (31). Briefly, cells transfected with 2 μg of DNA in 60-mm dishes, and 2 μg of DNA in 24-mm dishes were washed twice with PBS. The cell medium was scraped and disrupted by 10 strokes with a Dounce homogenizer in hypotonic buffer (10 mM β-glycerophosphate, pH 7.4, 1 mM MgCl2, 2 mM EGTA, 1 mM dithiothreitol) containing protease inhibitors (200 μl/dish). The homogenate was loaded onto 200 μl of 2 x sucrose in hypotonic buffer and centrifuged at 15,000 × g for 30 min to pellet intact cells and nuclei. The supernatant from above the sucrose cushion was centrifuged at 100,000 × g for 30 min, and the resultant supernatant was designated as the cytosolic fraction. The pellet was solubilized in hypotonic buffer containing 0.5% Triton X-100 and 0.1% sodium deoxycholate and was designated as the membrane fraction. For Dvl-1-V5, Rfz9-Myc, or Rfz9 mutant detection in immunoblot, transfected cells in a 6-well plate were scraped and centrifuged in lysis buffer containing 50 mM Tris, pH 7.4, 1.2% Nonidet P-40, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% SDS) containing protease inhibitors at 4 °C for 20 min to remove cell debris. Proteins concentrations were determined by the BCA assay (Pierce). Samples were electrophoresed in SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBS with 0.1% Tween 20 and probed with anti-Myc, anti-V5, anti-β-catenin (Transduction Laboratories), or anti-actin antibody (Sigma). Peroxidase-conjugated secondary antibodies against rabbit IgG and mouse IgG (Jackson ImmunoResearch Laboratories) were used and visualized by enhanced chemiluminescence.

**Luciferase Assay**—Transfections were performed in 6-well plate cultures with 0.3 μg of an Rfz9 construct or vector, 0.6 μg of a Wnt-β-HA (32) or vector, 0.05 μg of a reporter construct TOPFLASH or FOPFLASH (gifts from G. Watanabe) (33), and 0.05 μg of the β-galactosidase expression construct pCDNA3.1/LacZ (Invitrogen). After 48 h, proteins were extracted using the reporter lysis buffer of Luciferase Assay System (Promega), and a luminometer (Bio-Rad) was used to measure luciferase activity. β-galactosidase activity was measured to normalize transfection efficiency. All experiments were performed in triplicate unless indicated otherwise.

**RESULTS**

**Cloning of Rfz9**—We screened a rat cortex cDNA library to clone Rfz9, and four positive clones were identified. Overlapping regions of their sequences match completely, and one of them has a full-length cDNA. The sequence encodes a protein of 592 amino acids, which is identical to Mfz9 except for a threonine at position 490 that is substituted with an alanine. The corresponding position in Hfz9 is also alanine. The coding region is 96 and 87% identical at the DNA level to Mfz9 and Hfz9, respectively. As is the feature of other Frizzled family members, Rfz9 contains an extracellular cysteine-rich domain (CRD) at the N terminus, seven putative transmembrane domains, and a cytoplasmic tail at the C terminus.

**Rfz9 Functions in Wnt/β-Catenin Signaling**—Most Frizzled members that activate Wnt/β-catenin signaling relocalize Dishevelled from the cytoplasm to the cell membrane (34). Thus, we tested whether Rfz9 can also relocalize Dishevelled, using a GFP fusion of Dvl-1 (Dvl-1-GFP). In 293T cells, Dvl-1-GFP has a cytoplasmic speckled expression pattern (Fig. 1A, a–c), similar to that found in Xenopus embryos injected with Dshd (25, 35) or Drosophila Dishevelled (Ddsh) (8). When Dvl-1-GFP was coexpressed with Rfz9-Myc, all cells that express both proteins show plasma membrane localization of Dvl-1-GFP (Fig. 1A, d–f), as has been reported with Dshd co-injected with Rfz1 or Xenopus Dishevelled homologs (25, 35) or Ddsh co-injected with Drosophila Dishevelled homologs into Xenopus embryos (8). Rfz9-Myc localized primarily to the cell membrane, although some intracellular expression was seen, most likely reflecting retention in the endoplasmic reticulum.

Dishevelled relocalization by Frizzled correlates with the hyperphosphorylation of Dishevelled (35). When V5-tagged Dvl-1 (Dvl-1-V5) was expressed alone, two bands were detected by Western blot with similar band intensities (Fig. 1B, upper panel). The slower migrating (upper) band most likely reflects a mobility shift caused by phosphorylation as has been reported previously (36). With Rfz9 coexpression, the faster migrating (lower) band dephosphates, and a band that migrates even slower than the upper band appears with a dose-dependent manner of the transfected Rfz9 plasmid. Alkaline phosphatase treatment of Dvl-1-V5 shifted all Dvl-1-V5 to the lower band, confirming that the upper bands were indeed caused by phosphorylation (Fig. 1B, lower panel).

**Xenopus embryo studies** have shown that some Frizzled proteins can activate Wnt/β-catenin signaling without ectopic Wnt...
mulation in the cytosolic fraction was significantly higher than vector-transfected cells (Fig. 1C). The membrane β-catenin level was not affected by Rfz9 expression. To further confirm that Rfz9 activates Wnt/β-catenin signaling, luciferase assays were performed using a reporter construct, TOPFLASH, which contains multimeric TCF-binding sites upstream of c-fos promoter driving luciferase expression (33). Rfz9 increased TCF transcription activity ~2-fold as compared with vector-transfected cells (Fig. 1D).

Wnt-2 Can Activate Rfz9-dependent TCF Transcription—To identify possible physiological ligands for Rfz9, a number of Wnt homologs were tested for their ability to enhance Rfz9-dependent TCF transcription. When Rfz9 was coexpressed with each of the Wnt proteins, a 10-fold increase of TCF activity was observed in the cells cotransfected with Wnt-2 as compared with Rfz9 alone (Fig. 2A). This was a striking observation as all other Wnt, including Wnt-1, -3, -3a, -4, -5a, -5b, -7a, and -7b, tested with Rfz9 showed no significant increase of TCF activity, and Wnt-2 alone did not induce TCF activity higher than vector transfection (Fig. 2A and data not shown). The differences in luciferase activity could not be accounted for by differences in Wnt expression levels since all of these HA-tagged Wnt constructs expressed comparable levels of proteins as reported previously (data not shown, and Refs. 27 and 32). Luciferase activity was not significantly increased with TOPFLASH, the reporter plasmid with mutated TCF-binding sites, confirming that Wnt-2 and Rfz9 specifically activates TCF (Fig. 2B).

To confirm that there is a correlation between TCF activity and β-catenin accumulation induced by Wnt-2 and Rfz9, cytosolic β-catenin levels were analyzed in the cotransfected cells. As expected, they showed higher β-catenin levels than those that expressed Wnt-2 or Rfz9 alone (Fig. 2C). Although the synergy between Wnt-2 and Rfz9 is not very evident with the β-catenin levels, it is most likely due to the presence of the cells that were untransfected and those transfected with Wnt-2 or Rfz9 alone in the sample. In the TCF reporter assays, on the other hand, most cells transfected with the reporter gene were also transfected with Wnt-2 and Rfz9.

There is an apparent discrepancy between the β-catenin accumulation and TCF activity data for the cells transfected with Wnt-2 and Rfz9 separately (Fig. 2, A and C). Whereas Wnt-2 accumulated β-catenin more than Rfz9, the TCF assay results show that the cells transfected with Wnt-2 did not show any TCF activity, as opposed to Rfz9 with 2-fold increase when compared with vector control. Although it is not clear why Wnt-2 alone can accumulate β-catenin but cannot activate TCF activity, one possible explanation is that a major pool of cytosolic β-catenin is bound to β-catenin inhibitory proteins such as Dsh (39, 40), preventing β-catenin from activating TCF, and Rfz9 but not Wnt-2 alone releases this inhibition.

Most of the Rfz9 C-terminal Tail Is Dispensable for Dvl-1 Relocalization and Hyperphosphorylation—To identify Rfz9 domains that are responsible for Wnt/β-catenin signaling activation, a number of Rfz9 C-terminal deletion mutants (Rfz9ΔC) were generated (Fig. 3A). They were generated as Myc tagged fusion proteins so that the expression of the mutants could be confirmed. These constructs expressed comparable levels of proteins (Fig. 3B). The lower one of the two bands that were detected for Rfz9Δ26 may be a degradation product. In Rfz9ΔN-Myc, the band that appears at a similar size to the other mutants may possibly be endogenous c-Myc, which is also detected faintly in vector-transfected cells (Fig. 3B). All the Rfz9 mutants were localized to the cell membrane, although there was some endoplasmic reticulum expression detected (Fig. 4A, g–i, for Rfz9ΔC38 as the representation of the Rfz9ΔC mutants).

**Fig. 1.** Rfz9 functions in Wnt/β-catenin signaling. Dvl-1 is relocalized by Rfz9 (A). Dvl-1-GFP was cotransfected with vector (a–c) or with Rfz9-Myc (d–f). The Myc-tagged Rfz9 was detected with anti-Myc antibody in image d but not in image a. Dvl-1-GFP was directly detected by fluorescence (b and e). Merged image c is from a and b, and merged image f is from d and e. Dvl-1 is hyperphosphorylated by Rfz9 (B). Upper panel, Dvl-1-V5 (0.3 μg) was cotransfected with an indicated amount of Rfz9 and vector to make up a total of 1.0 μg of DNA. The proteins were detected with anti-V5 antibody. Lower panel, Dvl-1-V5 was cotransfected with Rfz9 or vector, and cells were immunoprecipitated with anti-V5 antibody followed by alkaline phosphatase treatment. The proteins were detected with anti-V5 antibody. Rfz9 induces the accumulation of cytosolic β-catenin (C). Cells transfected with Rfz9 or vector were fractionated. The proteins were detected with anti-β-catenin and anti-actin antibodies. Rfz9 activates TCF transcription (D). Cells were transfected with 0.3 μg of Rfz9 (right bar) or vector (left bar), 0.05 μg of TOPFLASH, 0.05 μg of pcDNA3.1/LacZ, and 0.6 μg of vector. The relative values represent the means from six independent experiments.
When Dvl-1-GFP was coexpressed with each of the Rfz9ΔC constructs, Dvl-1-GFP was relocalized to the plasma membrane, which is similar to the effect of the wild-type Rfz9 (Fig. 4A, g–i, for Rfz9ΔC38 as the representation). Deletion of 38 amino acids, which is more than half of the C-terminal tail, did not affect the ability of the receptor to relocalize Dvl-1-GFP, suggesting that most of the Rfz9 C-terminal tail is dispensable for Dvl-1 relocalization. In some cells, which showed apparent low levels of Rfz9 wild-type or mutant expression, Dvl-1-GFP tended to show the membrane localization, suggesting that Dvl-1 relocalization does not require high levels of Rfz9 expression (Fig. 4A, d–f). Deletion of more than 38 amino acids resulted in poor protein expression (data not shown), preventing evaluation of their effect on Dvl-1 localization.

The C-terminal tail deletions were also examined for their effect on Dvl-1 hyperphosphorylation. Coexpression of Dvl-1-V5 with each of the Rfz9ΔC mutants showed that they have the same effect on Dvl-1-V5 as the wild-type Rfz9 (Fig. 4B). Cotransfection of 0.3 μg of each Rfz9 mutant resulted in the disappearance of the lower band (Fig. 4B, upper panel), whereas 0.7 μg of a mutant transfection caused a third band to appear at the highest molecular weight (Fig. 4B, lower panel). These results suggest that all Rfz9ΔC mutants were equally effective at inducing Dvl-1-V5 phosphorylation as the wild-type Rfz9 and that most of the Rfz9 C-terminal tail is not required for Dvl-1 hyperphosphorylation.

Most of the Rfz9 C-terminal Tail Is Required for β-Catenin Stabilization and TCF Transactivation—The β-catenin stabi-
transfected cells (Fig. 5A). Cells were transfected with vector (a–c), Rfz9-Myc (d–f), Rfz9ΔC38 (g–i), or Rfz9ΔN (j–l) into the cells. Full-length Rfz9 and deletion mutants were detected with anti-Myc antibody (d, g, and j); no Myc-tagged protein was detected in vector-transfected cells (a). Dvl-1-GFP was detected directly by fluorescence (b, e, h, and k). Merged images c, f, i, and l are from images a and b, d and e, g and h, and j and k, respectively. Rfz9ΔC mutants can hyperphosphorylate Dvl-1 (B). Upper panel, Dvl-1-V5 (0.3 µg) was cotransfected with 0.3 µg of Rfz9 wild type, Rfz9ΔC (ΔC5, ΔC15, ΔC26, or ΔC38), or vector, and 0.4 µg of vector to make up a total of 1.0 µg of DNA. The proteins were detected with anti-V5 antibody. Lower panel, Dvl-1-V5 (0.3 µg) was cotransfected with 0.7 µg of Rfz9 wild type, Rfz9ΔC (ΔC5, ΔC15, ΔC26, or ΔC38), or vector, and the proteins were detected with anti-V5 antibody.

Axin Is Relocalized by Rfz9 in the Presence of Dvl-1—Axin has been known to be a negative regulator of Wnt/β-catenin signaling that becomes relocalized from the cytoplasm to cell membrane by LRPS overexpression. To determine whether Rfz9 is involved in this process, a GFP fusion of Axin (Axin-

in cytosolic β-catenin levels, only Rfz9ΔC5 could increase TCF activity (Fig. 5B). Taken together with the effects of the C-terminal tail deletions on Dvl-1 relocalization and hyperphosphorylation, these data suggest that there is a difference in the Rfz9 C-terminal amino acids that are required for the induction of the Dvl-1 modifications as compared with those required for β-catenin stabilization and TCF-dependent transcription.
GFP) was expressed in the cells together with and without Rfz9-Myc. Rfz9-Myc overexpression had no effect on Axin-GFP localization as it remained in the cytoplasm in a speckled pattern similar to Dvl-1-GFP (Fig. 7A). It has been reported that Dishevelled colocalizes with Axin in cells and that they directly interact with each other (11, 41). It was possible that Dvl-1 relocalization by Rfz9 would also change Axin localization. Thus, Dvl-1-V5 was coexpressed with Axin-GFP in the presence and absence of Rfz9-Myc. Dvl-1-V5 localization shows the cytoplasmic speckled pattern in the absence of Rfz9-Myc and the membrane-associated pattern in the presence of Rfz9-Myc as expected (Fig. 7B, a and d). Axin-GFP localization followed Dvl-1-V5 localization, and in the presence of Rfz9-Myc, it was associated with the cell membrane (Fig. 7B, a–f). Cotransfection of Axin-GFP and Dvl-1-V5 with each of the Rfz9ΔC mutants or Rfz9ΔN resulted in the same membrane localization pattern of Axin-GFP (Fig. 7B, g–l, for Rfz9ΔC38 as the representation of Rfz9ΔC mutants), suggesting that Axin relocalization is not dependent on most of the Rfz9 C-terminal tail or the Wnt-binding domain. These results also suggest that Axin relocalization is not sufficient to activate Wnt/β-catenin signaling as Rfz9ΔC38, ΔC26, and ΔC15 relocalized Axin-GFP in the presence of Dvl-1-V5, but they did not accumulate β-catenin nor activate TCF activity as compared with either wild type or Rfz9ΔC5 (Fig. 7B, g–i, for Rfz9ΔC38 as the representation, and Fig. 5, respectively).

**DISCUSSION**

Although there have been a number of studies on Wnt-Frizzled interactions and signaling, most of them have involved *Drosophila* genetics and *Xenopus* embryo studies. Even the
characterization of ligand binding and signaling properties of mammalian Frizzled proteins has been carried out mostly with Drosophila and Xenopus Wnt homologs, and many of these studies have been conducted in Drosophila cell lines or Xenopus embryos. Although these studies were helpful in characterizing common protein structures and signaling mechanisms, they could neither address specificities of mammalian Wnt-Frizzled interactions nor effects of Frizzled activation in mammalian cells. In addition to the previous reports on Hfz1 and Mfz8 (27, 28), the present study on Rfz9 sheds new light on these issues and uncovers a few novel aspects of the receptor function.

Previous studies on Hfz9 and Mfz9 have shown that Frizzled-9 is highly expressed in the brain (29, 30). Although these studies did not show that Frizzled-9 expression is brain-specific, we found that Rfz9 expression is highest in the brain.² It has also been shown that Frizzled-9 is expressed in neural precursor cells in the developing nervous system (42). Hfz9 was identified by searching genes deleted in patients with Williams syndrome, a developmental neurological disorder with deficits in visuospatial cognition (29). Although it is still an open question whether Hfz9 is responsible for some aspects of Williams syndrome pathology, its high expression in the brain makes it likely to be involved in normal brain development. Although Hfz9 has been shown to bind Drosophila Wg (29), it was not known whether Frizzled-9 functions in Wnt/β-catenin signaling. As a first step in characterizing Frizzled-9 functions, we determined that Rfz9 activates Wnt/β-catenin signaling and can be specifically activated by Wnt-2. Interestingly, a recent study suggested that the WNT2 gene is an autism susceptibility gene (43). The present study suggests that Wnt-2 is a possible physiological ligand for Frizzled-9, although whether the functional loss of either protein leads to developmental disorders in humans remains to be determined.

With the deletion mutant analysis of Rfz9, we showed that most of the C-terminal tail domain is not required for the induction of Dvl-1 hyperphosphorylation and relocalization. We also demonstrated that Rfz9 can relocalize Axin in the presence of Dvl-1. Although the Dishevelled relocalization may have other roles, it is likely that Dishevelled acts as a carrier protein that brings Axin to the plasma membrane, where Axin is destabilized by LRP5/6 (7). The Axin relocalization and destabilization might be required for inhibiting the negative regulatory function of Axin, which leads to β-catenin stabilization and accumulation.

The cell fractionation and reporter gene assays demonstrated that most of the Rfz9 C-terminal tail is necessary for the receptor-mediated accumulation of β-catenin and activation of TCF transcription. This finding is in contrast to a previous study on Xenopus Frizzled-3 (Xfz3) in which the deletion of most of the C-terminal tail did not compromise the expression of the β-catenin responsive gene, Stamos (38). The C-terminal conserved motif Lys-Thr-X-X-Trp was implicated in mediating Wnt/β-catenin signaling (38). Although this motif may be necessary for the Wnt/β-catenin signaling, whether or not it actually mediates the signaling remains to be clarified since this motif is conserved even among Frizzled members that have not been shown to function in Wnt/β-catenin signaling. Thus, the motif may be only required for maintaining proper receptor conformation. The apparent discrepancy between Rfz9 and Xfz3 in functional requirements for the presence of C-terminal tails could be explained by structural differences between Rfz9 and Xfz3. Importantly, whereas the study on Xfz3 did not find a difference in the requirements for the inductions of the Dishevelled modifications and those of β-catenin stabilization and TCF transactivation, the present study determined that there is such a difference in Rfz9. Additional studies are necessary to determine whether this difference is also found with other Frizzled members. Since Axin relocalization by Rfz9 and Dvl-1 is not dependent on most of Rfz9 C-terminal tail, it is likely that Axin relocalization is not sufficient to accumulate β-catenin. The Axin destabilization mediated by LRP5/6 may be required for the β-catenin accumulation, and it is possible that this process also requires most of the Rfz9 C-terminal tail. Although we did not observe any Axin destabilization by Rfz9 overexpression or coexpression of Rfz9 with Wnt-2 and/or Dvl-1, it could be because endogenous LRP5/6 activity was not high enough to induce detectable levels of the Axin destabilization.

In this study, we also discovered that, even in the absence of the Wnt-binding domain, Rfz9N not only relocalizes and hyperphosphorylates Dvl-1 but can also induce the accumulation of cytosolic β-catenin and activate TCF transcription. The fact that it cannot be further activated by Wnt-2 is explained by the lack of the Wnt-binding domain. Whether or not Dishevelled relocalization and hyperphosphorylation induced by Frizzled are dependent on Wnt activity remains unclear, although there have been some indications that Wnt activity is responsible for these Dishevelled modifications (9, 36, 44). It is possible that there is some endogenous Wnt that can bind and activate Rfz9 in these cells, and the deletion in Rfz9N leads to a conformation that is similar to activated wild-type Rfz9, thus inducing the same effect on Dvl-1. This active conformation could also be responsible for the cytosolic β-catenin accumulation and TCF transactivation by Rfz9N. Another possibility is that Rfz9 isomerizes between two different states, an inactive and an active conformation even in the absence of Wnt. Although the equilibrium between the inactive and active states lies toward the inactive state, overexpression of Rfz9 could cause the absolute amount of Rfz9 protein in the active conformation to increase to the point at which basal signaling can be detected. The deletion in Rfz9N could adopt a conformation that shifts the equilibrium toward the active state. The finding that Rfz9N acts as a weak constitutively active mutant could be explained by an inability to interact with LRP5/6 through Wnt, making the Axin destabilization mechanism inefficient. An important function of Wnt may be to bring Frizzled and LRP5/6 together so that β-catenin becomes sufficiently accumulated, thereby inducing TCF-dependent transcription required for the cellular activities.

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