Interaction of Frizzled Related Protein (FRP) with Wnt Ligands and the Frizzled Receptor Suggests Alternative Mechanisms for FRP Inhibition of Wnt Signaling*

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Frizzled related proteins (FRPs) comprise a family of secreted molecules that contain an N-terminal cysteine-rich domain (CRD) highly similar to the CRDs of the frizzled family of membrane-anchored Wnt receptors. FRPs have been shown to interact with Wnt proteins and antagonize Wnt signaling in a Xenopus developmental model. We demonstrated that FRP antagonizes the Wnt-induced increase in uncomplexed β-catenin in both transient cotransfection and stable transformation models, where Wnt-induced morphological alterations are inhibited as well. We showed further that FRP inhibits Wnt signaling in a paracrine mode using a T-cell factor luciferase reporter to measure Wnt function. Investigation of the mechanisms responsible for FRP inhibition revealed that FRP forms complexes with WNT-1 or WNT-2 through its CRD domain. Transfection analysis with FRPs containing different tags revealed that FRP itself forms complexes and that this ability is conferred by its CRD domain. Finally, we demonstrated by cotransfection that FRP forms complexes with a prototype frizzled. All of these findings are consistent with a model by which FRP inhibits Wnt signaling through interactions with Wnt and/or formation of nonfunctional complexes with the frizzled receptor.

The Wnt proteins comprise a highly conserved, multimember ligand family, which play important roles in patterning and cell fate determination (1, 2). A number of downstream components of Wnt signaling have been identified by a combination of genetic and biochemical approaches. Wnts act through the cytoplasmic protein Dishevelled to inhibit the activity of the serine-threonine kinase GSK3. GSK3 appears to bind through the transmembrane portion (17). More evidence implicating fz CRDs highly similar to the fz CRD but then diverge and lack any transmembrane domain, has recently been identified. The latter form can interact with TCF/LEF transcription factors and, after translocation to the nucleus, activate target genes (6).

There is evidence that activation of Wnt signaling can contribute to the neoplastic process (7, 8). Inappropriate expression of these ligands due to promoter insertion of the mouse mammary tumor virus (1) or targeted expression in transgenic mice causes mammary tumor formation (9). Moreover, in cell culture, several Wnt family members have been shown to induce altered morphology and increased saturation density of certain epithelial (10, 11) and fibroblast (12, 13) cell lines. Finally, genetic alterations affecting APC or β-catenin, associated with increased uncomplexed β-catenin levels, have been observed in human colon cancers (14), melanomas (15), and hepatocellular carcinomas (16), indicating that aberrations of Wnt signaling pathways are critical to the development of these and possibly other human cancers.

Recent studies have identified the products of the multimer frizzled family as Wnt receptors (17). These proteins are characterized by a large cysteine-rich extracellular domain (CRD), a seven-transmembrane spanning domain, and a cytoplasmic tail. Exogenous expression of Drosophila frizzled 2 (Dfz2) in a suitable recipient insect cell line conferred the Drosophila Wnt phenotype, Wingless (Wg), the ability to bind to the cell surface and to signal by increasing intracellular levels of β-catenin. Furthermore, transfection of Dfz2 or several different mammalian fz cDNAs into human 293T cells conferred the ability to bind Wg. The fz CRD has been shown to be its Wnt binding domain by the ability of Wg to bind to a glycosphatidylinositol-anchored Dfz CRD in the absence of the transmembrane portion (17). More evidence implicating fz as the Wnt receptor has come from experiments performed in the Xenopus embryo system in which Wnt induced axis duplication when cojected with members of the fz family (18).

A family of related molecules, which contain N-terminal CRDs highly similar to the fz CRD but then diverge and lack any transmembrane domain, has recently been identified. When coexpressed with Wnt family members in Xenopus embryos, such proteins including human frizzled related protein (FRP) and Frzb-1 were shown to antagonize Wnt-induced duplication of the dorsal axis (19–21), indicating that these proteins may function as inhibitors of Wnt action during development. Frzb-1 has been further shown to coimmunoprecipitate with Wnt proteins in vitro and after cotransfection of COS7 cells (20). Moreover soluble Frzb-1 was shown to bind to 293 cells expressing a WNT-1 transmembrane chimera (19). In addition, mouse members of this family expressed as glycosphatidylinositol-anchored fusion proteins conferred to Wg the ability to bind 293 cells (22). More recently Lin et al. (23) have provided evidence that the bovine Frzb-1 can prevent
WNT-1-induced cytosolic accumulation of β-catenin in 293 cells and have localized sites within the CRD, which are critical for WNT-1 binding and inhibition of WNT-1-mediated axis duplication in Xenopus embryos. The present studies were undertaken to investigate the mechanisms by which FRP antagonizes Wnt function. The results have implications with respect to how FRP modulates the complex functions of the Wnt multimember family of developmentally regulated signaling molecules.

MATERIALS AND METHODS

Plasmid Construction—pcEV/WNT-1-HFc and pcEV/WNT-2-HFc, in which the IgG-HFc open reading frame (24) was fused in frame downstream of the coding region, were previously described (12). The pcDNA/WNT-2-HFc was constructed by inserting WNT-2-HFc into pcDNA3 (Invitrogen). pBabe/WNT-2-HFc containing the puromycin resistance gene was constructed by inserting the WNT-2-HFc fragment into pBabe, under the control of the cytomegalovirus transcriptional unit. pLNCX/Wnt-1-FA was kindly provided by Dr. J. Kitaigewski (Columbia University, New York). In pMTRTFRP, the FRP coding region was introduced into MMTeo (24). To construct pcDNA/FRP-HA or pcDNA/FRP-FLAG, the FRP coding region lacking its stop codon (21) was PCR-amplified using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) as described previously (12), in the presence of a forward BamHI-flanked primer, GGAAGATCCGCGGCGCAT-GGCATCGGGCGCA, that included a Kozak consensus sequence and a reverse EcoRI-flanked primer, GCAGGGATCTTTAACAACGCAGCT-GAAAGTGGG. The FRP PCR product was inserted in frame into an EcoRI-flanked HA (YPYDVPDYA) or M2 (DYKDDDDK) (Eastman Kodak Co.) epitope-encoding sequence. To construct pcDNA/CRD-HFc-FLAG, the N-terminal fragment of FRP was PCR-amplified using the above mentioned forward BamHI-flanked primer and a reverse EcoRI-flanked primer, GGAAGATCCGCGGCGCAT-GGCATCGGGCGCA, and the PCR product was introduced in frame upstream of the HA- or M2-encoding sequence, as detailed above. To construct pcDNA/CRD-H96-FLAG, the N-termi nal fragment of FRP was PCR-amplified using the above mentioned forward HindIII-flanked primer, GGAAGATCCGCGGCGCAT-GGCATCGGGCGCA, and the PCR product was integrated in frame upstream of the HA- or M2-encoding sequence as described previously (12). Two TCF/Luc reporter constructs, pGL3-OT containing TCF-responsive domains and pGL3-OF containing mutant-binding sites, were kindly provided by B. Vogelstein (Johns Hopkins Oncology Center, Baltimore). A schematic diagram of the constructs generated for this study is shown in Fig. 1.

Subconfluent NIH3T3 cells were plated at 1.5 × 10^5 cells per 100-mm dish. After 24 h cells were transfected with 1 μg of each plasmid DNA, by the calcium phosphate coprecipitation method (25) as described (26). Cultures were incubated with the DNA precipitates for 24 h and then washed in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (Life Technologies, Inc.). To ensure selection of cells coexpressing WNT-2 and FRP, different selectable markers were utilized in each construct. Cotransfectants were doubly selected by addition of 750 μg/ml genetin (Life Technologies, Inc.) and 2 μg/ml puromycin (Calbiochem) to the medium. Cells expressing WNT-2 or FRP individually were cotransfected with control vectors containing the other selectable marker.

Transient Transfection—For transient transfection 70–80% confluent cultures of 293T cells were transfected by the calcium phosphate method using 3 μg of each plasmid DNA. When necessary the amounts of DNAs used for cotransfection were adjusted to yield similar levels of protein expression. Cells were exposed for 5–6 h to the DNA precipitates and then washed in growth medium. After 48 h, cells were harvested and cell lysates prepared for analysis as indicated below.

GST-E-cadherin Binding Assay—GST-E-cadherin was expressed in bacteria and purified from the bacterial lysates by binding to glutathione-Sepharose beads as described previously (12). Cells were lysed in immunoprecipitation buffer (10 mM sodium phosphate, pH 7, 0.15 μM NaCl, 1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride), and cell extracts were clarified by centrifugation. In order to perform a quantitative analysis, two different amounts of total protein (0.1 and 1 mg) were analyzed for each sample by incubation with Sepharose beads (Amerisham Pharmacia Biotech) bound to the GST-E-cadherin. After 1 h of incubation with rotation at 4 °C, the beads were collected for centrifugation, washed, and dissolved in Laemmli buffer. Samples were subjected to SDS-PAGE followed by immunoblotting with 0.5 μg/ml anti-β-catenin antibody (Transduction Laboratories). Detection was performed using an [35S]labeled protein A (Amersham Pharmacia Biotech).

Paracrine Assay for Wnt Signaling—The paracrine assay was performed as described. Briefly, 293T cells transiently transfected with 1 μg of pLNCX/Wnt-1-FA or with an empty vector were cocultured with 293T cells transiently transfected with 1 μg of pcDNA/FRP-HA, pGL3-OT, or pGL3-OF and 0.1 μg of β-galactosidase expression vector. After 48 h, β-galactosidase units (RLU) were measured using the Promega luciferase assay system according to the manufacturer’s protocol. The activities were normalized for transfection efficiency using β-galactosidase activity. TCF/LEF-dependent luciferase activity was calculated by subtracting RLU levels obtained with the pGL3-OF reporter from those obtained by pGL3-OT (27).

Western Blot and Coimmunoprecipitation—Cultures were solubilized in lysing buffer (0.01 M phosphate buffer, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% NaCl, 5 μg/ELISA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride). Around 100 μg of total cell lysates were resolved by SDS-PAGE, and proteins were transferred to Immobilon-P membranes. After blocking with 5% bovine serum albumin in PBS, filters were incubated with the specific primary antibody (2.6 μg/ml horseradish peroxidase-conjugated rabbit anti-mouse HFc, Dako; 10 μg/ml anti-FLAG M2, Kodak; 15 μg/ml anti-HA-antibody obtained from the Hybridoma Center, Mount Sinai School of Medicine, New York). When the anti-FLAG or the anti-HA primary antibodies were used, membranes were washed in PBS/Tween 20 and incubated for 1 h with a secondary horseradish peroxidase-conjugated rabbit anti-mouse antibody. After washing 6 times in PBS/Tween 20, membranes were subjected to ECL analysis (Amersham Pharmacia Biotech). For coimmunoprecipitation analysis, cell lysates were solubilized in lysing buffer (see above), and proteins were centrifuged at 10,000 × g for 20 min at 4 °C. Around 1.5–3 mg of total cell lysates were incubated with the specific antibody (24 μg of goat anti-mouse HFc, Pierce; 10 μg of anti-FLAG M2, 15 μg of anti-HA) for 1 h on ice and then incubated with rotation with protein A beads (Pierce) for 1 h at 4 °C. The beads were collected by centrifugation, washed three times in immunoprecipitation buffer, dissolved in Laemmli buffer, and analyzed by SDS-PAGE, followed by detection with each specific antibody. The extracellular matrix fraction was prepared as follows. Cultures were washed twice with PBS containing 1 mg/ml aminocaproic acid and 1 mM phenylmethylsulfonyl fluoride and with PBS containing 0.5 mM EDTA. The supernatant containing 15–18 μg protein was centrifuged at 15 000 × g at 37 °C. Cells were removed, and the plates were washed 2–3 times with PBS solution and twice with distilled H2O. The extracellular matrix was then solubilized in 200 μl of Laemmli buffer.

Metabolic Labeling—Subconfluent 293T cultures were transiently transfected with 3 μg of each plasmid DNA. Forty hours later, cells were washed with PBS and incubated for 30 min in methionine- and methionine- and cysteine-free DMEM in the absence of serum. Cells were then labeled for 3 h with 3.5 μl of the same medium containing 200 μCi of [35S]methionine (–1,175 Ci/mmol, NEN Life Science Products) or 200 μCi of [35S]methionine and 200 μCi of [35S]cysteine (–1,075 Ci/mmol, NEN Life Science Products). Labeled cells were rinsed with cold PBS, lysed in 0.5 ml of lysing buffer, and clarified by centrifugation. Cell extracts were incubated with 24 μg of goat anti-mouse HFc (Pierce), 15 μg of anti-HA, 10 μg of anti-FLAG, or 10 μg of anti-FLAG. Immunoprecipitates were recovered with protein A (Pierce) beads, dissolved in Laemmli buffer, and analyzed by SDS-PAGE. After electrophoresis, the gels were treated with Enhance (DuPont), dried, and autoradiographed.

RESULTS

FRP Inhibits Wnt Function in Both Autocrine and Paracrine Modes—Studies to date showing that FRPs antagonize Wnt action have generally utilized in vivo models, with the exception of one recent report in which cotransfection of 293 cells with Frzb-1 and WNT-1 resulted in the inhibition of Wnt-induced accumulation of cytosolic β-catenin (23). To confirm and extend these findings with respect to FRP, we cotransfected 293T cells with WNT-1 and FRP. This transient expression system has the advantage of inducing high levels of pro-
tein expression. However, we observed that cotransfection led to variable levels of expression of the exogenous proteins. To overcome this difficulty, WNT-1-HFc (12) was cotransfected with the vector control or HA-tagged FRP (Fig. 1) at different DNA ratios in order to ensure that similar WNT-1 protein levels could be obtained in the presence or absence of FRP expression.

Under conditions in which 293T cells transfected with WNT-1, in the presence or absence of FRP, expressed similar WNT-1 levels (Fig. 2A), FRP was also specifically detected as a 36-kDa species in WNT-1/FRP-cotransfected cells (Fig. 2B). Total and uncomplexed β-catenin levels (12) were quantitated in the same cells using a GST-E-cadherin binding assay as described under “Materials and Methods.” Although total β-catenin levels were not appreciably altered (data not shown), Fig. 2C shows that WNT-1 induced an increase of around 10-fold in the amount of uncomplexed β-catenin over that of the vector control. Of note, expression of FRP dramatically inhibited this increase (Fig. 2C). Similar results were obtained with WNT-2 (data not shown). These findings established, under carefully controlled conditions, that FRP acts to inhibit Wnt signaling functions responsible for increased free β-catenin levels.

We next investigated the ability of FRP to inhibit Wnt functions in a stable transformation model. To do so, WNT-2 and FRP genes were cotransfected using different selectable markers into NIH3T3 cells, in which both WNT-1 and -2 induce morphologic alterations that include increased saturation density as well as higher steady state levels of uncomplexed β-catenin (12). Whereas WNT-2 transfectants exhibited abnormal growth and achieved higher cell density, double marker selected cultures expressing both FRP and WNT-2 showed little if any morphological differences from vector control or FRP transfectants (Fig. 3A). Similar results were obtained in multiple experiments. Moreover, inhibition of the transformed phenotype by FRP was correlated with inhibition of Wnt function as indicated by the substantially lower levels of WNT-2-induced free β-catenin in the stable cotransfectants (Fig. 3B).

Both Wnt and FRP proteins are processed through the secretory pathway but remain associated with cells that have been analyzed to date (21, 28, 29). The sites of interactions and mechanisms by which FRP exerts its inhibitory functions on Wnt signaling remain to be elucidated. To address this question, we first analyzed the localization of FRP in transfected 293T cells. As shown in Fig. 4, FRP was found to be present in cell lysates and the extracellular matrix but was not detected in the medium using conditions known to optimize FRP release into the medium of cultured fibroblasts (21). When the results were normalized for the amount of cell lysate, culture fluid, or extracellular matrix in the same Petri dish, FRP was found to be predominantly cell-associated (90%) with the remainder present in the extracellular matrix (Fig. 4). These results indicate that FRP remains tightly cell-associated when expressed by 293T cells.

Experiments were next performed to analyze the ability of FRP to inhibit Wnt actions in a paracrine mode. To do so we utilized a recently developed transient assay involving a luciferase reporter for TCF transcriptional activation (27) as an indirect marker of Wnt-1 function. 293T cells have previously
been shown to respond to autocrine Wnt-1 by stimulating TCF-dependent transcription (13). We established a cocultivation assay in which cells transiently transfected with Wnt-1 are able to stimulate the activation of the TCF reporter in target cells in a paracrine mode. 293T cells were transfected with Wnt-1 or vector control and then cocultivated with 293T cells, which were cotransfected with the TCF reporter and either the vector control or FRP. As shown in Fig. 5, TCF reporter activity was increased over 20-fold in 293T cells in response to cocultivation with Wnt-1-expressing cells. However, the responsiveness of FRP expressing 293T cells to paracrine-acting Wnt-1 was not significantly different from those expressing vector alone. These results indicate that Wnt-1 function can be inhibited by FRP in a paracrine mode.

FRP Interacts with Wnts—Previous studies have shown that Frzb-1 coimmunoprecipitates several members of the Wnt family (20, 23). To confirm and extend these findings with respect to FRP, we performed immunoprecipitation experiments with lysates of 293T cells cotransfected with WNT-1-HFc or WNT-2-HFc in combination with either FLAG-tagged FRP or FLAG-tagged C-terminally truncated FRP encoding only its CRD (Fig. 1). Antiserum against the HFc tag was used for immunoprecipitation followed by immunoblotting with anti-FLAG to detect FRP or CRD-FRP. As shown in Fig. 6A, FRP and CRD-FRP could be immunoprecipitated in complexes with either Wnt-1 or -2. The specificity of these interactions was demonstrated by the fact that anti-HFc failed to immunoprecipitate FRP or CRD-FRP in the absence of Wnt-1 coexpression and by the fact that Wnt with a different tag, HA, was similarly able to immunoprecipitate FRP-FLAG (data not shown).

To investigate further the nature of Wnt-FRP interactions as well as to examine the stoichiometry of the complex, we performed biosynthetic experiments. Thus, 293T cells were transfected with WNT-2-HFc in the presence or absence of FRP. Around 40 h later cultures were labeled for 3 h with 35S-methionine followed by immunoprecipitation analysis of cell lysates with anti-HFc. Fig. 6B shows that anti-HFc specifically immunoprecipitated the radiolabeled 66-kDa WNT-2-HFc from lysates of WNT-2-transfected 293T cells. In contrast, both
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WNT-2-HFc and a 36-kDa species corresponding to FRP were coimmunoprecipitated from cotransfected cultures. No other major bands were observed, arguing against the possibility that other protein(s) participated in FRP-WNT-2 binding interactions. Of note, the intensity of the band corresponding to FRP was significantly greater than the signal associated with WNT-2-HFc in these complexes (Fig. 6B). Similar results were obtained in several independent experiments. The ratio between the intensity of the FRP and the WNT bands was around 1.7 as calculated by densitometry. As a similar number of methionines are present in both proteins, these results raised the possibility that Wnt-2 might be present in a complex containing more than one FRP molecule.

In order to establish the specificity of the Wnt-FRP interactions, we performed biosynthetic experiments using PDGFB as a control for binding interactions. PDGFB, like Wnt, contains multiple cysteine residues and is also processed through the secretory pathway (30, 31). FRP-HA and WNT-2-HA were cotransfected with PDGFB, and cells were labeled with [35S]methionine and [35S]cysteine. Cell lysates were subjected to immunoprecipitation with either anti-HFc, followed by immunoblotting with anti-FLAG to detect FRP and CRD-FRP (Fig. 6B). Analysis of FRP-WNT-2 interactions in metabolically labeled cells. 293T cells were transfected with either vector control HFc-tagged pcDNA/WNT-2 alone or in the presence of pcDNA/FRP. After labeling, cell lysates were immunoprecipitated with anti-HFc, followed by SDS-PAGE analysis. PDGF does not form complexes with Wnt or FRP. Lysates from labeled 293T cells transfected with vector, pcDNA/PDGFB, or pcDNA/PDGFB cotransfected with HA-tagged WNT-2 or FRP were subjected to immunoprecipitation with either anti-PDGFB or anti-HA and analyzed by SDS-PAGE.

Analysis of FRP Structure—We took advantage of the availability of both HA- and FLAG-tagged FRP and CRD-FRP constructs (Fig. 1) to investigate FRP structure. Thus, 293T cells were transfected individually or cotransfected with the FLAG- and HA-tagged versions of FRP or CRD-FRP, respectively. Fig. 7A shows the analysis of transfected cell lysates by immunoblotting with anti-FLAG (top) or following immunoprecipitation with the same antibody (bottom). The results revealed that each of the FLAG-tagged proteins was expressed well and was immunoprecipitable by anti-FLAG. Fig. 7B further shows that anti-HA detected FRP-HA and CRD-FRP-HA in cells transfected with these expression vectors (Fig. 7B, top). Of note, FRP-HA was specifically immunoprecipitated by anti-FLAG from cells coexpressing FRP-FLAG. Similarly, CRD-FRP-HA was specifically immunoprecipitated from lysates of cells cotransfected with CRD-FRP-FLAG (Fig. 7B, bottom). Furthermore, antibody specificity controls indicated that FRP-HA and CRD-FRP-HA were immunoprecipitated by anti-FLAG only in the presence of their FLAG-tagged versions. These findings indicate that FRP, itself, can form complexes and that its CRD is sufficient to mediate these interactions.

We next investigated whether FRP was capable of forming such complexes following secretion. Because FRP remains tightly cell-associated (Fig. 4), experiments were performed by independently transfecting HA or FLAG-tagged FRPs in 293T.
cells, followed by cocultivation of the transfectants for 48 h. Immunoprecipitation of cell lysates revealed FRP-HA-FRP-FLAG complexes indicating that these interactions can occur following secretion from cells (Fig. 8).

**FRP Forms Complexes with the Hfz6 Receptor**—Evidence that the CRD of FRP has a predicted structure similar to that of fz (21) suggested the possibility that fz itself might interact with FRP. We have cloned several members of the fz family including Hfz6 from a human cDNA library. For the present studies, we generated an expression vector encoding a FLAG-tagged C-terminal truncated version of Hfz6 containing its CRD region but lacking the entire transmembrane and cytoplasmic domains (Fig. 1). This construct, designated CRD-Hfz6-FLAG, was cotransfected into 293T cells with either vector control, FRP-HA, or CRD-FRP-HA. Immunoblotting analysis with anti-FLAG revealed expression (Fig. 9A, top) and specific immunoprecipitation (Fig. 9A, bottom) of FLAG-tagged CRD-Hfz6. The expression of HA-tagged FRP and CRD-FRP in transfectants can be seen in Fig. 9B (top). When anti-HA immunoblotting analysis of anti-FLAG immunoprecipitates was performed, the results showed the specific interaction of CRD-Hfz6 with both FRP and CRD-FRP (Fig. 9B, bottom). Immunoprecipitation experiments performed following cocultivation of cells independently transfected with CRD-Hfz6 or FRP confirmed these interactions (data not shown). These findings establish the ability of FRP to form complexes with a prototype fz and provide evidence that these interactions are mediated by their homologous CRD regions.

To assess the stoichiometry of the FRP-CRD-Hfz6 complexes, we performed biosynthetic experiments. 293T transfectants expressing FRP-HA and CRD-Hfz6-FLAG individually or together were labeled with [35S]methionine and [35S]cysteine. Cell lysates were then immunoprecipitated with anti-HA or anti-FLAG. As shown in Fig. 10, each protein was readily detected in singly transfected cultures, with FRP expressed at higher levels than CRD-Hfz6. When cotransfectants were subjected to coimmunoprecipitation with anti-HA or anti-FLAG, FRP-CRD-Hfz6 complexes were detectable in both cases. When the lysates were immunoprecipitated with anti-HA, the band corresponding to FRP was stronger than that corresponding to CRD-Hfz6, consistent with the higher level of FRP expression. However, when anti-FLAG was used to immunoprecipitate CRD-Hfz6, present in limiting amounts, the coprecipitated FRP band showed a very similar intensity. The ratio between the intensity of the signal for FRP and the CRD-Hfz6 band was found to be 1.0 when analyzed by densitometry. Since the

**Fig. 7.** FRP complex formation in transfected 293T cells. A, following transfection or cotransfection with the HA and FLAG-tagged versions of pcDNA/FRP and pcDNA/CRD-FRP, cell lysates were analyzed by immunoblotting with anti-FLAG directly (top) and after immunoprecipitation (IP) with the same antibody (bottom). B, the same cell lysates were immunoblotted with anti-HA directly (top) or after immunoprecipitation with anti-FLAG (bottom).

**Fig. 8.** FRP complex formation in autocrine and paracrine modes. In the autocrine assay, 293T cells were cotransfected with either the HA- and FLAG-tagged versions of pcDNA/FRP or with the FLAG-tagged pcDNA/FRP and the vector. In the paracrine assay, the pcDNA/FRP-FLAG transfectants were cocultivated with the 293T cells transfected with either the vector or the pcDNA-FRP-HA. Cell lysates were analyzed by immunoblotting with anti-FLAG directly (top) or after immunoprecipitation (IP) with anti-HA antibody (bottom).

**Fig. 9.** FRP interaction with Hfz6. 293T cells were transfected with pcDNA/CRD-Hfz6 FLAG-tagged, pcDNA/FRP-HA, or pcDNA/CRD-FRP-HA or cotransfected with CRD-Hfz6-FLAG together with FRP-HA or CRD-FRP-HA. A, transfected cell lysates were then subjected to anti-FLAG immunoblotting directly (top) or following immunoprecipitation (IP) with the same antibody. B, the same cell lysates were immunoblotted with anti-HA directly (top) or after immunoprecipitation with anti-FLAG (bottom).
number of cysteines and methionines in the two molecules is similar, these observations support the heterodimeric interaction between the two proteins. No other species was consistently detectable in both anti-HA and anti-FLAG coimmunoprecipitations. However, we cannot rigorously exclude the presence of other molecules in the complex. To confirm the specificity of the FRP-CRD-Hfz6 interactions, cotransfection of the FRP-HA, FRP, and Hfz6, which are processed through the same secretory pathway, could form illegitimate complexes by improper disulfide bond formation under conditions of exogenous coexpression. However, cotransfection with another cysteine-rich, secreted molecule, PDGFB (30, 31), yielded no evidence of complexes with any of the same molecules. Moreover, FRP complexes were detected when cells were independently transfected with FRPs containing different tags and cocultivated. All of these findings strongly support the specificity of the complexes involving FRP and either Wnts or Hfz6. The strength of the binding interactions for each of these complexes was sufficient to survive exposure to 0.1% SDS. Such conditions do not impair antigen-antibody binding but do disrupt interactions between heterodimers of tyrosine kinase growth factor receptors (34, 35). Continued investigation of the physical and biochemical characteristics of the complexes involving FRP with Wnts and the fz receptors should provide further insights into the nature of these interactions.

Our present studies are consistent with at least two models by which FRP may function as a naturally occurring antagonist of Wnt signaling. According to the first, FRP binds Wnt and exerts its inhibitory function by competing for the ability of the Wnt ligand to interact with the fz receptor. However, a second model emerges from our results showing that FRP forms complexes with the fz receptor and that these interactions are mediated by their homologous CRDs. Such findings support the possibility that fz, itself, may form complexes which function as the signaling and/or binding receptor for Wnt. According to this model, FRP could act by a dominant negative mechanism, interacting with the fz receptor and forming nonfunctional complexes that are incapable of transmitting the Wnt signal.

In several assay systems, FRP acted to inhibit Wnt-induced alterations of β-catenin regulation and TCF transcription. Moreover, we showed that FRP inhibited Wnt function in both autocrine or paracrine modes. Similar conclusions were derived from microinjection of Xenopus embryos (20). Although as shown here and in previous studies (21), secreted FRP remains predominantly associated with the cell or extracellular matrix, it could be postulated to inhibit paracrine-acting Wnts by either of the above models. Lin et al. (23) reported that certain Frzb deletion mutants that retained the ability to bind WNT-1 failed to block WNT-1-induced axis duplication. This lack of a strict correlation between the ability of Frzb to interact physically with Wnts and its ability to inhibit Wnt signaling suggests that the mechanism of antagonism is more complex than can be explained solely by Wnt binding. Genetic analysis aimed at localizing FRP domains required for Wnt binding, as well as for formation of complexes with the fz receptor, should be helpful in assessing the proposed models for FRP inhibition of Wnt signaling. These models are not mutually exclusive, and cooperation of the two mechanisms might be necessary to ensure more complete functional inhibition of Wnt signaling.

The FRP family is not the only example of naturally occurring Wnt antagonists. Recently, dickkopf-1 (dkk-1), which encodes Dkk-1, a secreted inducer of Spemann’s organizer in Xenopus was isolated by an expression cloning strategy (36). This 40-kDa secreted protein contains two cysteine-rich domains and represents a new multigene family. Dkk-1 and Frzb have overlapping patterns of expression during development. Genetic analysis indicates further that Dkk-1 inhibits Wnt signaling upstream of dishevelled (36). It will be of interest to determine whether Dkk-1 acts like FRP/Frzb to bind Wnt ligands and/or their fz receptors or, indirectly, by activating an independent Wnt inhibitory pathway.

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