Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 6770–6775, June 1997
Developmental Biology

Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action

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Contributed by Harold E. Varmus, April 15, 1997

ABSTRACT **Frizzled polypeptides are integral membrane proteins that recently were shown to function as receptors for Wnt signaling molecules. Here, we report the identification of a novel, secreted 36-kDa protein that contains a region homologous to a putative Wnt-binding domain of Frizzleds. This protein, called Frizzled-related protein (FRP), was first identified as a heparin-binding polypeptide that copurified with hepatocyte growth factor/scatter factor in conditioned medium from a human embryonic lung fibroblast line. Degenerate oligonucleotides, based on the NH_2-terminal sequence of the purified protein, were used to isolate corresponding cDNA clones. These encoded a 313-amino acid polypeptide, containing a cysteine-rich domain of ≈110 residues that was 30–40% identical to the putative ligand-binding domain of Frizzled proteins. A 4.4-kb transcript of the *FRP* gene is present in many organs, both in the adult and during embryogenesis, and homologs of the gene are detectable in DNA from several vertebrate species. In biosynthetic studies, FRP was secreted but, like Wnts, tended to remain associated with cells. When coexpressed with several Wnt family members in early *Xenopus* embryos, FRP antagonized Wnt-dependent duplication of the embryonic dorsal axis. These results indicate that FRP may function as an inhibitor of Wnt action during development and in the adult.**

Extracellular signaling molecules have essential roles as inducers of cellular proliferation, migration, differentiation, and tissue morphogenesis during normal development. They also participate in many of the aberrant growth regulatory pathways associated with neoplasia. Among the molecules involved in these activities are the Wnt glycoproteins. In vertebrates, this family consists of more than a dozen structurally related molecules, containing 350–380 amino acid residues of which >100 are conserved, including 23–24 cysteine residues (1, 2). *Wnt-1*, the first Wnt-encoding gene to be isolated, was identified as an oncogene expressed as a result of insertional activation by the mouse mammary tumor virus (3, 4). Subsequently, transgenic expression of *Wnt-1* confirmed that constitutive expression of this gene caused mammary hyperplasia and adenocarcinoma (5). Targeted disruption of the *Wnt-1* gene revealed an essential role in development, as mouse embryos had severe defects in their midbrain and cerebellum (6–8). *Wingless* (*Wg*), the *Drosophila* homolog of *Wnt-1*, was independently identified as a segment polarity gene (9). Gene targeting of other *Wnt* genes demonstrated additional important roles for these molecules in kidney tubulogenesis and limb bud development (10, 11).

Several aspects of Wnt signaling have been illuminated by studies in flies, worms, frogs, and mice (12, 13), but until recently little was known about key events that occur at the external cell surface. Identification of Wnt receptors was hampered by the relative insolubility of the Wnt proteins, which tend to remain tightly bound to cells or extracellular matrix (14, 15). However, several observations now indicate that members of the Frizzled (FZ) family of molecules (16) can function as receptors for Wnt proteins or as components of a Wnt receptor complex (17–19). The prototype of this family, *Drosophila frizzled* (*Dfz*), was first identified as a tissue polarity gene that governs orientation of epidermal bristles (20). Cells programmed to express a second *Drosophila Fz* gene, *Fz2*, bind Wg and transduce a Wg signal to downstream components of the signaling pathway (17).

Each *Fz* gene encodes an integral membrane protein with a large extracellular portion, seven putative transmembrane domains, and a cytoplasmic tail (16, 21). Near the NH_2 terminus of the extracellular portion is a cysteine-rich domain (CRD) that is well conserved among other members of the FZ family (16). The CRD, comprised of ≈110-amino acid residues, including 10 invariant cysteines, is the putative binding site for Wnt ligands (17).

Given the potential complexity of interactions between the multiple members of Wnt and FZ families (1, 16–19), additional mechanisms might exist to modulate Wnt signaling during specific periods of development or in certain tissues. Here we report evidence for such a mechanism, namely the identification of a novel secreted gene product that is closely related to the FZ CRD and antagonizes Wnt action. We propose that this FRP is a prototype for molecules that function as endogenous regulators of Wnt activity.

MATERIALS AND METHODS

Purification and Physical Characterization. Conditioned-medium collection, ultrafiltration, heparin-Sepharose affinity chromatography, and SDS/PAGE were performed as described (22). Hepatocyte growth factor/scatter factor (HGF/SF)-containing fractions were identified by immunoblotting (23). Occasionally heparin-Sepharose fractions were processed by reverse-phase C_4 HPLC (22) to enhance purity of FRP. Gels were fixed and silver-stained using the reagents and protocol from Bio-Rad.

Microsequencing. Approximately 30 μg of protein was loaded onto an Applied Biosystems gas-phase protein sequenator. Forty rounds of Edman degradation were carried out, and phenylthiohydantoin amino acid derivatives were identified with an automated on-line HPLC column (model 120A, Applied Biosystems).

Abbreviations: FRP, Frizzled-related protein; FZ, Frizzled; CRD, cysteine-rich domain of Frizzled proteins; HGF/SF, hepatocyte growth factor/scatter factor.

Data deposition: The sequence in this paper has been deposited in the GenBank database (accession no. AF001900).

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Molecular Cloning and Analysis. Four pools of 26-base degenerate oligonucleotides were synthesized on the basis of either of two segments of amino acid sequence determined by microsequencing of purified FRP. Two pools corresponding to the sequence NVGYKKMVL contained all possible codon combinations except for the substitution of inosine residues in the third positions of the codons for the first Val and Gly; one subset terminated with bases CT and the other with TT. Two additional pools, corresponding to the sequence FYTKPPQXV, contained all possible codon combinations except for the substitution of inosine residues in the third positions of the codons for both Pro residues; one subset contained four codon options for serine in the X position, while the other had the remaining two. Oligonucleotide pools were labeled and used to screen an oligo(dT)-primed M426 cDNA library as described (24).

Selected cDNA inserts were analyzed by restriction endonuclease digestion. The nucleotide sequence of the FRP cDNAs was determined by the dideoxy chain-termination method. To search for homology between FRP and any known protein, we analyzed the GenBank, Protein Data Base, SwissProt, and Protein Identification Resource protein sequence databases. Alignments were generated with the program PILEUP 8 from the Wisconsin Package (Genetics Computer Group, Madison, WI).

Northern and Southern Blotting Analyses. RNA from cell lines was isolated, transferred to nitrocellulose filters, and hybridized with labeled probes as described (24). Northern blots containing ≈ 2 μg of poly(A)$^+$ RNA isolated from a variety of different organs were purchased from Clontech. Labeled probes were hybridized in Express Hyb hybridization solution (Clontech) according to the manufacturer's protocol. The FRP *Not*I–*Sma*I cDNA fragment and human β-actin cDNA probe provided by Clontech were ^{32}P-labeled with random hexamers and used at a concentration of $1–2 \times 10^6$ cpm/ml (specific activity $>8 \times 10^8$ cpm/μg DNA).

Southern blot analysis was performed as described (25), except for variation in formamide concentration during hybridization, as noted in the text. FRP cDNA probes were ^{32}P-labeled with the nick-translation kit from Amersham.

Chromosomal Localization. A 4.1-kb *FRP* genomic fragment obtained from a human fibroblast genomic DNA library (Stratagene) was labeled with biotin or digoxigenin and used as a probe for *in situ* hybridization to locate the *FRP* gene in chromosomal preparations of methotrexate-synchronized normal peripheral human lymphocyte cultures. The conditions for hybridization, detection of fluorescent signal, digital-image acquisition, processing, and analysis were as described (26). The identity of the chromosomes with specific signal was confirmed by rehybridization using a chromosome 8-specific probe, and the signal was localized on G-banded chromosomes.

Biosynthetic Studies. M426 cells grown in T-25 flasks were incubated for 30 min in methionine-free DMEM in the presence or absence of 50 μg/ml heparin (bovine lung, Sigma; when present, heparin was included in all subsequent media), which was subsequently replaced with medium containing [^{35}S]methionine (1 mCi/5 ml per dish; 1 Ci = 37 GBq). After 30 min, the radioactive medium was removed, and monolayers washed with medium containing unlabeled methionine, then incubated for varying intervals in fresh nonradioactive medium. At the specified times, the conditioned media and cell lysates were collected and processed as described (27). Immunoprecipitations were performed with a rabbit polyclonal antiserum (100 μg/ml) raised against a synthetic peptide corresponding to FRP amino acid residues 41–54, in the presence or absence of competing peptide (50 μg/ml). Immune complexes adsorbed to GammaBind (Pharmacia) were pelleted by centrifugation and washed; labeled proteins were resolved by SDS/PAGE and detected by autoradiography.

Microinjection of mRNAs into *Xenopus* Embryos. Wnt-1, wg, Xwnt-3a, and Xwnt-8 plasmids were used as described (28–31). The FRP *Nae*I–*Sal*I cDNA fragment, which includes the full coding sequence, was subcloned into the *Stu*I and *Xho*I sites of pCS2+ (32). All mRNAs for injection were synthesized as capped transcripts *in vitro* with SP6 RNA polymerase (Ambion Megascript Kit). Embryo preparation and staging were performed as described (33). Transcripts were injected into the two blastomeres near the equatorial midline region at the 4-cell stage.

RESULTS

Purification and Molecular Cloning of a Novel Heparin-Binding Protein. During the isolation of HGF/SF from human embryonic lung fibroblast culture fluid, we identified a 36-kDa polypeptide that copurified with HGF/SF following a variety of chromatography procedures (27). Because the comigration of this protein and HGF/SF suggested that it might regulate growth factor activity, a preparative scheme was devised to obtain sufficient quantities for study. This was accomplished by conservative pooling of fractions eluting from heparin-Sepharose resin with 1.0 M NaCl, once it became evident that a portion of the 36-kDa protein emerged after the HGF/SF-containing fractions. Protein obtained in this manner was sufficiently pure and abundant for structural and limited functional analysis (Fig. 1*A*).

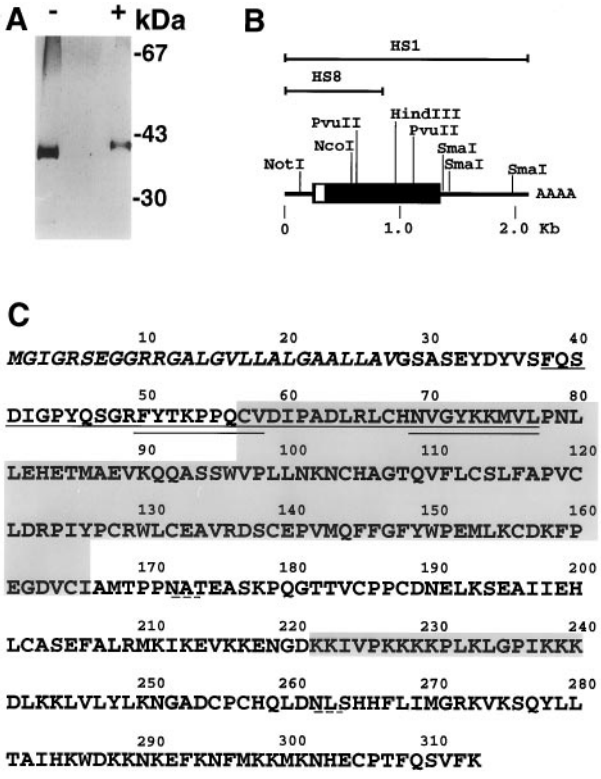

FIG. 1. (*A*) SDS/PAGE analysis of heparin-Sepharose purified FRP. Approximately 200 ng of protein was resolved in a 4–20% polyacrylamide minigel (Novex) under reducing (+) or nonreducing (−) conditions, and subsequently stained with silver. The position of molecular mass markers is indicated at the right. (*B*) Representation of human FRP cDNA clones. Overlapping clones HS1 and HS8 are shown above a diagram of the complete coding sequence and the adjacent 5′ and 3′ untranslated regions. The coding region is boxed; the open portion corresponds to the signal sequence. Untranslated regions are represented by a line. Selected restriction sites are indicated. (*C*) Predicted FRP amino acid sequence (standard single-letter code). The peptide sequence obtained from the purified protein is underlined. Double-underlined sequences were used to generate oligonucleotide probes for screening of the M426 cDNA library. The putative signal sequence is italicized. The large shaded region is the cysteine-rich domain homologous to CRDs in members of the FZ family. The small shaded region is a lysine-rich segment that fulfills the criteria for a consensus hyaluronic acid-binding sequence. The dashed underlining denotes two potential asparagine-linked glycosylation sites.

Microsequencing of the purified 36-kDa protein yielded two NH_2-terminal sequences, one beginning three residues downstream from the other. Positive identifications were made in 37 of the first 40 cycles of Edman degradation, as follows: FQSDIGPYQSGRFYTKPPQXVDIPADLRLXXNVGYKKMVL (X denotes inability to make an amino acid assignment). Degenerate oligonucleotides corresponding either to sequence FYTKPPQXV or NVGYKKMVL were used to probe a M426 cDNA library. An initial screening of 10^6 plaques yielded ≈350 clones recognized by probes derived from both peptide segments. Restriction digestion of several plaque-purified phage DNAs revealed two classes of inserts. Mapping (Fig. 1*B*) and sequence analysis (Fig. 1*C*) of a representative from each class, designated HS1 and HS8, demonstrated that they were overlapping cDNAs. HS1 was ≈2 kb in length and contained a 942-bp ORF; HS8 encoded a portion of the 942-bp ORF as well as ≥0.3 kb of cDNA extending upstream of the ATG start codon. The putative start codon, located at position 303 in the HS1 sequence, was flanked by sequence that closely matched the proposed GCC(G/A)CCATGG consensus sequence for optimal initiation by eukaryotic ribosomes (34). An upstream in-frame stop codon was not present.

As expected for a secreted protein, a hydrophobic 27-amino acid segment at the NH_2-terminus likely functions as a signal peptide. The experimentally determined protein sequence begins 11 residues downstream from the presumptive signal sequence, suggesting additional processing or incidental proteolysis. There was complete agreement between the predicted and observed amino acid sequences; the three undefined residues in the latter corresponded to Cys-57, Cys-67, and His-68, residues that typically are undetectable or have low yields following Edman degradation. Two overlapping sequences in the COOH-terminal region fulfill the criteria for a consensus binding site to hyaluronic acid (35) (Fig. 1*C*). Two potential asparagine-linked glycosylation sites are also present. A consensus polyadenylylation signal was not identified in the cDNA sequence, raising the possibility that the cDNA clones from this oligo(dT) primed library resulted from internal priming at an adenine-rich region.

Relationship to FZ. Search of several protein databases revealed significant homology of a portion of the predicted amino acid sequence to a specific region conserved among members of the FZ family (Fig. 2). The observed homology is confined to the extracellular CRD of FZ, a region consisting of ≈110 amino acid residues that includes 10 cysteines and a small number of other invariant residues. This domain has special importance because it is a putative binding site for Wnt ligands (17). The FRP CRD is 30–42% identical to the CRD of the other FZ proteins.

In addition to the plasma membrane-anchored FZ proteins and FRP, three other molecules have been described that also possess a FZ CRD motif. An alternatively spliced isoform of mouse collagen XVIII was the first such protein to be reported (38). The two other molecules, mouse SDF5 (40) and human FRZB (39), resemble FRP in that each consists of ≈300 amino acid residues, including a signal peptide, CRD near its NH_2-terminus and a hydrophilic COOH-terminal moiety. FRP and SDF5 have 58% identities in their CRDs, while FRP and FRZB are only 32% identical in this region. Elsewhere, these molecules are only 15–20% identical. Thus, FRP, SDF5, and FRZB may constitute a subfamily of small, FZ-related proteins that lack the seven transmembrane motif responsible for anchoring FZ proteins to the plasma membrane and are presumably secreted.

FRP Gene is Expressed in Multiple Organs and Cell Types. Using the 1081-bp *Not*I–*Sma*I fragment of HS1 (Fig. 1*B*) as probe, a single 4.4-kb transcript was detected in poly(A)$^+$ RNA from several human organs (Fig. 3). In adult tissues, the highest level of expression was observed in heart, followed by kidney, ovary, prostate, testis, small intestine, and colon. Lower levels were seen in placenta, spleen and brain, while transcript was barely detectable in skeletal muscle and pancreas. No hybridization signal was evident in mRNA from lung, liver, thymus, or peripheral blood leukocytes. In poly(A)$^+$ RNA from a small sample of human fetal organs, the 4.4-kb transcript was highly

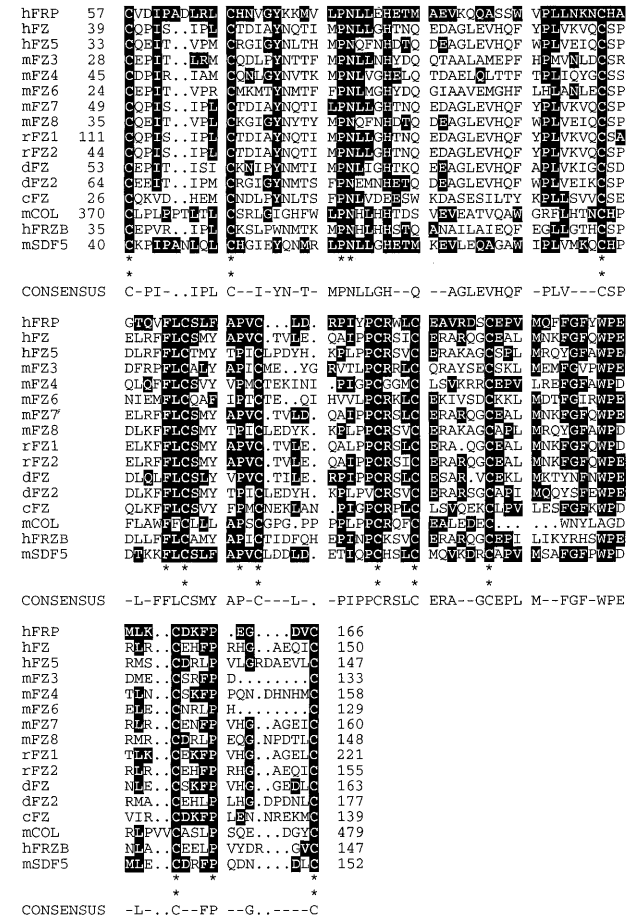

FIG. 2. Comparison of the CRDs of FRP and other members of the FZ family. Solid black shading highlights identities present in human FRP and any other FZ family member. The consensus sequence indicates residues present in at least 8 of the 16 FZ or FZ-related proteins. **, The ten invariant cysteine residues; single asterisks indicate other invariant residues. hFRP, human FZ-related protein; hFZ (36); hFZ5 (16); mFZ3-mFZ8 (16); rFZ1 and rFZ2 (37); dFZ (21); dFZ2 (17); cFZ (16); mCOL, mouse collagen XVIII (38); hFRZB (39); and mSDF5 (40).

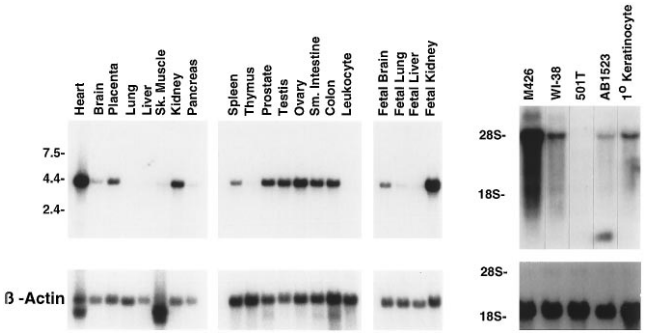

FIG. 3. FRP mRNA expression in normal human adult and embryonic tissues, and in cultured cells. Blots containing ≈2 μg of poly(A)$_+$ RNA from each of the indicated tissues or 10 μg of total RNA from different human cell lines were probed with radiolabeled FRP and β-actin cDNA fragments, as described in the Methods. The position of DNA size markers, expressed in kb, is indicated at the left of the tissue blots; the position of 28S and 18S ribosomal RNA is shown at the left of the cell line blot.

represented in kidney, at moderate levels in brain, barely detectable in lung, and undetectable in liver.

Northern analysis of total RNA from various human cell lines demonstrated the 4.4-kb transcript and, occasionally, additional faint bands not further analyzed (Fig. 3 *Right*). While the transcript was detected in RNA from embryonic lung (M426 and WI-38) and neonatal foreskin (AB1523) fibroblasts, it was not observed in a sample of adult dermal fibroblasts (501T). In addition to fibroblasts, the transcript was seen in RNA from primary keratinocytes, indicating that expression was not limited to cells of mesenchymal origin. Considering that the cumulative size of the overlapping FRP cDNAs was only 2.1 kb, detection of a 4.4-kb transcript reinforced the suggestion that the cDNAs were generated by internal priming at adenine-rich regions.

Chromosomal Localization and Detection of the FRP Gene in Different Species. Using a fluorescent-labeled 4.1-kb genomic fragment containing a portion of the FRP coding sequence, *in situ* hybridization revealed a single locus at chromosome 8p11.1-12 (Fig. 4). This site may be near the putative locus of the *hFZ3* gene, based on homology with the location of *mFz3* in the mouse genome (16). Radiation hybrid analysis yielded results consistent with the fluorescent *in situ* hybridization analysis (unpublished observations).

To determine whether the *FRP* gene was present in other species, genomic DNAs from various sources were fully digested with *Eco*RI and hybridized with an *Nco*I–*Sma*I cDNA fragment (Fig. 1*B*) under varying conditions of stringency (Fig. 5). Multiple bands were observed under highly stringent conditions (50% formamide) in DNA from human, rhesus monkey, mouse, and chicken. With moderate stringency (35% formamide), no additional fragments were seen in the DNA from these species but fragments were detected in *Xenopus* DNA. No hybridization signal was observed with DNA from *Drosophila* or yeast (*Saccharomyces cerevisiae*) in these experiments. At low stringency (20% formamide), the background was too high to detect specific signals (data not shown). These results strongly suggested that the *frp* gene is highly conserved among vertebrates. Although these experiments did not detect an *FRP* homolog in the invertebrates, the existence of such homologs was not rigorously excluded, due to the limitations of the method.

Southern blotting performed either with the *Not*I–*Nco*I cDNA fragment (Fig. 1*B*) or with synthetic oligonucleotide probes corresponding to different portions of the FRP coding sequence, hybridized to subsets of genomic fragments detected with the *Nco*I–*Sma*I probe (data not shown). This finding and the lack of additional bands detected only under relaxed conditions (Fig. 5) suggested that highly related *FRP*-like sequences are not present

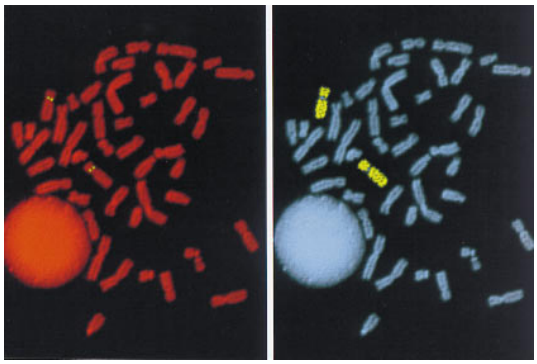

FIG. 4. Chromosomal localization of the *FRP* gene by fluorescent *in situ* hybridization. To localize the *FRP* gene, 100 sets of metaphase chromosomes were analyzed. In 80 metaphases, a double fluorescent signal was observed with the *FRP* genomic probe in 8p11.1-12 on both chromosome homologs (*Left*). The identity of the chromosomes was confirmed by hybridization with a probe specific for chromosome 8 (*Right*).

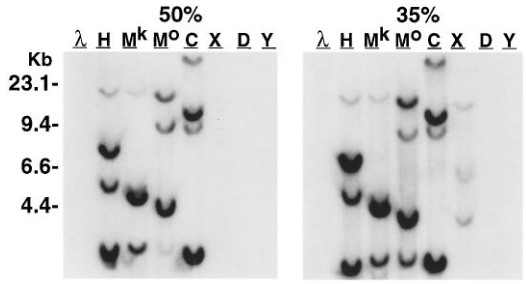

FIG. 5. Southern blot analysis of *FRP* genomic sequences in different species. After fractionation by agarose gel electrophoresis and transfer to filters, *Eco*RI-digested genomic DNAs were hybridized in the presence of either 50% or 35% formamide. Specimens were from the following species: λ, lamda phage; H, human; M^k, rhesus monkey; M^o, mouse; C, chicken; X, *Xenopus laevis*; D, *Drosophila melanogaster*; Y, yeast (*S. cerevisiae*).

in the human genome. Thus, the multiple genomic fragments hybridizing to the FRP cDNA in Southern blots are likely to reflect the presence of several exons in the *hFRP* gene.

Biosynthetic Studies Show that FRP is Secreted, but Primarily Cell-Associated in the Absence of Exogenous Heparin. To study the synthesis and processing of FRP protein, a pulse-chase experiment was performed with [^{35}S]methionine labeled M426 cells either in the absence or presence of added heparin. As shown in Fig. 6, a 36-kDa protein band was specifically immunoprecipitated with antiserum raised against a synthetic peptide corresponding to a portion of the FRP NH_2-terminal sequence. In the absence of soluble heparin, after either 1 h (lanes 1 and 5) or 4 h (lanes 9 and 13) FRP was much more abundant in the cell lysate than in the conditioned medium. However, after 20 h, the amount of FRP protein in the medium (lane 21) was comparable to that which remained cell-associated (lane 17). At this last time point, the combined band intensity in the two compartments had decreased relative to that observed earlier, suggesting significant protein turnover during the experiment. Moreover, after 20 h the FRP-specific signal appeared as a doublet, providing additional evidence of proteolysis. In the presence of soluble heparin (50 μg/ml), most of the FRP was detected in the medium at all three time points (compare lanes 3 and 7, 11 and 15, 19 and 23). Heparin also appeared to stabilize FRP, as the band intensity was stronger when heparin was present, and there was no evidence of partial proteolysis. Interestingly, others have shown that heparin can release Wnt-1 from the cell surface in a similar manner (14, 15, 41). Taken together, our results demonstrate that FRP is secreted, although it tends to remain cell-associated and relatively susceptible to degradation unless released into the medium by soluble heparin.

FRP Antagonizes Wnt Action in *Xenopus* Embryo Assay. Because FRP possesses a potential binding site for Wnt molecules and appears to partition among cellular compartments like Wnt-1, it seemed possible that FRP might modulate the signaling activity of Wnt proteins. We envisioned two alternatives: FRP might antagonize Wnt function by binding the protein and blocking access to its cell surface signaling receptor, or FRP might enhance Wnt activity by facilitating the presentation of ligand to the FZ receptors, analogous to the action of soluble interleukin 6 receptors (42).

To test these possibilities, we examined the effect of FRP on Wnt-dependent dorsal axis duplication during *Xenopus* embryogenesis. Previous studies have demonstrated that microinjection of mRNA encoding certain Wnt molecules, such as mouse Wnt-1, Wg, XWnt-8, or XWnt-3a, into early *Xenopus* embryos can induce the formation of an ectopic Spemann organizer and, subsequently, duplication of the dorsal axis (28–31, 43, 44). As illustrated in Fig. 7, injection of suboptimal doses of Wnt-1, Wg, or XWnt-8 mRNA into embryos induced partial or complete

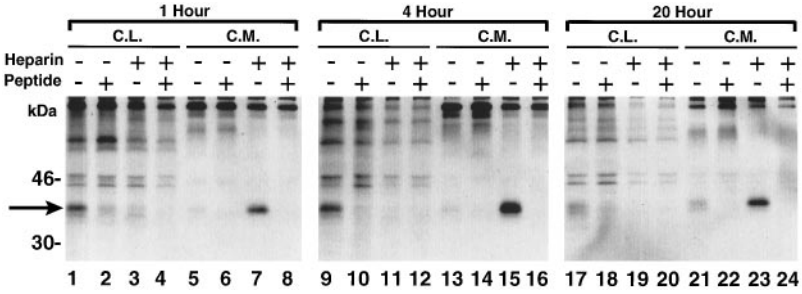

FIG. 6. Biosynthesis of FRP in M426 cells. A pulse-chase experiment was performed with metabolically labeled cells incubated either in the absence or presence of heparin. Proteins were immunoprecipitated from cell lysates (C.L.) or conditioned medium (C.M.) with FRP peptide antiserum in the absence or presence of competing peptide, and resolved in a SDS/10% polyacrylamide gel. Cells and media were harvested 1, 4, or 20 h after a 30 min labeling period. Lanes 1–24 are labeled at the bottom. The protein band corresponding to FRP is indicated by an arrow. The position of molecular mass markers is shown at the left.

duplication in at least 75% of the animals. Suboptimal doses were used to enable us to detect enhancement of the axis duplication phenotype, if the role of FRP was to facilitate Wnt signaling. However, when similar quantities of FRP and Wnt RNA were coinjected, the incidence and extent of axial duplication were significantly reduced (Fig. 7). The effect was dose-dependent, as the number of animals with an abnormal phenotype was even lower when the relative amount of FRP RNA was increased 5- to 10-fold. Injection of FRP RNA alone at a higher dose (100 pg) into the dorsal side of the embryo did not affect the endogenous dorsal axis formation (data not shown).

Surprisingly, FRP was much less effective in antagonizing XWnt-3a, suggesting a degree of specificity regarding interactions with different members of the Wnt family. The Wnt signaling pathway is thought to proceed through suppression of the activity of glycogen synthase kinase-3, a cytoplasmic serine-threonine kinase (13). Axis duplication induced by a dominant-negative, kinase-inactive mutant of glycogen synthase kinase-3β (33, 45, 46) was not affected by FRP (data not shown), consistent with the assumption that FRP directly interferes with Wnt signaling at the cell surface, not by indirectly interfering with a late step in the Wnt signaling pathway.

DISCUSSION

In the present report, we describe a novel human gene product that resembles FZ proteins in that it possesses a conserved FZ CRD, a putative binding domain for Wnt ligands. In contrast to the original members of the FZ family, FRP lacks any transmembrane region or cytoplasmic domain required to transduce Wnt signaling inside the cell. Because it is preferentially distributed to the cell surface or matrix, it is well-positioned to interact with Wnt proteins. Theoretically, binding of FRP to Wnts might stabilize the latter, and consequently enhance Wnt signaling. However, our findings indicate that in *Xenopus* embryos FRP inhibits Wnt-dependent axial duplication when various Wnts and FRP are coexpressed. These results suggest that FRP behaves like a dominant-negative receptor in this model system, similar to the effect of the secreted NH_2-terminal ectodomain of human FZ5 on axis duplication by XWnt-5A and hFZ5 (19). Further elucidation of the interaction between FRP and Wnt proteins will require study of the proteins in isolated form.

The existence of other molecules besides FRP that have a FZ CRD but lack the seven transmembrane motif and cytoplasmic tail suggests that there is a subfamily of proteins that function as regulators of Wnt activity. Little is known about the activity of SDF5, which was cloned using the signal sequence trap method (40). FRZB is a heparin-binding molecule thought to be involved in skeletal morphogenesis (39). Recently Rattner *et al.* (47) cloned cDNAs encoding the murine homologs of SDF5, FRZB, and FRP. They showed that, when artificially linked to the plasma membrane via a glycolipid anchor, SDF5 and FRZB conferred cellular binding to Wg. Thus, it now appears likely that all three molecules can interact with Wnt proteins and modulate their activity. Future studies should define the activities and unique functions of these different FZ-related proteins, especially with regard to Wnt regulation.

Recently Zecca *et al.* (48) demonstrated that Wg functions as a gradient morphogen. Just as the formation of stable Wnt gradients may rely on their ability to bind proteoglycan (41), the heparin-binding property of FRP implies that it might also function in a graded manner. In fact, gradients of interacting proteins might be a general mechanism of Wnt regulation. A detailed analysis of the expression patterns of FRP and interacting Wnt molecules would help to address the viability of this model. If FRPs and Wnts are expressed in proximity to each other, then targeted disruption of FRP expression might result in uncontrolled Wnt activity. This could be manifested by developmental abnormalities in tissue morphogenesis or perhaps by neoplasia.

The large size of the FZ and Wnt families and the potential for mutual binding between several members of the two groups make it difficult to predict which receptor/ligand interactions are likely to be affected by FRP. For example, Wg protein binds to cells expressing a variety of FZ proteins (17), yet Wnt-5A induces axis duplication only in *Xenopus* embryos expressing FZ5 (19). Furthermore, a comparison of patterns of FRP expression with published reports concerning FZs and

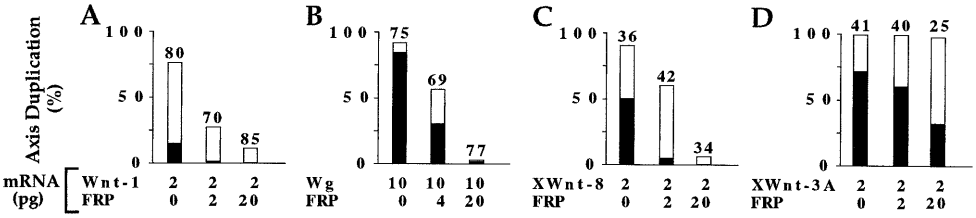

FIG. 7. Dorsal axis duplication in *Xenopus* embryos in response to varying combinations of Wnt and FRP transcripts. Each bar represents the percentage of axis duplication; the solid portion within each bar represents the percentage of extensive duplication, which is defined by the presence of the cement gland and at least one eye in the duplicated axis. The total number of embryos injected in two to four independent experiments is indicated by the number above each bar. The amount of mRNA injected per embryo is shown below the bars.

Wnts indicates that many of the *Fz* and *Wnt* genes are expressed in tissues where FRP transcripts also are seen. For instance, the heart, kidney, intestines, and reproductive organs have high levels of FRP mRNA. These organs also are among the highest in expression of FZ1, FZ2, and FZ4 transcripts (16, 37). Wnt-2, Wnt-4, Wnt-5A, and Wnt-5B have been detected at these sites as well (1). These data provide indirect evidence that FRP might have an impact on several Wnt/FZ interactions. However, knowledge about the affinity of FRP for different Wnt family members will be required to fully assess its effect on Wnt signaling. As shown in Fig. 7, FRP may inhibit only a subset of Wnt proteins.

Although our discovery of FRP was dependent on an apparent association of this molecule with HGF/SF, subsequent experiments have failed to confirm significant interaction of the two molecules. While these negative results are not definitive, they suggest that the copurification of these proteins from the same conditioned medium may have been fortuitous. Despite this apparent coincidence, the FRP and HGF/SF signaling pathways may intersect in a mutually reinforcing fashion. HGF/SF stimulates the tyrosine phosphorylation of β-catenin, which may reduce the binding of β-catenin to cadherins (13, 49). This might contribute to the disruption of cell-cell adhesion that characterizes the cell scattering activity of HGF/SF, and may antagonize aspects of Wnt-dependent cell-cell contact. In a mammary gland model, concomitant with a stimulation of branching morphogenesis, HGF/SF inhibited the expression of Wnt-5A (50). Thus, it is possible that the actions of FRP and HGF/SF complement each other in some respects, even though the proteins do not appear to have a direct interaction.

Note. While this manuscript was being reviewed, two groups reported that FRZB was a secreted antagonist of Wnt signaling expressed in the Spemann organizer (51, 52).

We thank Alex Cech, David Hirshfield, and Jared Rosenberg for technical assistance and/or help in preparing figures; and Jeremy Nathans for providing us with a copy of his manuscript (47) prior to publication. We also are grateful to Igor Dawid and Jean-Pierre Saint-Jeannet for their support and helpful discussions. P.W.F. is supported by Grant DK 47102 from the National Institutes of Health; X.H. was supported by the U.S. Army Breast Cancer Research Program.

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