Secreted Frizzled-related Protein-1 Binds Directly to Wingless and Is a Biphasic Modulator of Wnt Signaling*

Aykut Üren‡, Frieda Reichsман§, Vasiliki Anest‡, William G. Taylor‡, Kanae Muraiso‡, Donald P. Bottaro‡, Susan Cumberledge§, and Jeffrey S. Rubin‡¶

From the ‡Laboratory of Cellular and Molecular Biology, Division of Basic Sciences, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the §Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01002

Secreted Frizzled-related protein-1 (sFRP-1) contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzleds. To facilitate the biochemical and biological analysis of sFRP-1, we developed a mammalian recombinant expression system that yields ~3 mg of purified protein/liter of conditioned medium. Using this recombinant protein, we demonstrated that sFRP-1 and Wg (wingless) interact in enzyme-linked immunosorbent and co-precipitation assays. Surprisingly, a derivative lacking the cysteine-rich domain retained the ability to bind Wg. Cross-linking experiments performed with radiiodinated sFRP-1 provided definitive evidence that sFRP-1 and Wg bind directly to each other. Besides detecting a cross-linked complex consistent in size with 1:1 stoichiometry of sFRP-1 and Wg, we also observed a larger complex whose size suggested the presence of a second sFRP-1 molecule. The formation of both complexes was markedly enhanced by an optimal concentration of exogenous heparin, emphasizing the potential importance of heparan-sulfate proteoglycan in Wnt binding and signaling. sFRP-1 exerted a biphasic effect on Wg activity in an armadillo stabilization assay, increasing armadillo level at low concentrations but reducing it at higher concentrations. These results provide new insights about the Wnt binding and biological activity of sFRPs.

Wnt proteins comprise a large family of structurally related, extracellular agents that have a variety of important functions during embryonic development (reviewed in Refs. 1 and 2). They specify cell polarity and fate, stimulate proliferation, and contribute to the patterning of tissue in many animal models. Wnt signaling also has been strongly implicated in the development of neoplasia. Although the prevalence of wnt overexpression in human cancer is still an open question, constitutive activity of Wnt signaling because of mutations in downstream effectors or modulators occurs with a high frequency in certain malignancies. In particular, mutations in APC (encoded by the tumor suppressor gene) and β-catenin (21). However, there have been no cross-linking experiments to verify that Wnt proteins bind directly to Fzs. Nonetheless, a specific portion of the Fz extracellular domain was reported to be responsible for the observed cellular binding of Wg and transduction of Wnt signaling (21). This region consists of ~120 amino acid residues and was designated the cysteine-rich domain (CRD) because it contained 10 cysteine residues that were conserved in all the known Fz family members (eight different fz genes in vertebrates and additional genes in various invertebrate species (29). A set of secreted Fz-related proteins (sFRP or FRP) recently have been described (30–41). These proteins consist of approximately 300 amino acids, including a CRD that is typically ~30–50% identical to the CRDs of Fz family members. The carboxyl-terminal portion of these proteins often contains segments rich in positively charged residues, and two (sFRP-1 and FrzB/sFRP-3) were reported to bind tightly to heparin (33, 42). When engineered to remain anchored to the cell membrane via a glycolipid tag, sFRP-2 and sFRP-3 conferred Wg binding to the cell surface (32). Moreover, co-expression of sFRP family members with selected Wnts in early Xenopus embryos caused inhibition of nuclear-3β to phosphorylate β-catenin and earmark it for rapid degradation. A block in this process, either because of mutations or Wnt activity, causes an elevation in cytosolic β-catenin, which accumulates in the nucleus where it can combine with DNA binding proteins of the TCF-LEF family (8–11). These complexes up-regulate the expression of specific genes such as c-myc and cyclin-D1 that promote cell proliferation and predispose to malignant transformation (12–14). An abundance of regulatory mechanisms have evolved to tightly control the cellular responses elicited by Wnts (15).

The identification of Wnt receptors was hampered for many years because of the inability to purify Wnt proteins and label them for classical binding experiments. This is still a problem, because the proteins tend to remain associated with the cell surface or extracellular matrix (16, 17). Fortunately, there are now sources of soluble, biologically active Wnt proteins available for binding studies (18–20). During the past few years a number of studies have established that Fz (frizzled) seven-pass transmembrane proteins can function as Wnt receptors or components of a receptor complex (21–28). For instance, ectopic expression of particular Fz family members resulted in binding of Wg to the cell surface and in Wnt signaling as indicated by increases in armadillo (Arm), the Drosophila ortholog of β-catenin (21). However, there have been no cross-linking experiments to verify that Wnt proteins bind directly to Fzs. Nonetheless, a specific portion of the Fz extracellular domain was reported to be responsible for the observed cellular binding of Wg and transduction of Wnt signaling (21). This region consists of ~120 amino acid residues and was designated the cysteine-rich domain (CRD) because it contained 10 cysteine residues that were conserved in all the known Fz family members (eight different fz genes in vertebrates and additional genes in various invertebrate species (29). A set of secreted Fz-related proteins (sFRP or FRP) recently have been described (30–41). These proteins consist of approximately 300 amino acids, including a CRD that is typically ~30–50% identical to the CRDs of Fz family members. The carboxyl-terminal portion of these proteins often contains segments rich in positively charged residues, and two (sFRP-1 and FrzB/sFRP-3) were reported to bind tightly to heparin (33, 42). When engineered to remain anchored to the cell membrane via a glycolipid tag, sFRP-2 and sFRP-3 conferred Wg binding to the cell surface (32). Moreover, co-expression of sFRP family members with selected Wnts in early Xenopus embryos caused inhibition of nuclear-3β to phosphorylate β-catenin and earmark it for rapid degradation. A block in this process, either because of mutations or Wnt activity, causes an elevation in cytosolic β-catenin, which accumulates in the nucleus where it can combine with DNA binding proteins of the TCF-LEF family (8–11). These complexes up-regulate the expression of specific genes such as c-myc and cyclin-D1 that promote cell proliferation and predispose to malignant transformation (12–14). An abundance of regulatory mechanisms have evolved to tightly control the cellular responses elicited by Wnts (15).

The identification of Wnt receptors was hampered for many years because of the inability to purify Wnt proteins and label them for classical binding experiments. This is still a problem, because the proteins tend to remain associated with the cell surface or extracellular matrix (16, 17). Fortunately, there are now sources of soluble, biologically active Wnt proteins available for binding studies (18–20). During the past few years a number of studies have established that Fz (frizzled) seven-pass transmembrane proteins can function as Wnt receptors or components of a receptor complex (21–28). For instance, ectopic expression of particular Fz family members resulted in binding of Wg to the cell surface and in Wnt signaling as indicated by increases in armadillo (Arm), the Drosophila ortholog of β-catenin (21). However, there have been no cross-linking experiments to verify that Wnt proteins bind directly to Fzs. Nonetheless, a specific portion of the Fz extracellular domain was reported to be responsible for the observed cellular binding of Wg and transduction of Wnt signaling (21). This region consists of ~120 amino acid residues and was designated the cysteine-rich domain (CRD) because it contained 10 cysteine residues that were conserved in all the known Fz family members (eight different fz genes in vertebrates and additional genes in various invertebrate species (29). A set of secreted Fz-related proteins (sFRP or FRP) recently have been described (30–41). These proteins consist of approximately 300 amino acids, including a CRD that is typically ~30–50% identical to the CRDs of Fz family members. The carboxyl-terminal portion of these proteins often contains segments rich in positively charged residues, and two (sFRP-1 and FrzB/sFRP-3) were reported to bind tightly to heparin (33, 42). When engineered to remain anchored to the cell membrane via a glycolipid tag, sFRP-2 and sFRP-3 conferred Wg binding to the cell surface (32). Moreover, co-expression of sFRP family members with selected Wnts in early Xenopus embryos caused inhibition of nuclear-3β to phosphorylate β-catenin and earmark it for rapid degradation. A block in this process, either because of mutations or Wnt activity, causes an elevation in cytosolic β-catenin, which accumulates in the nucleus where it can combine with DNA binding proteins of the TCF-LEF family (8–11). These complexes up-regulate the expression of specific genes such as c-myc and cyclin-D1 that promote cell proliferation and predispose to malignant transformation (12–14). An abundance of regulatory mechanisms have evolved to tightly control the cellular responses elicited by Wnts (15).
of Wnt-dependent duplication of the dorsal axis (30, 31, 33, 39). Co-precipitation studies suggested that they could associate with Wnts and thereby block ligand interaction with Fzs, the presumptive signal-transducing membrane receptor (31, 39, 43). However, because these experiments were performed with unpurified reagents, the evidence of sFRP/Wnt binding was indirect.

The present study was undertaken to test the hypothesis that sFRP-1 binds directly to Wnt protein, and if so, to determine what portions of sFRP-1 were required for this interaction. Given that both sFRP-1 and Wnt proteins bind heparin (33, 44) and that lowering tissue proteoglycan content, either by genetic or biochemical manipulation, impaired Wnt signaling (45–48), we also investigated the impact of heparin on sFRP-1/Wnt interactions. To facilitate this work, we developed a recombinant expression system that yielded large quantities of purified, sFRP-1 protein and utilized a previously described source of soluble, biologically active Wg (19). Results from ELISA, co-precipitation, and cross-linking experiments provided compelling evidence of direct binding between sFRP-1 and Wg. This binding was strongly influenced by heparin. Surprisingly, the Fz-related CRD was not required for binding. Moreover, sFRP-1 exhibited a biphasic effect on Wg signaling, enhancing Wg-dependent stabilization of Arm at low concentrations but inhibiting stabilization at high concentrations. These findings establish that sFRPs can regulate Wnt activity and provide a new perspective regarding their biological effects and mode of action.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—MDCK cells** (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum (Colorado Serum Company) in 5% CO₂ at 37 °C. *Drosophila* S2 cells and SH-SW-G cells transfected with a heat shock promoter/Wg construct (18, 19), and S2 cells expressing DP2 (21) were kindly provided by the Nusse lab. All three S2 lines were cultured in Schneider’s Drosophila medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 25 °C in atmospheric air. Wg-containing and S2 control media were concentrated as described previously (44).

**Immunoblotting and Immunoprecipitation—** Proteins resolved by SDS-PAGE were transferred to Immobilon-P membranes (Millipore). Unless stated otherwise, all subsequent steps were performed at room temperature. After brief washing in phosphate-buffered saline (PBS), membranes were blocked with 3% nonfat dry milk in TTBS (20 mM Tris-HCl, pH 9.0, 0.05% Tween-20, 150 mM NaCl) for 2 h. Following five washes with TTBS, membranes were incubated for 2 h with primary antibodies diluted 1:1000 (for a typical final concentration of 1–2 μg/ml) in 0.5% bovine serum albumin (BSA)/TTBS. sFRP-1 rabbit antiserum was raised either against a synthetic amino-terminal peptide (33) or the full-length, purified protein. Monoclonal antibody to Wg, mAb 4D4, first prepared in Dr. Stephen Cohen’s lab, was a gift from the Nusse lab; antibodies to the c-Myc and polyhistidine epitopes were raised from Invitrogen. After five washes with TTBS, membranes were incubated for 1 h with horseradish peroxidase conjugated to anti-mouse or anti-rabbit secondary antibodies (Amersham Pharmacia Biotech) diluted 1:2000 in 0.5% BSA/TBTS. Following five more washes with TBTS, bound antibodies were visualized by chemiluminescence (Amersham Pharmacia Biotech) using X-Omat AR film (Kodak).

For immunoprecipitation, Wg-containing medium (80 μl) was preincubated with individual sFRP-1 derivatives (300 μl/30 min) for 10 min at room temperature. Subsequently, anti-Myc (0.2 μg) was added to the samples, which were then incubated overnight at 4 °C. Sample volumes were adjusted to 500 μl with lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 6.7 mM Na₃PO₄, 1 mM NaVO₃, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 180 μM of a 50% merosin G-Sepharose slurry (Amersham Pharmacia Biotech) was added. After 1 h of incubation at 4 °C in a rotary shaker, samples were washed three times with 1 ml of lysis buffer. Final pellets were resuspended in 2× SDS sample buffer and boiled for 4 min, and the proteins were resolved by SDS-PAGE.

**Expression, Purification, and Analysis of Recombinant sFRP-1 and Its Derivatives—** The human sFRP-1 N-terminal cDNA fragment (33) was subcloned into an XhoI site in the pcDNA3.1 expression vector (Invitrogen). To prepare derivatives containing c-Myc and polyhistidine epitopes at their carboxyl termini, cDNAs encoding full-length sFRP-1 or deletion mutants were generated by polymerase chain reaction with primers that introduced EcoRI and HindIII restriction sites at the 5’ and 3’ ends, respectively. The sequences comprising the various derivatives are indicated in Fig. 2A. Purified polymerase chain reaction products were ligated into the pcDNA3.1Myco/His, C(−) expression vector (Invitrogen), and plasmid samples prepared from transformed DH5α competent cells (Life Technologies, Inc.). The fidelity of cDNAs was verified by sequence analysis.

MDCK cells (1.5 × 10⁶) transfected with 10 μg of DNA of the various sFRP-1 constructs, using the calcium phosphate precipitation method. Mass cultures were selected with Geneticin (500 μg/ml) for 21 days. To isolate clonal cell lines, mass cultures were subcultured at a 1:50,000 dilution in collagen-coated wells and subsequently transferred to culture dishes for further analysis. Expression of recombinant protein was determined by immunoblotting equal quantities of total protein from conditioned medium and/or cell lysates. For large scale preparations, sFRP-1/MDCK transfectants were grown in T75 flasks until confluent. After washing with PBS, the cells were maintained in serum-free Dulbecco’s modified Eagle’s medium, and conditioned media were collected every 3 days for five to seven consecutive harvests. Media were clarified by centrifugation at 10,000 × g for 10 min at 4 °C and filtration (pore size: 0.2 μm; Corning). Subsequently, media were concentrated 40-fold by ultrafiltration in a stirred chamber apparatus (Amicon M2000) using a Millipore YM membrane with either a 10- or 3-kDa molecular mass cut-off. Concentrated samples were snap-frozen for subsequent purification.

Native sFRP-1 was purified with HiTrap-Heparin columns (Amersham Pharmacia Biotech) equilibrated with PBS/0.3 mM NaCl. After applying the sample to the column, the resin was washed with 10 column volumes of equilibration buffer. Protein was eluted with a step gradient of increasing NaCl concentration. Aliquots from representative fractions were resolved by SDS-PAGE and analyzed by immunoblotting or silver staining (Bio-Rad). sFRP-1 derivatives containing Mychistidine epitopes were purified in a similar manner, only using HiTrap Chelating Affinity columns (Amersham Pharmacia Biotech).

The resin (1.0 ml) was washed with 5.0 ml of water, charged with 0.5 ml of 0.1 M NiSO₄, and washed again with 5.0 ml of water. Following equilibration with 50 mM phosphate/10 mM imidazole buffer (pH 7.4), protein was eluted with a step gradient of increasing imidazole concentration. Selected fractions were analyzed by immunoblotting and silver staining. Typically, sFRP-1 derivatives were recovered with 0.1 mM imidazole. Aliquots of sFRP-1 protein were subsequently microsequenced with an Applied Biosystems protein sequencer (model 476). For sFRP-3CRD, 30 rounds of Edman degradation were carried out to ensure that the entire CDR was deleted.

**sFRP-1/Wg ELISA Binding Assays—** sFRP-1 diluted in 0.2% NaNO₂/ PBS was incubated in 96-well Falcon ELISA plates (300 ng/50 μl/well) for 2 h at 37 °C. After decanting, all wells were filled with 4% BSA/0.02% NaN₃. Briefly, 10 μl of 100 μg/ml was incubated in an ELISA plate reader (Bio-Rad). After 2 h of incubation, the wells were washed with TAPS (0.05% Tween-20 in 0.02% NaN₃/PBS), 50-μl aliquots of Wg-containing or S2 control medium diluted in 1% BSA/TAPS were incubated overnight at room temperature. After five washes with TAPS, 50 μl of Wg αbigen diluted in 1% BSA/TAPS was added. After washing five times with TAPS (0.05% Tween-20 in 0.02% NaNO₂/PBS), 50-μl aliquots of Wg-containing or S2 control medium diluted in 1% BSA/TAPS were incubated overnight at room temperature. After five washes with TAPS, 2 mg/ml p-nitrophenolphosphate (Sigma) in carbonate buffer (0.1 M Na₂CO₃, 1 mM MgCl₂, pH 9.8) was added. Absorbance at 405 nm was measured with an ELISA plate reader (Bio-Rad). When the solid phase assay was performed with the various sFRP-1 derivatives, wells were coated with 60 ng solutions of the respective derivatives. ELISA competition experiments were performed as described above, except the indicated concentrations of sFRP-1 derivatives were preincubated with Wg conditioned medium for 45 min at room temperature prior to addition to wells that had been coated with native sFRP-1.

**Covalent Cross-linking—** sFRP-1 was iodinated as described previously (49). Briefly, 10 μg of sFRP-1 was reacted with 125I in 50 μl of a 30% chloramine T suspension at room temperature for 30–60 s. After addition of 80 μg/ml sodium metabisulfite, the reaction mixture was applied to a heparin-Sepharose column (bed volume, 0.3 ml) equilibrated in 0.1% BSA/PBS. Labeled sFRP-1 was eluted with equilibration buffer containing 1 mM NaCl and stored in frozen aliquots. Approximately 50 μl of Wg-containing or control medium was incubated with 1 μCi of 125I-sFRP-1 for 40 min at room temperature. In some experiments, varying
concentrations of heparin (12 kDa from porcine intestine; Fisher) or unlabeled sFRP-1 were also present (see figures for details). After addition of 1 mM bis(sulfosuccinimidyl) suberate (BS²) cross-linking agent (Pierce), the incubation continued for 20 min. The reaction was quenched with 20 mM glycine/1 mM Tris-HCl, and the mixture was incubated with Wg mAb (10 µg/ml) overnight at 4 °C. After addition of 0.5 ml of lysis buffer and 50 µl of a 50% protein G-Sepharose slurry, samples were incubated for 1 h at 4 °C. Beads were pelleted by centrifugation at 1000 × g for 3 min at 4 °C and washed three times with 1 ml of lysis buffer. The final pellets were resuspended in 2× SDS sample buffer, boiled for 4 min, and briefly microfuged to facilitate transfer. Protein samples were resolved in 8% polyacrylamide gels by SDS-PAGE. After fixation in 20% methanol/10% acetic acid for 45 min, the gel was dried and exposed to X-Omat AR film (Kodak) for autoradiography.

**Armadillo Stabilization Assay**—This assay was performed as described previously (44). The blots were probed with two primary antibodies, mouse monoclonal anti-Arm antibody N27A at 1:50 and mouse monoclonal anti-HSP70 at 1:200,000 and one secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad). Immunoreactive protein bands were visualized by treating the blots with ECL reagents (Amersham Pharmacia Biotech) and then exposing them to x-ray film. Equal loading of total protein was confirmed by inspection of the HSP70 protein band in each sample lane.

**RESULTS**

**Mammalian Expression System Provides an Abundant Source of Recombinant sFRP-1**—To generate a plentiful supply of sFRP-1 protein, we stably transfected MDCK cells with a pcDNA3.1 vector containing the coding sequence of human sFRP-1. MDCK cells have favorable properties for recombinant expression because they grow rapidly and, once confluent, can remain attached to plastic for several weeks in serum-free medium. Consequently, several sequential harvests of conditioned medium can be collected from the same monolayer. A one-step preparative scheme involving heparin-Sepharose affinity chromatography was sufficient to purify sFRP-1 from concentrated conditioned medium (Fig. 1A). Typically we recovered 0.25–0.50 mg of sFRP-1/liter of medium from the transfected mass culture. Silver staining and immunoblot analysis confirmed the purity and identity of the recombinant protein that eluted from heparin-Sepharose with 1.0 M NaCl (Fig. 1A). The protein band in both analyses usually was broad and occasionally resolved into two or three components (Fig. 1B, inset), indicative of microheterogeneity. This was borne out by microsequencing, which revealed that the majority of protein had an amino-terminal sequence beginning at Ser-31, one residue downstream from the proposed signal peptide cleavage site (33). Two other sequences, beginning at Asp-41 and Phe-50, also were obtained and presumably resulted from partial proteolysis. Glycosylation may account for additional heterogeneity.² To optimize the yield of recombinant protein, clonal lines were isolated from the mass culture, and their conditioned media were screened for sFRP-1 content (Fig. 1D). Clone 11 cells (lane 11 in Fig. 1D) were expanded for large scale preparations and yielded 2–4 mg of sFRP-1/liter of conditioned medium.

**sFRP-1 Deletion Mutants Define Heparin-binding Region**—We generated a series of deletion mutants that would allow us to correlate binding properties with particular regions of the sFRP-1 molecule. To facilitate detection and purification, c-Myc and polyhistidine epitope tags were attached to the carboxyl terminus of each derivative (Fig. 2A). The sFRP-Δ1 sequence extends through amino acid residue 171, a short distance beyond the CRD. sFRP-Δ2 and sFRP-Δ3 contain progressively more of the carboxyl-terminal region. Included within sFRP-Δ3 is a lysine-rich domain previously identified as a consensus binding site for hyaluronic acid (33). sFRP-ΔCRD lacks the CRD but contains the remaining amino-terminal and entire carboxyl-terminal sequences. All the sFRP-1 derivatives were readily secreted and remained in solution after ultrafiltration, chromatography, dialysis, and repeated freeze-thawing, suggesting that there were no gross defects in folding. The proteins were purified to homogeneity by using nickel resin chromatography (Fig. 2B). Initial characterization of these molecules focused on their heparin-binding properties because of the potential importance of this binding trait to the interaction with Wnt proteins. Although full-length sFRP-1 labeled with the c-Myc and histidine tags (sFRP-M/H) eluted from heparin-Sepharose in the same position as native sFRP-1, sFRP-Δ1 and sFRP-Δ2 were not retained on the resin (Fig. 2C). Inclusion of the lysine-rich segment in sFRP-Δ3 resulted in a protein with

² A. Uren and J. S. Rubin, unpublished observations.
sFRP-1 Binds Wg and Is a Biphasic Wnt Modulator

Intermediate heparin-binding capability, eluting with 0.5 M NaCl. This implied that the heparin-binding properties of intact sFRP-1 probably involve multiple sites distributed in the carboxyl-terminal third of the molecule. Consistent with this view, sFRP-ΔCRD bound heparin-Sepharose in a manner similar to that of the native protein (Fig. 2C).

Wg Binds to sFRP-1, but the CRD Is Not Required for This Interaction—We used several independent approaches to test the hypothesis that Wnt protein can bind to sFRP-1. First, we employed an ELISA to measure sFRP-1 binding to Wg. Wells were coated with purified full-length sFRP-1 and then blocked with an excess of BSA. Subsequently, conditioned medium from S2HSWg cells expressing soluble Wg was incubated in the wells overnight at room temperature. As a control, aliquots of the same medium were incubated in wells treated with BSA but not sFRP-1. In addition, other wells coated with sFRP-1 were incubated with medium from S2 cells that did not express Wg. As illustrated in Fig. 3A, Wg bound specifically to the wells coated with sFRP-1, and the amount of bound Wg varied with the dilution of Wg medium. In contrast, little Wg was detected in wells that had not been treated with sFRP-1, and no signal was observed when medium lacking Wg was used in the assay (Fig. 3, A and B). These results strongly suggest that sFRP-1 can bind Wg and presumably other Wnt proteins as well.

Based on these findings, we performed a similar analysis using wells coated with the various sFRP-1 deletion mutants (Fig. 3C). Surprisingly, the data indicated that the CRD was not required for Wg binding. In fact, the amount of Wg detected in wells coated with sFRP-ΔCRD matched that seen in wells treated with full-length, epitope-tagged sFRP-1. On the other hand, derivatives lacking portions of the carboxyl-terminal region showed reduced Wg binding. sFRP-Δ2 exhibited intermediate binding avidity, whereas sFRP-Δ1 and sFRP-Δ3 had only limited binding activity. No binding was observed in wells treated with BSA alone (data not shown). These results implied that although the CRD might confer a component of the binding capacity, the carboxyl-terminal region of sFRP-1 was primarily responsible for its ability to bind Wg.

In these experiments, wells were coated in parallel with the same molar concentration of the various sFRP-1 derivatives, and analysis indicated that comparable amounts of each derivative adhered to the well surface. Therefore, the contrasts in relative binding efficiency were not attributable to differences in the concentration of protein coating the wells. However, it was conceivable that the sFRP-1 derivatives could adsorb to the well surface in ways that would differentially mask a Wg binding site. Therefore, we also compared their ability to bind Wg in solution. To wells coated with native sFRP-1, we added Wg medium that had been preincubated for 45 min with varying concentrations of the sFRP-1 mutants. The ability of the mutants to interact with Wg was indicated by the extent to which they could inhibit Wg binding to the wells. The results of these experiments (Fig. 3D) were in good agreement with the previous pattern: sFRP-ΔCRD competed for Wg binding as effectively as sFRP-1 MH, whereas sFRP-Δ2 had a partial effect. sFRP-Δ1 and sFRP-Δ3 had little or no efficacy in the competition assay. Thus, the observed differences in Wg binding to the sFRP-1 derivatives were not caused by inadvertent masking of binding sites but were due to the intrinsic properties of the derivatives.

The association of sFRP-1 proteins with Wg was also examined in co-precipitation experiments. Following incubation of epitope-tagged sFRP-1 mutants with Wg medium, proteins were precipitated with anti-Myc and subsequently immunoblotted with anti-Wg (Fig. 4). Approximately 10–20% of Wg protein was precipitated with either sFRP-ΔCRD or sFRP-Δ1 MH. A weak association was detected with sFRP-Δ2, but none was observed with sFRP-Δ1 or sFRP-Δ3. Thus, both ELISA and co-precipitation experiments showed that the CRD was not required for Wg binding.

Cross-linking Establishes Direct Interaction of sFRP-1 with Wg That Is Enhanced by Optimal Dose of Heparin—All the binding studies described above provided evidence that sFRP-1 and Wg can associate with each other. However, because only one of the reagents, sFRP-1, was purified to homogeneity, we could not exclude the possibility that an unidentified factor in the Wg medium might mediate the binding interaction between sFRP-1 and Wg. To examine this possibility, covalent affinity cross-linking experiments were performed with radiolabeled sFRP-1 and conditioned medium from Wg-expressing and con-

Fig. 2. Identification of sFRP-1 heparin-binding domain. A, schematic of sFRP-1 and its derivatives. Numbers indicate amino acid residues in sFRP-1 sequence at boundaries of recombinant proteins. CRD (hatched boxes) borders also are shown. The white boxes correspond to lysine-rich segments. MH indicates the Myc-His epitope tags. B, anti-Myc immunoblot (left panel) and silver stain (right panel) analysis of purified sFRP-1 mutant proteins. The positions of molecular mass markers are indicated at the left. C, conditioned media from MDCK cells transfected with sFRP-1 derivatives were applied to heparin-Sepharose columns. Samples were eluted with indicated concentrations of NaCl, and fractions were analyzed by Western blotting with anti-Myc.
trol S2 cells. Following incubation of reactants as described under "Experimental Procedures," proteins were immunoprecipitated with anti-Wg and resolved by SDS-PAGE, and cross-linked complexes were detected by autoradiography (Fig. 5A).

No complexes were observed in the absence of cross-linker or Wg. In contrast, two distinct radiolabeled bands were evident when the cross-linking reaction was carried out in the presence of Wg. The lower band had an apparent molecular mass consistent with a complex comprised of one molecule each of sFRP-1 and Wg. This is the strongest evidence to date that the two proteins can interact directly with each other.

The difference in apparent size of the upper and lower bands was 35 ± 2.9 kDa (mean ± S.D., calculated from four experiments), which corresponds closely to the molecular mass of sFRP-1 and Wg. This suggests that the upper band might represent a ternary complex with a third unidentified partner linked to sFRP-1 and/or Wg. The absence of both bands when Wg was lacking from the cross-linking reaction, when anti-Wg immunoprecipitation was omitted, or in the presence of an excess of unlabeled sFRP-1 demonstrated that sFRP-1 and Wg were present in both complexes (Fig. 5A and data not shown). Comparable displacement of 125I-sFRP-1 by unlabeled sFRP-1 suggested that the binding affinity of tracer in the two complexes was similar (Fig. 5B). Unlabeled sFRP-D1 and sFRP-D2 also competed with tracer for binding in both complexes, although neither was as potent as full-length sFRP-1 (data not shown).

Because sFRP-1 and Wg are both heparin-binding proteins and because heparan-sulfate proteoglycan (HSPG) had been shown to regulate Wg/Wnt activity in vivo, we investigated the effect of heparin in the cross-linking experiment. Initial studies revealed that heparin at a concentration of 10 μg/ml caused a dramatic increase in the intensity of both bands corresponding to cross-linked complexes (Fig. 5A). Subsequently, a dose-response analysis indicated a biphasic pattern in which optimal stimulation was observed with 1–10 μg/ml of heparin (Fig. 5C). This effect was specific for heparin, because no stimulation was observed when chondroitin sulfate, keratan sulfate, or hyaluronic acid were used under similar conditions (data not shown).

These data indicated that heparin and presumably HSPG have a marked impact on the interaction of sFRP-1 and Wnt proteins, as represented by Wg in this study.

sFRP-1 Binds Wg and Is a Biphasic Wnt Modulator

![sFRP-1/Wg ELISA binding experiments.](https://example.com/sfrp-1.png)

**Fig. 3.** sFRP-1/Wg ELISA binding experiments. A, ELISA wells were coated with sFRP-1 or BSA alone and incubated with dilutions of Wg-containing or S2 control medium. Bound Wg protein was detected with anti-Wg and secondary immune reagents as described under “Experimental Procedures.” B, conditioned media from control S2 or Wg-expressing S2 cells were analyzed by immunoblotting with anti-Wg. The arrow at the right indicates primary Wg band. Positions of molecular mass markers are shown at the left. C, ELISA wells were coated with sFRP-1 derivatives and incubated with indicated dilutions of conditioned media containing Wg. D, ELISA wells were coated with sFRP-1 and incubated with Wg-containing media that had been pre-incubated with the indicated concentrations of sFRP-1 derivatives. Each panel is representative of several experiments.

![Co-precipitation of sFRP-1 derivatives and Wg.](https://example.com/co-precipitation.png)

**Fig. 4.** Co-precipitation of sFRP-1 derivatives and Wg. sFRP-1 mutant proteins were incubated with Wg-containing media, precipitated with anti-Myc, and immunoblotted with anti-Wg (upper panel) or anti-Myc (lower panel). Serial dilutions of Wg medium were also analyzed. Note that sFRP-Δ1 migrated near the bottom of the gel in the lower panel. The positions of molecular mass markers are shown at the right. IP, immunoprecipitation.
Indeed, high concentrations of sFRP-1 (10 and 25 μg/ml) blocked Wg activity (Fig. 6A). However, lower concentrations of sFRP-1 had the opposite effect: as little as 20 ng/ml (0.6 nM) of sFRP-1 incubated with Wg medium caused a significant increase in the amount of Arm protein relative to that observed with Wg medium alone. Maximal Arm response was seen with 100–500 ng/ml of sFRP-1. This potentiating effect was not attributable to a prolongation of the Wg half-life in solution, because Wg half-life was much longer than the duration of the assay, even in the absence of sFRP-1.3 sFRP-1 had no effect on Arm levels in the absence of Wg and no effect on S2 cells lacking DFz2 expression (data not shown). Thus, sFRP-1 activity presumably involved an interaction with Wg that required signaling through DFz2.

We also compared sFRP-M/H, sFRP-DCRD, and sFRP-D2 in the Arm assay. sFRP-M/H behaved like native sFRP-1 at the concentrations tested (0.02–2 μg/ml), enhancing Wg-dependent stabilization of Arm (Fig. 6B). This implied that the addition of Myc and histidine epitope tags did not alter its biological activity. sFRP-DCRD and sFRP-D2 also increased the activity of Wg in this assay, although their potency was reduced, especially that of sFRP-D2, relative to sFRP-M/H (Fig. 6, B–D). For technical reasons, we were unable to test their ability to inhibit Wg signaling at the highest concentrations. Nonetheless, taken together these results demonstrated that the recombinant proteins used in the binding analysis were biologically active. This reinforced the conclusions drawn above concerning the structural requirements for Wg binding. In particular, the CRD was not required either for binding or biological activity, although its absence reduced the specific activity of sFRP-1.

DISCUSSION

One of the primary objectives of the present study was to test the hypothesis that sFRP-1 and Wnt protein bind directly to each other. Previous reports described co-precipitation experiments in which various sFRP family members were shown to associate with one or more Wnt proteins. Although these results supported the idea that sFRP and Wnt molecules interact, they did not address the possibility that their association might

---

3 S. Cumberledge, unpublished observations.
be indirect, mediated by a factor that could bind both parties. This was a distinct possibility because neither protein was used in a purified state. In addition, some of the earlier observations were made with cells co-expressing both recombinant proteins such that association might occur during their synthesis and would not reflect a normal pattern of interaction. We endeavored to minimize the contribution of indirect effects by using purified preparations of sFRP-1 and an independent source of Wg. sFRP-1/Wg binding was demonstrated both in solid phase and solution assays, utilizing ELISA and co-precipitation formats. Covalent cross-linking of $^{125}$I-sFRP-1 with Wg provided the strongest evidence of a direct interaction between the two proteins. Surprisingly, besides detecting a cross-linked complex consistent in size with one sFRP-1 and one Wg molecule, we also observed a larger complex whose size suggested the presence of a second sFRP-1 molecule. Although the exact nature of this larger entity is currently unknown, taken together these results established that sFRP-1 is a direct binding partner for Wnt protein.

**Role of Proteoglycan in Binding of sFRP-1 and Wg**—The $^{125}$I-sFRP-1/Wg cross-linked complexes were detected in the absence of added heparin but were more abundant when the reaction was performed with an optimal concentration of exogenous heparin. Heparin or endogenous HSPG might promote sFRP-1/Wg binding by serving as a scaffold to facilitate interaction between sFRP-1 and Wg. Alternatively, heparin/HSPG might promote binding by stabilizing a conformation of either sFRP-1 or Wg that would increase their mutual affinity or by enhancing ligand or receptor oligomerization. Of note, the ability to bind heparin was not itself sufficient for cross-linking to Wg; similar experiments conducted with Wg medium and a control heparin-binding polypeptide did not yield cross-linked Wg complexes (data not shown). Moreover, the spacer arm of the cross-linking agent was only 11.4 Å long, reinforcing the conclusion that sFRP-1 binds directly to Wg and presumably other Wnt proteins.

Although the effect of heparin on sFRP-1/Wg binding was observed in an artificial, cell-free setting, these results are consistent with other findings suggesting an important role for HSPG in Wnt signaling in vivo. In *Drosophila*, mutations in genes encoding enzymes involved in proteoglycan biosynthesis disrupt Wg signaling (46–48). Heparitinase treatment of mouse kidney primordia in organ culture similarly inhibited Wnt-dependent developmental processes (45). Although our data indicate that heparin/HSPG can have a strong effect on sFRP/Wnt binding, it is also conceivable that they would have an impact on Fz/Wnt interactions. Indeed recent reports suggest that Dally, a cell surface core protein containing heparan sulfate modifications, might be a component of a Wg/Wnt receptor complex (50, 51). In short, our present findings complement the evidence from in vivo studies that HSPG has a profound effect on Wnt activity and specifically suggest that HSPG can regulate Wnt binding interactions with sFRP proteins.

**CRD Is Not Required for Wg Binding to sFRP-1**—Among the most unexpected findings in the present study was the observation that the CRD was not required for Wg binding. The prevailing view that the CRD is the Wnt binding site is based on several experiments in which the Fz CRD conferred Wnt binding and/or responsiveness (20, 21, 23). In addition, carboxyl-terminally truncated Fz derivatives containing the CRD had dominant negative effects on Wg signaling and altered Wg distribution in vivo (52, 53). Although in some instances, adjacent non-CRD sequence was also present and conceivably could have contributed to the interaction with Wnt protein. Nonetheless, the use of constructs that corresponded more precisely to the boundaries of the CRD provided compelling evidence that this domain can bind Wnts. However, these studies do not exclude the possibility that additional Wnt binding sites might exist elsewhere in Fz or sFRP molecules. The recent description of another soluble Wnt antagonist that lacks a Fz CRD but is thought to bind Wnt protein provides support for the idea that there are other Wnt binding domain(s) (54). With regard to sFRPs, a small deletion in the CRD of FrzB/sFRP-3 did not eliminate binding to Wnt-1, although it did eliminate the ability to modulate Wnt-1 activity (43). However a larger deletion in the CRD did disrupt Wnt-1 binding, although no information was presented regarding the overall stability of this engineered sFRP-3 derivative (43).

In the present study, we have examined the binding of human sFRP-1 with *Drosophila* Wg. Although there is a high degree of conservation between Wg and vertebrate Wnts, it is possible that the interactions we observed were not entirely representative of the interactions that occur between sFRP-1 and Wnts within the same species. Perhaps the relative contributions of the CRD and non-CRD sequences to sFRP/Wnt binding would differ in a homologous system.

Evidence that sFRP-ΔCRD can bind Wg came from multiple experimental models and was highly reproducible. The proteins were shown to interact both in a solid phase assay and in solution. Lacking from these results was proof that the proteins bind directly to each other. Our attempts to document direct binding of sFRP-ΔCRD and Wg by covalent cross-linking have thus far been unsuccessful. Although this might be attributable to a variety of technical issues, it also is consistent with the idea that their binding is indirect. In this regard, it is noteworthy that sFRP-ΔCRD retained the full heparin-binding capacity of the native protein. Therefore, it is possible that this sFRP-1 derivative associated with Wg via soluble HSPG, whose presence in Wg-containing S2 conditioned medium had been previously inferred (44). Such a complex would not likely be detected in our experiments based on the cross-linking properties of BS²; correspondingly, we have not observed heparin cross-linked by BS² to $^{125}$I-sFRP-1 or a number of other heparin-binding tracer proteins (Fig. 5). Although the details of their interaction have not been fully defined, the ability of sFRP-ΔCRD to enhance the activity of Wg in the Arm stabilization assay distinguished it from another heparin-binding protein (data not shown) and indicated that its association with Wg has biological relevance.

The carboxyl-terminal deletion mutants that retained the CRD were remarkable for their relatively weak association with Wg. In principle their limited binding might be attributed to the improper folding of artificially engineered proteins, although there was no evidence that this was the case. While our manuscript was in preparation, Bafico *et al.* (55) reported that a sFRP-1 truncation mutant retaining the CRD was able to co-purify with Wnt-1 and Wnt-2. Because their experiments were performed with whole cell lysates from cells co-transfected with sFRP-1 and either of the Wnt proteins, high local concentrations and/or cofactors might have contributed to the observed interactions. Our studies demonstrated that of all the truncation mutants, sFRP-Δ2 exhibited an intermediate capacity to interact with Wg. This implies that sFRP-Δ2 shares a portion of a Wnt binding epitope with sFRP-ΔCRD or that it contains another binding site involving the CRD that was perturbed in the Δ1 and Δ3 mutants.

**Biphasic Modulation of Wg Signaling by sFRP-1**—Previous studies involving co-expression of sFRP and Wnt proteins in the same cells indicated that sFRP family members can inhibit Wnt signaling. This was true in early *Xenopus* embryos because co-injection of mRNA encoding sFRP and Wnt molecules blocked Wnt-dependent duplication of the dorsal axis (30, 31,
sFRP-1 binds Wg and is a biphasic Wnt modulator

33, 39), and in transfected cells in culture where stabilization of β-catenin was inhibited (43, 55). In these instances, high local concentrations of the proteins would have been likely, corresponding to the high end of the sFRP-1 dose-response experiment in the present report that also resulted in Wnt inhibition. Our work is the first to show that sFRP can enhance Wnt signaling under certain conditions. Biphasic regulation by sFRP-1 provides a mechanism to facilitate the position-dependent properties of Wnt signaling; cells in close proximity to sources of sFRP-1 would be more refractory to Wnts, whereas cells at a greater distance would have their response to Wnts potentiated by a lower sFRP-1 concentration. Such a pattern has recently been described for short gastrulation (Sog), which reportedly binds and inhibits Decapentaplegic near the source of Sog where its concentration is high but enhances Decapentaplegic activity at a distance through a diffusion-dependent mechanism (56). Regulation by overlapping Wnt and sFRP gradients could generate a high degree of spatial specificity to Wnt responses, a well recognized characteristic of Wnt signaling during development (1).

The molecular mechanism responsible for biphasic modulation of Wg signaling by sFRP-1 is open to speculation. We postulate that this effect is due to the presence of two distinct binding sites for sFRP-1 on Wg that vary in their affinity: binding to the high affinity site would promote Wnt signaling, whereas binding to the low affinity site would inhibit it. Perhaps a higher affinity interaction of Wg with the carboxy-terminal domain of sFRP-1 promotes signaling by presenting a favorable Wg conformation to Fz, whereas additional lower affinity binding via the CRD competes with Fz. This might involve a single sFRP-1 molecule binding to one Wg molecule, but it also could entail two sFRP-1 molecules interacting with one Wg. The cross-linking data raise the possibility that sFRP-1 and Wg might interact with both 1:1 and 2:1 stoichiometry. Only a very small percentage of sFRP-1 tracer was detected as a homodimer in our cross-linking experiments, suggesting that 2:1 stoichiometry probably is not due to binding of an sFRP-1 homodimer to Wg. Although displacement experiments with unlabeled sFRP-1 did not indicate an obvious difference in tracer affinity in the two complexes, relative affinities of the two hypothetical sFRP-1/Wg binding sites might vary with local factors in vivo, such as specific HSPG composition. Even if the larger cross-linked complex does not contain two sFRP-1 molecules, the basic hypothesis that there are two distinct sFRP-1 binding sites with different affinities might still be valid; tracer concentrations in the cross-linking experiments might have been too low for detection of binding to the proposed lower affinity site. Alternative mechanisms also could account for a biphasic pattern of regulation. For instance, sFRP-1/Wg might act as an agonist at low sFRP-1 concentrations, but at high concentrations sFRP-1 could interact with Fz or another cell surface component and block signaling. Bafico et al. (55) recently presented evidence that sFRP-1 and Fz protein can associate with each other. Further investigation is necessary to distinguish between these possibilities.

Quantitative Analysis of sFRP-1/Wg Interactions—The experimental designs used in this study did not allow for a direct determination of an sFRP-1/Wg binding affinity constant. The inability to ascertain the concentration of Wg in conditioned medium prevented us from quantifying the ratio of bound versus free ligand in the ELISA. Use of soluble sFRP-1 derivatives in the ELISA competition model did enable us to compare the relative affinities of these molecules in this setting. However, we could not readily estimate absolute affinity from the competition data because the protein concentration required for displacement of Wg binding was dependent on the number of binding sites in the wells. Moreover, even the relative Wg binding affinities of the sFRP-1 derivatives in our various assay systems could be affected by differences in experimental conditions, such as HSPG/heparin content. Estimation of affinity by titrating the displacement of an sFRP-1 tracer with unlabeled ligand in the cross-linking experiment was complicated by the presence of heparin, which can bind sFRP-1 and thereby alter the concentration of the free sFRP-1 pool. Ignoring this effect, the apparent affinity was in the range of 10–30 nM, rather close to the ~9 nM affinity recently calculated for the interaction of XWnt8 and mFz8 (20). In addition to these approximations, the Arm stabilization assays showed that recombinant sFRP-1 elicited a biological response at a subnanomolar concentration and activation was maximal at ~15 nM. The higher concentrations required for inhibition of Wnt signaling might occur in restricted locations near the sites of sFRP-1 synthesis.

Implications for sFRP Research—The results of the present study establish that sFRP-1 can bind Wg and regulate Wnt signaling. We infer that other members of the sFRP subfamily have similar properties, although much work will be required to define the specific relationships that govern the interactions of the many Wnts, sFRPs, and Fzs. The availability of purified recombinant protein, binding, and biological assays as described in this report will facilitate this effort. Of particular interest will be the role of proteoglycan in the various protein-protein interactions that characterize Wnt/Fz/sFRP signaling and the generality of biphasic regulation by sFRP molecules. With regard to sFRP-1, recent reports suggest that it has pro-apoptotic activity (35) and is up-regulated in certain settings following serum withdrawal (57). Its chromosomal locus at 8p11–12 (33) is a site associated with loss of heterozygosity for a variety of malignancies, and sFRP-1 expression is absent from a high percentage of human breast tumor specimens (58). Taken together, these observations suggest that sFRP-1 might function as a tumor suppressor, consistent with its ability to inhibit Wnt signaling at high concentrations. Future investigation will address the potential role of sFRP-1 and related molecules in the pathogenesis of cancer as well as in developmental processes mediated by Wnt proteins.

Acknowledgments—We thank Veena Kapoor for performing transfections with sFRP-1 constructs and the Neuse lab for providing s2 cells and S2 cells expressing Wg (S2HISWg) as well as Wg mAb 4D4. Anti-Arm (mAb N27A1) was kindly provided by Dr. Eric Wieschaus.

REFERENCES
1. Cadigan, K. M., and Nusse, R. (1997) Genes Dev. 11, 3286–3305
2. Dan, T. C. (1996) Biochem. J. 329, 299–231
3. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) Science 275, 1784–1787
4. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Science 275, 1787–1790
5. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) Science 275, 1790–1792
6. de la Coste, A., Romagnolo, B., Billuart, P., Renard, C. A., Buendia, M. A., Soubran, O., Fabre, M., Chelly, J., Beldjord, C., Kahn, A., and Perret, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8847–8851
7. Devereux, T. R., Anna, C. H., Foley, J. P., White, C. M., Sills, R. C., and Barrett, J. C. (1999) Oncogene 18, 4726–4733
8. Behrens, J., von Kries, J. P., Kuhl, M., Brunn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996) Nature 382, 638–642
9. Molenaar, M., van de Wetering, M., Oosterwold, M., Peterson-Maduro, J., Godsau, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996) Cell 88, 391–399
10. van de Wetering, M., Cavallio, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hush, D., Jones, T., Bejooske, A., Peifer, M., Morin, M., and Clevers, H. (1997) Cell 88, 789–799
11. Peifer, M. (1997) Science 275, 1722–1725
12. He, T. C., Sparks, A. B., Katzen, S., Hecox, J., and Potten, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15081–15086
13. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D’Amico, M., Pestell, R., and Ben-Ze’ev, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5522–5527
14. Tetsu, O., and McCormick, F. (1999) Nature 396, 422–426
15. Pennisi, E. (1998) Science 281, 1438–1441
