Journal of Cellular Biochemistry 102:1519–1528 (2007)

Structure–Function Analysis of Secreted Frizzled-Related Protein-1 for its Wnt Antagonist Function

Ramesh A. Bhat,* Barbara Stauffer, Barry S. Komm, and Peter V.N. Bodine

Women's Health & Musculoskeletal Biology Division, Wyeth Research, Collegeville, Pennsylvania 19426

Abstract Secreted frizzled-related proteins (sFRPs) are glycoproteins that are recognized as Wnt antagonists. To identify the functional domains that are involved in Wnt antagonist function, several sFRP-1 mutants and sFRP-1/sFRP-2 chimeras were generated. These mutants were characterized in an optimized T-cell factor (TCF)-luciferase based assay in U2OS human osteosarcoma cells. Deletions of the sFRP-1 cysteine rich domain (CRD) lead to the complete loss of Wnt antagonist function. A region between amino acids 73–86 within the second loop of the CRD of sFRP-1 was necessary for the optimal Wnt inhibitory function. Within this region, a conserved tyrosine residue played a critical role, and its change to neutral or polar amino acids lead to decreased Wnt inhibitory activity. The sFRP-1/sFRP-2 chimeras with the netrin domain of sFRP-1 replaced by corresponding sFRP-2 sequences showed 40–70% loss of Wnt antagonist function. The sFRP-1/sFRP-2 chimera with the replacement of C-terminal 19 amino acids of sFRP-1 with 11 amino acids of sFRP-2 resulted in 70% loss of activity indicating that carboxyl-terminal region of sFRP-1 is important for its Wnt inhibitory activity. The structure–function analysis studies of sFRP-1 clearly demonstrate the interaction of several functional domains for its optimal Wnt antagonist function. J. Cell. Biochem. 102: 1519–1528, 2007.

Key words: sFRP-1; sFRP-2; sFRP-3; frizzled receptor; Wnt, cysteine rich domain

The secreted frizzled–related proteins (sFRPs) are ~32–40 kDa glycoproteins that were identified as antagonists of Wnt signaling [Finch et al., 1997; Melkonyan et al., 1997; Rattner et al., 1997; Uren et al., 2000; Kawano and Kypta, 2003]. In mammals, there are five sFRPs, grouped into two subfamilies based on sequence homology. sFRP-1 is most closely related to sFRP-5 and sFRP-2 (56% and 36% amino acid similarity, respectively) and is more distantly related to sFRP-3 and sFRP-4 (19% and 17% amino acid similarity, respectively). The sFRPs contain three structural units: an amino terminal signal peptide, a frizzled (FZD) type cysteine-rich domain (CRD) and a carboxy-terminal netrin domain. The CRD spans ~120 amino acids, contains 10 conserved cysteine residues and has 30–50% sequence similarity to the CRD of FZD receptors. The disulphide linkage and cysteine spacing of human sFRP-1 has been determined, and the cysteine spacing of the CRD is highly conserved throughout the homologs and orthologs [Chong et al., 2002]. Crystallographic data of the CRD of mouse sFRP-3 and mFZD 8 has been resolved, and the structures have revealed the potential for the CRD to homodimerize or heterodimerize between sFRP and FZD [Dann et al., 2001]. Several biochemical studies have shown the interaction of the sFRP CRD and Wnt and also complex formation of the CRDs of FZD and sFRPs [Bafico et al., 1999]. Such findings suggest that sFRP inhibition of Wnt signaling may operate through at least two mechanisms: (i) by competition with FZD for Wnt ligands or (ii) in a dominant-negative fashion by direct formation of non-signaling inactive complexes with FZD receptors [Bafico et al., 1999].

The carboxyl-terminal half of sFRPs contains a domain that shares some sequence similarity with the axon guidance protein, netrin [Serafini et al., 1994]. This netrin domain is defined by six cysteine residues and several conserved segments of hydrophobic residues and secondary structures. Such a structural domain has also been found in tissue inhibitors of metalloproteinases, Type1 procollagen

*Correspondence to: Ramesh A. Bhat, Women's Health & Musculoskeletal Biology Division, Wyeth Research, 500 Arcola Road, Collegeville, PA 19426.
E-mail: Bhatr@Wyeth.com

Received 13 March 2007; Accepted 15 March 2007

DOI 10.1002/jcb.21372

C-proteinase enhancer proteins and complement proteins C3, C4 and C5 [Banyai and Patthy, 1999]. The netrin domain in sFRP-1 and sFRP-5 contains a highly charged hyaluronan-binding domain that is responsible for the heparin-binding properties of the protein [Uren et al., 2000]. The hyaluronan binding region is shown to be involved in the interaction of sFRP-1 with wingless, the drosophila ortholog of mammalian Wnt-1 [Uren et al., 2000].

The biological activity of sFRPs is largely attributed to their role as regulators of Wnt function. Several studies have suggested a role in the regulation of apoptosis [Melkonyan et al., 1997; Kawano and Kypta, 2003; Han and Amar, 2004; Bodine et al., 2005]. In a knockout mouse model, deletion of sFRP-1 lead to decreased osteoblast and osteocyte apoptosis, increased osteoprogenitor differentiation, enhanced bone formation and elevated bone mineral density [Bodine et al., 2004]. Recently, using a phage display peptide library, a peptide-binding motif that bound to sFRP-1 has been identified [Chuman et al., 2004], and the interaction of sFRP-1 with RANKL that contains the peptide motif has been demonstrated. Such an interaction of sFRP-1 and RANKL lead to the inhibition of osteoclast formation [Hausler et al., 2004]. Thus, the biological role of sFRP-1 has expanded into new avenues beyond its role as a regulator of Wnt action.

Wnt proteins constitute a family of secreted, glycosylated lipoproteins that play essential roles during embryonic development as well as maintenance of tissues [Wodarz and Nusse, 1998; Miller, 2002; Logan and Nusse, 2004; Moon et al., 2004]. Wnt and lipoprotein related protein (LRP) 5/6 binds to the cell surface receptors encoded by the FZD gene family. Receptor activation in turn activates the cytosolic Dishellved (Dsh) protein. In the canonical Wnt signaling, Dsh is involved in turning off a protein complex dedicated to the degradation of β-catenin, resulting in the cytoplasmic accumulation of β-catenin. The β-catenin enters the nucleus and along, with the T-cell factor/lymphoid enhancer factor (TCF/LEF) and other transcription factors, interacts with the target genes and regulates their transcription. A canonical Wnt response is generally measured in transient systems by utilizing a reporter gene linked to either a minimal promoter with the TCF binding element upstream of it or the promoter of the Wnt target genes. In such a functional assay, Wnt upregulates the reporter gene, and this activation of the reporter gene is inhibited by sFRPs [Lin et al., 1997; Bhat et al., 2004]. In the present work, we have generated several sFRP-1 mutants and chimeras of sFRP-1 and sFRP-2 and characterized the mutants for their Wnt antagonist activity using an optimal TCF-luciferase reporter assay. The studies with the sFRP-1 mutants have identified several critical domains important for its Wnt antagonist function.

MATERIALS AND METHODS

Expression Plasmids

Human sFRP-1 cDNA was cloned as a *Bam*HI and *Xba*I fragment into the corresponding sites in pcDNA 3-expression plasmid (Invitrogen, Carlsbad, CA). Human sFRP-3 was cloned as an Asp 718-*Xho*I fragment, and hsFRP-2 was cloned as a *Hin*dIII and *Xba*I fragment into the pcDNA 3-expression vector.

sFRP-1 CRD Deletion

The CRD deletion mutant (amino acids Δ115–163) was generated by PCR amplification of sFRP-1 with 5′ primers with a *Bam*HI site:AAAGGATCCGGCATGGGCATCGGGCGCAGC and 3′ primers with an *Aat*II site: CGGGCGCGACGTCCGAGCAGAGGAAGA. The CRD deletion mutant (amino acids Δ71–163) was generated using the 5′ primer (as above) and 3′ primer with *Aat*II site: CTTCTTGACGTCCACGTTGTGGCACAG. The HotStar Taq DNA polymerase (Qiagen, Valencia, CA) was used for PCR under the following conditions: 94°C, 15′; denaturation at 94°C, 30 s; annealing at 55°C, 30 s; extension at 72°C, 45 s for 5 cycles, 94°C, 30 s; 58°C, 30 s; 72°C, 45 s; for 20 cycles and final extension at 72°C for 7 min. The purified PCR fragments were digested with *Bam*HI and *Aat*II, fractionated on an agarose gel, purified and cloned along with *Aat*II to *Xba*I fragment from sFRP-1 plasmid into a *Bam*HI and *Xba*I fragment of pcDNA 3 expression vector.

sFRP-1 and sFRP-2 Chimera

sFRP-1/sFRP-2 (amino acids 1–226/214–295) chimera was generated by PCR amplification of the respective fragments and cloning the *Bam*HI–*Xho*I fragment of sFRP-1 and XhoI and *Xba*I fragment of sFRP-2 into *Bam*HI–*Xba*I portion of pcDNA3 expression vector. The

following primers were used for the amplification. sFRP-1 5′ (see above) sFRP-1 3′ TATCTCGAGGACAATCTTCTTGTCGCCA, sFRP-2 5′ primer ATCCTCGAGACCAAGAGCAAGACCA, sFRP-2 3′ primer TAATCTAGACTAGCACTGCAGCTTGCGGAT. sFRP-1/sFRP-2 (amino acids 1–248/238–295) chimera was isolated by amplifying the *Hin*dIII–*Xba*I fragment of sFRP-2 using the following primers: sFRP-2 5′ primer AAGAAGCTTGTGCTGTGGCTCAAAGACAGC, sFRP-2 3′ primer (see above), and cloning the *Hin*dIII–*Xba*I fragment into the corresponding sites of the sFRP-1 expression plasmid. The sFRP-1/sFRP-2 (amino acids 1–295/285–295) chimera was generated by creating a unique *Eco*RI site in sFRP-1 at amino acid 295 (sFRP-1 primers 5′ see above), sFRP-1 3′ TTTGAATTCCTTGTTTTTCTTTGTCCCA and cloning the PCR amplified portion of sFRP-2 corresponding to 285–295 amino acids (5′ primer GGGCAGAGAGAATTCAAG, 3′ primer see above) into the *Eco*RI site. The sFRP-2/sFRP-1 (amino acids 1–284/296–314) chimera was generated by cloning the *Bam*HI–*Eco*RI fragment of sFRP-2 (PCR 5′ primer AAAGGATCCGGCATGCTGCAGGGCCCTGGCTCG, 3′ primer TGCGGGAGATGCGCTTGAATTCTCTCTG) and *Eco*RI–*Xba*I of sFRP-1 (PCR primer 5′ TGGGACAAGAAAAACAAGGAATTCA and 3′ primer see above) fragment into the *Bam*HI and *Xba*I sites of pcDNA3 expression vector.

sFRP-1 CRD Mutation

The single point mutation and changes in the small functional domains were generated by two step PCR using primers containing the desired mutation and amplifying with the 5′ or 3′ primer to generate the PCR fragment with the desired mutation and cloning them into a pcDNA 3 vector along with the rest of the sFRP-1 fragment derived either by PCR or by restriction digestion of the sFRP-1 expression plasmid. To mutate the tyrosine to phenylalanine the following primers were used: 5′ TGCCACAACGTGGGC**TTC**AAGAAGATG and sFRP-1 3′ CCCTCTAGAATCACTTAAACACGGACTGAAAGGTG. The PCR amplified product was digested with *Dra*III and *Xba*I, and the purified fragment was cloned into *Dra*III and *Xba*I fragment of sFRP-1 in pcDNA3 plasmid. Other sFRP-1 tyrosine mutants were generated by replacing the tyrosine codon sequence of the 5′ primer with the desired amino acid codon and cloning the PCR amplified product as indicated above.

sFRP-1 Hyaluronan Binding Domain Mutation

sFRP-1 coding sequence contains a unique *Apa*I site after the hyaluronan-binding region. PCR primers with changes that convert the lysine codon into alanine were obtained and used to generate the sFRP-1 fragment with a mutation of the hyaluronan-binding region. This fragment was cloned along with the *Apa*I–*Xba*I fragment of sFRP-1 into pcDNA 3-expression vector.

All of the plasmid constructs were verified by DNA sequencing.

Expression Studies With sFRP-1 Mutants

sFRP-1 and mutant plasmids were transfected into U2OS (ATCC, Rockville, MD) cells plated in 60 mm dishes using lipofectamine. After 24 h, the cells were washed with PBS and lysed using RIPA buffer. The clarified cell lysates were fractionated on a 10% NuPAGE gel (Invitrogen), and the proteins were transferred onto a nitrocellulose membrane. Western blot was performed following the ECL plus detection system (Amersham, London) using a sFRP-1 specific polyclonal (H90, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody and a goat anti-rabbit /HRP conjugate as secondary antibody (Rockland, Gilbertsville, PA).

Cell Line and Transfection. The osteosarcoma cell line U2OS (ATCC) was maintained in growth media consisting of McCoy's 5A medium (Invitrogen) containing 10% fetal calf serum (Hyclone, Logan, UT), 2 mM glutamax-1 (Invitrogen) and 1× penicillin and streptomycin (Invitrogen) and incubated at 37°C with 5% CO_2/95% humidified air. For transfection studies, the cells were plated in a 96-well tissue culture plate in growth media without antibiotics and incubated overnight in the incubator. The growth medium was removed, and the cells were washed once with OPTI-MEM I medium (Invitrogen) and were then fed with 100 μl of OPTI-MEM I. The cells were transfected with the following DNA's using lipofectamine as recommended by the manufacturer (Invitrogen). For each transfection, the following DNA's were diluted together in OPTI-MEM I medium: (100 ng of 16× TCF-luciferase [Bhat et al., 2004; Chuman et al., 2004], 20 ng of Wnt3 (Upstate Biotechnology, Lake Placid, NY), 75 ng of sFRP-1 and 25 ng of β-Galactosidase (Clontech, Palo

Alto, CA) and 0.4 μl of lipofectamine 2000 (Invitrogen) in a total volume of 50 μl. The DNA–lipofectamine mixture was then added to each well, and the plates were incubated in a 37°C incubator for 4 h. The medium was then removed, and the cells were washed with 150 μl of phenol red free RPMI 1640 medium (Invitrogen), refed with 100 μl of RPMI medium supplemented with 2% fetal calf serum, 2 mM glutaMAX-1 and 1% penicillin–streptomycin, and incubated in a 37°C incubator overnight. The next day, the cells were washed twice with 150 μl/well of PBS without Ca++ and Mg++ (Invitrogen) and then lysed with 50 μl/well of cell culture lysis reagent (Promega, Madison, WI). The cell lysates were assayed for luciferase (Promega) and β-galactosidase (Tropix, Bedford, MA) activity using a microlumatPLUS luminometer (EG&G Berthhold, BadWildbad, Germany). The luciferase activity was normalized with β-gal to offset the transfection efficiency, and the data was analyzed using the JMP program (SAS Institute, Cary, NC). The antagonist activity of sFRP-1, and its mutants were presented as the percent Wnt activity.

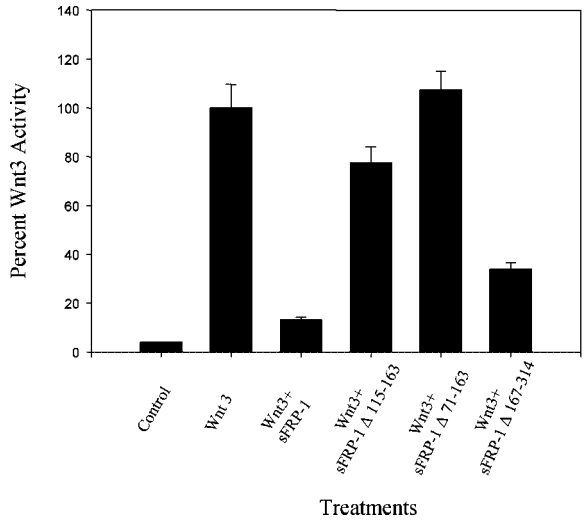

Fig. 1. The CRD domain is critical for Wnt antagonist function of sFRP-1. sFRP-1 and its CRD mutants were co-transfected along with 16× TCF-luciferase reporter, CMV-β-gal and Wnt3 plasmids into U2OS cells. After 20 h, the luciferase and β-gal activities in the cell lysates were measured and the results were normalized with β-gal and presented as percent Wnt3 activity.

RESULTS

The CRD Domain is Critical for Wnt Antagonist Function of sFRP-1

In U2OS cells, transfection of a Wnt3 expression plasmid along with an optimized 16× TCF-luciferase reporter lead to a 30-fold increase in luciferase activity compared to the reporter alone. Co-transfection of wild-type full-length sFRP-1 lead to about 90% inhibition of the Wnt-mediated upregulation of the TCF reporter (Fig. 1). In order to identify the role of the sFRP-1 CRD in Wnt3 antagonist function, deletion of the CRD was undertaken, and the resulting sFRP-1 mutants were assayed for their Wnt antagonist function (Fig. 1). A deletion of 48 amino acids (△115–163) within the CRD lost about 75% of Wnt3 inhibitory activity, while, a larger deletion of 92 amino acids (△71–163) totally abolished the ability of sFRP-1 to antagonize the Wnt-mediated upregulation of the TCF-reporter. These results indicate that the CRD domain is critical for the Wnt antagonist function of sFRP-1 and confirms previous report [Uren et al., 2000]. In support of these data, a truncated sFRP-1 (△167–314) containing the CRD alone was about 80% as active as full-length sFRP-1. These results clearly demonstrate that the sFRP-1 CRD is critical in mediating its Wnt antagonist function.

The sFRP CRD Contains Multiple Functional Domains Required for Optimal Wnt Antagonist Function

The CRD of sFRPs contains 10 conserved cysteine residues, and they form five disulphide linkages [Chong et al., 2002]. Sequence alignment of the CRD domains of sFRP-1 and sFRP-3 indicates about 30% amino acid identity and shows several pockets that differ in their amino acid sequence (Fig. 2a). sFRP-3 is about 60% less active compared to sFRP-1 in its Wnt3 antagonist function (Fig. 3a), and this difference in the Wnt inhibitory activities could be due to amino acid differences within the CRD of sFRP-3. We therefore hypothesized that replacement of sFRP-1 sequence with sFRP-3 would lead to reduced Wnt antagonist activity if the CRD is involved in its function. Several sFRP-1 mutants with amino acid changes in the CRD that correspond to the sFRP-3 sequence were generated and tested for their Wnt antagonist function (Fig. 2b). The change of amino acids HNVGY to KSLPW did not affect the sFRP-1 Wnt antagonist function; however, the change of the sFRP-1 CRD segment from KKMVL to

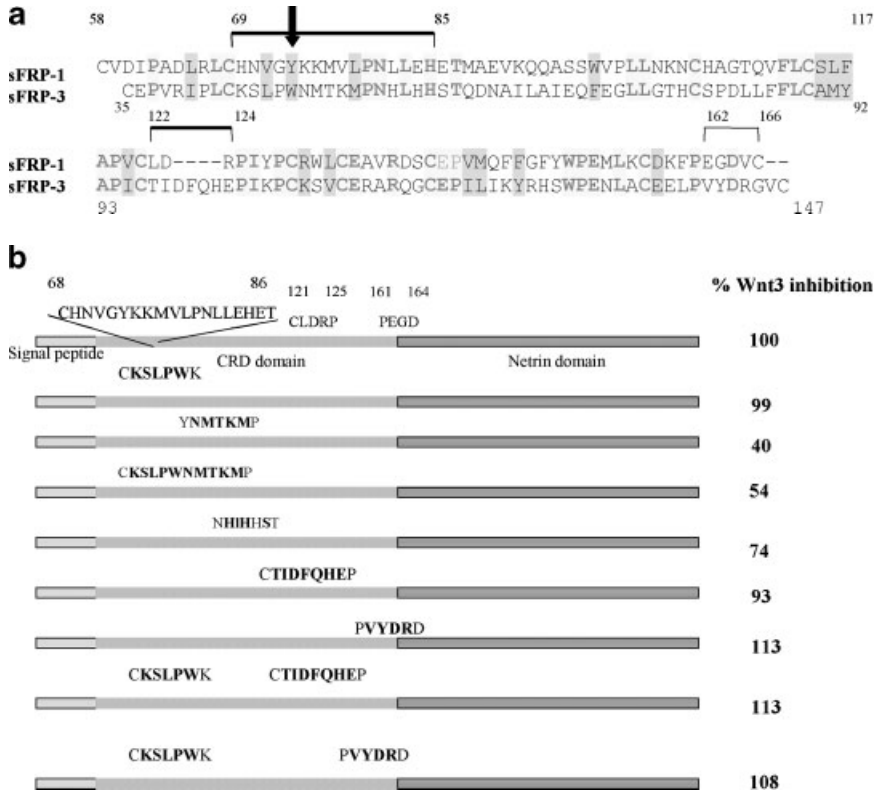

Fig. 2. The sFRP-1 CRD contains distinct domains required for its Wnt antagonist function. **a**: Alignment of sFRP-1 and sFRP-3 CRD sequences. The portion of CRD that is replaced or mutated is shown in brackets with numbers corresponding to sFRP-1. The tyrosine (aa 73) in sFRP-1 is shown by an arrow. Identical amino acids are shown in bold letters, the conserved amino acids are shown in shaded rectangles. **b**: sFRP-1 CRD mutants. The sFRP-1 CRD sequence is replaced by the sFRP-3 amino acids in the mutants. The sequence of sFRP-1 targeted for replacement is shown on the top with amino acid numbers. The amino acid residues in bold letters correspond to the sFRP-3 sequence. The amino acids in normal font represent the sFRP-1 amino acids on either side of sFRP-3 sequence. The Wnt antagonist activity of sFRP-1 and its mutants are shown as percent of Wnt3 inhibition on the right.

NMTKM (as in sFRP-3) resulted in a 60% loss of Wnt3 antagonist activity. Combining the two above changes, i.e. HNVGYKKMVL to KSLPWNMTKM, resulted in a 46% loss of activity. These results clearly indicate that the region between 74 and 78 amino acids plays an important role in the Wnt antagonist function of sFRP-1. Similarly, a change of amino acids LLEHE (81–85) to HLHHS resulted in about a 26% loss of activity. The results clearly indicate that the second loop of the CRD between cysteine 2 and 3 is critical for its Wnt inhibitory function, and amino acids 74–85 play an important role in the Wnt antagonist function of sFRP-1. Replacement of the sFRP-1 sequence LDR with TIDEFQHE residues resulted in a slight decrease in Wnt antagonist activity. However, replacing the HNVGY and LDR with KSLPW and TIDFQHE resulted in greater Wnt antagonist function. A change of sequence from EG to VYDR resulted in greater Wnt3 antagonist activity. The above results indicate that there are multiple distinct regions within the CRD domain that are required for optimal Wnt3 antagonist function of sFRP-1.

A Tyrosine/Tryptophan Residue Within the Second Loop of the CRD is Critical for the Wnt Antagonist Function of sFRP-1

A tyrosine residue (amino acid 73) within the second loop of the CRD is conserved in sFRP 1, 2, and 5 and is replaced by tryptophan in sFRP-3 and sFRP-4 (Fig. 3a). The tyrosine residue is also conserved in all of the 10 FZD receptors [Nusse, 2007]. In order to determine the role of tyrosine in the Wnt antagonist function of sFRP-1, a number of sFRP-1 mutants were generated with changes in the tyrosine residue and assayed for their ability to antagonize Wnt3-mediated upregulation of the TCF-luciferase reporter (Fig. 3b). A change of tyrosine to tryptophan in sFRP-1 did not affect its ability to

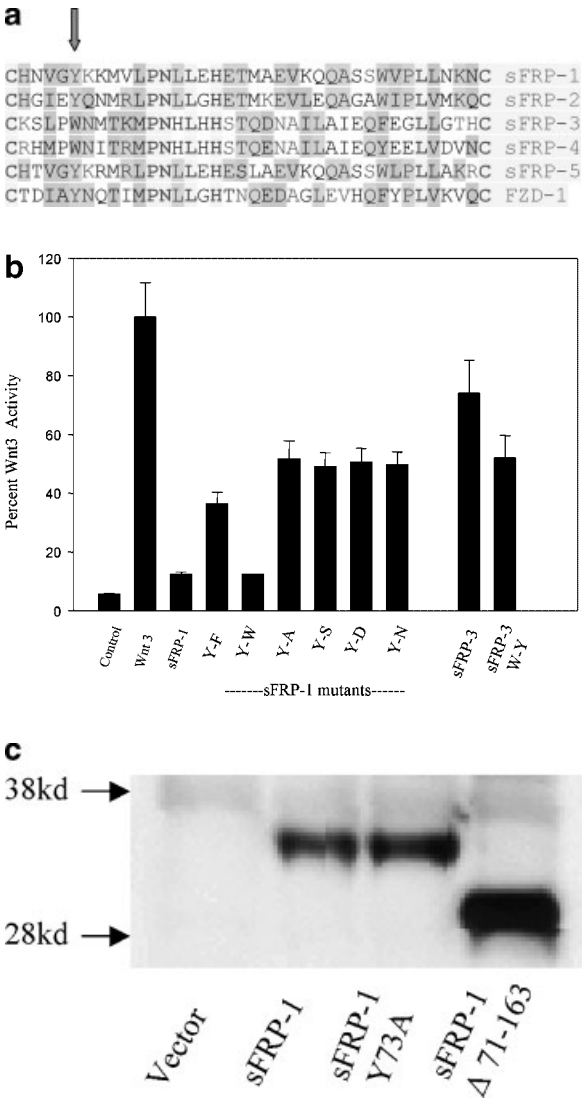

Fig. 3. A tyrosine residue in the second loop of CRD is critical for Wnt antagonist function of sFRP-1. **a**: Alignment of amino acid sequence of sFRPs and FZD1 CRD second loop. Bold letter indicates identical amino acids. The shaded rectangles correspond to the conserved amino acids. The arrow represents the tyrosine residue in sFRP-1, -2, -5, FZD1 and tryptophan in sFRP-3, -4. **b**: Functional analysis of sFRP-1, sFRP-3 mutants. sFRP-1 and sFRP-3 mutants were assayed for their Wnt inhibitory function and their antagonist activity is presented as percent Wnt activity. The amino acids that are mutated are presented in single letters—Y: tyrosine, F: phenyl alanine, W: tryptophan, A: alanine, S: serine, D: aspartic acid, N: asparagine. **c**: sFRP-1 protein expression. sFRP-1 wild type and mutant plasmids were transiently expressed in U2OS cells and the cell lysates were analyzed for sFRP-1 protein by Western blot using sFRP-1 specific antibody.

inhibit Wnt3 action; conversely, a change of tryptophan to tyrosine in the CRD of sFRP-3 resulted in increased Wnt3 antagonist activity. The conservative change of the tyrosine residue in sFRP-1 to phenylalanine reduced its Wnt3 antagonist activity by 27%. Moreover, the change of tyrosine to alanine, serine, aspartate or asparagine further reduced the Wnt antagonist function to about 40% compared to the wild type sFRP-1. The expression levels of the sFRP-1 mutants were similar to those of the wild type protein (Fig. 3c), suggesting that the loss of function is not due to the difference in protein levels, but rather is due to the disruption/alteration of the sFRP-1 structure. The functional analysis of the sFRP-1 mutants clearly demonstrates the key role of tyrosine/tryptophan in the overall architecture of sFRP-1 and in its biological function.

The Netrin Domain Plays a Role in the Optimal Wnt Antagonist Function of sFRP-1

In order to address the role of the netrin domain in the Wnt antagonist function of sFRP-1, chimeras of closely related sFRPs such as sFRP-1 and sFRP-2 were characterized for their Wnt3 antagonist activity. sFRP-2 exhibits about 36% homology with sFRP-1 and has pockets of differences within the netrin domain (Fig. 4a). sFRP-2 is about 25% less active in inhibiting Wnt3-mediated upregulation of 16× TCF-luciferase activity (Fig. 4b). Three sFRP-1/sFRP-2 chimeras with the netrin domain sequences replaced by the corresponding sFRP-2 sequences were generated and tested for their Wnt3 antagonist activity in the transfection assay (Fig. 4b). All three chimeras exhibited less Wnt3 antagonist activity compared to sFRP-1. The sFRP-1 chimera with the replacement of the carboxyl-terminal 88 and 66 amino acids with 82 and 58 amino acids of sFRP-2 resulted in about 40% loss of activity. The chimera with the replacement of the carboxyl-terminal 19 amino acids with 11 amino acids of sFRP-2 resulted in the loss of about 70% of the activity. These results clearly indicate that the carboxyl-terminal sequence of sFRP-1 is important for optimal Wnt antagonist function of sFRP-1.

The Hyaluronan-Binding Domain is Important for Optimal Wnt Antagonist Function of sFRP-1

The sFRP-1 netrin domain contains clusters of multiple lysine residues referred to as the hyaluronan-binding domain [Uren et al., 2000]. The effect of the mutation of lysine clusters in sFRP-1 into alanine on Wnt3 antagonist function is shown in Figure 5. Mutation of the

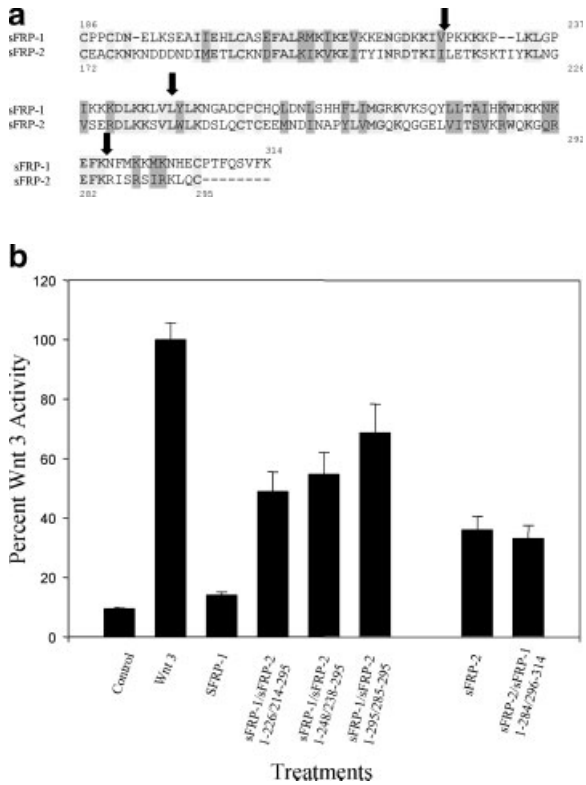

Fig. 4. The netrin domain of sFRP-1 is critical for the optimal Wnt antagonist function. **a**: Alignment of sFRP-1 and sFRP-2 amino acid sequences corresponding to the netrin domain. Bold letters indicate identical amino acids. The shaded rectangles correspond to the conserved amino acids in sFRP-1 and sFRP-2. The arrow represents the crossover in sFRP-1/sFRP-2 chimera. **b**: Wnt inhibitory activity of sFRP-1, sFRP-2 and their chimeras. The Wnt3 inhibitory activity is presented as percent Wnt3 activity.

proximal 5 lysine residues into alanine has a minimal effect on the Wnt antagonist function, whereas, the mutation of distal lysine residues resulted in about a 15% loss of activity. The

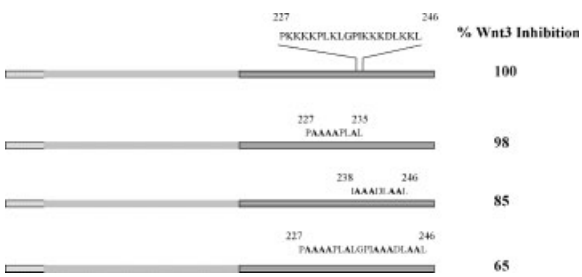

Fig. 5. The hyaluronan binding region in sFRP-1 is required for its optimal Wnt antagonist function. Schematic representation of sFRP-1 and its hyaluronan-binding domain mutants. The sFRP-1 region targeted for mutation is shown on the top. Bold letters represent the mutated amino acids, and the letters in uppercase represent the sFRP-1 amino acids. The Wnt3 inhibitory activity is shown on the right as percent Wnt3 inhibition.

sFRP-1 mutant with a total of 10 lysine residues in the hyaluronan domain changed into alanine resulted in a loss of about 35% of the activity, suggesting that the hyaluronan-binding domain is also important for the sFRP-1 Wnt antagonist function.

DISCUSSION

Wnt signaling is initiated by the binding of Wnt to a membrane receptor complex composed of the FZD receptor and LRP, leading to the activation the canonical or Wnt/β-catenin pathway. The activation of Wnt signaling can be measured either by the increase in the cytoplasmic accumulation of β-catenin or the activation of TCF/LEF-reporter genes [Wodarz and Nusse, 1998; Miller, 2002]. In such assays, sFRP-1 is shown to decrease the Wnt-mediated accumulation of cytoplasmic β-catenin and inhibit the activation of the TCF-reporter. Microinjection of mRNA into xenopus embryos is generally used to validate Wnt signaling, and in such a system, Wnt1-induced axis duplication is inhibited by sFRPs [Lin et al., 1997]. Biochemical studies utilizing the co-immunoprecipitation or ELISA methods are also used to identify the interaction of Wnt and sFRPs; however the results of such physical interaction studies did not correlate with in vivo functional studies using xenopus embryo axis duplication [Lin et al., 1997].

Structure–function studies using sFRP-3 mutants have revealed that the complete removal of the CRD abolishes the Wnt1/sFRP-3 interaction in vitro and the inhibition of the Wnt1-mediated axis duplication in xenopus embryos [Lin et al., 1997]. Removal of the carboxyl-terminal portion of the molecule preserves both the Wnt–sFRP-3 interaction and reduced functional inhibition of axis duplication. In contrast, studies utilizing human sFRP-1 and Wg (wingless, a drosophila Wnt) have shown that the sFRP mutants lacking the CRD retained the ability to bind to Wg, and the deletion of the carboxyl terminal resulted in the reduction or loss of Wg binding. These studies have concluded that the CRD might confer a component of the binding capacity, but the carboxyl-terminal region of sFRP-1 is primarily responsible for its ability to bind Wg [Uren et al., 2000]. Although the above methods provided the insight into the potential mechanism of the Wnt antagonism of sFRP-1, the

studies did not identify the critical regions that are essential to the biological function of sFRPs.

We have used an optimized TCF-luciferase reporter-based assay for measuring the Wnt signaling and Wnt antagonist function of sFRPs. A luciferase-based reporter plasmid containing 16 copies of the TCF-element upstream of a tk promoter was developed [Bhat et al., 2004]. Several cell lines were analyzed for the optimal Wnt response, and the U2OS cells reproducibly showed a good Wnt response with nearly 30-fold activation when co-transfected with a Wnt3 expression plasmid. The amount of Wnt and sFRP-1 transfected were optimized to obtain nearly 90% inhibition with sFRP-1. This optimized transfection method allowed us to characterize the sFRP-1 mutants and to identify the critical regions that are required for Wnt antagonist function. Using this assay system, sFRP-3 is less efficient in inhibiting Wnt3 compared to sFRP-1, which could be due to the differences in the CRD sequences. A change of the sequences in the second loop of-sFRP-1 to those of sFRP-3 have revealed that the amino acids between 73–86 play an important role in the Wnt antagonist function of sFRP-1. In particular, the changes of KKMVL to NMTKM lead to a substantial loss of the antagonist activity. The results correlate very well with studies of alanine scanning mutants of sFRP-3 CRD and its subsequent binding to XWnt8-AP. Mutations around NMTKM lead to either reduced or total loss of the binding of the sFRP-3 mutants to X-Wnt-AP chimera [Dann et al., 2001]. Similarly, a change of LLEHE to HLHHS (as in sFRP-3) affected the Wnt antagonist function of sFRP-1. In sFRP-3, the HHHS residues are exposed based on fractional solvent solubility studies [Dann et al., 2001], and it is possible that subtle changes in the amino acids of sFRPs may alter its secondary and tertiary structures affecting the Wnt antagonist function of sFRP. It is intriguing to note the critical role of tyrosine (amino acid 73) in sFRP-1 for its Wnt antagonist function. This tyrosine is conserved in closely related sFRPs like sFRP-1, sFRP-2 and sFRP-5 and is replaced by tryptophan in sFRP-3 and sFRP-4. The tyrosine is also conserved in all of the FZD receptors [Nusse, 2007]. The change of tyrosine to tryptophan in sFRP-1 did not affect its Wnt antagonist function, whereas a change to phenylalanine did result in about a 20% loss of Wnt antagonist function. A more drastic effect on Wnt antagonist function is seen when the aromatic amino acid, tyrosine, is changed into neutral or polar amino acids such as alanine, serine, and aspartic or asparagine residues. In crystal structure studies with the sFRP-3 CRD and mFZD8 CRD, the tryptophan/tyrosine residue is buried within the CRD structure. In many proteins, tyrosine residues are generally involved in H bonding, either with other amino acid side chains or with water molecule resulting in the stabilized protein structure. The change of the aromatic amino acid tyrosine into neutral or polar amino acids may disrupt such bonding, altering the folding of the molecule and resulting in the loss of Wnt antagonist function of sFRPs. Preliminary studies with the FZD receptor have shown that the tyrosine residue in the second loop indeed is critical for the activation of canonical signaling by Wnt ligand (unpublished results). The change of tryptophan to tyrosine in sFRP-3 results in a gain of Wnt antagonist activity, suggesting that the tyrosine residue is the favored amino acid residue for the optimal Wnt antagonist function of sFRPs.

The chimera of sFRP-1/sFRP-2 showed reduced Wnt antagonist function suggesting that the 3′ netrin domain is important for optimal Wnt3 inhibitory activity. In particular, the chimera with the last 19 amino acids of sFRP-1 replaced by 11 amino acids of sFRP-2 showed only about 30% of sFRP-1 activity, clearly demonstrating the role of the 3′ sequences in the overall structure of sFRP-1. In contrast, the replacement of the sFRP-2 sequence with sFRP-1 did not result in a significant change in its Wnt3 antagonist activity. These results suggest that the amino acid residues interacting with the carboxyl-terminal region may be different, and a low amino acid homology between sFRP-1 and sFRP-2 (36% similarity) supports that notion. Additional functional studies of the carboxyl-terminal mutants of sFRP-1 will help to identify the amino acid residues involved in the optimal Wnt antagonist function. A crystal structure of full-length sFRP-1 is necessary to confirm the role of the carboxyl- terminal region of sFRPs in the structural organization of sFRP-1 and its Wnt antagonist function.

The sFRP-1 3′ region contains multiple lysine residues, and this hyaluronan binding region is shown to be involved in the binding of sFRP-1 to heparin or heparin sulphate proteoglycan

(HSPG) [Uren et al., 2000]. The role of heparin/HSPG in the sFRP1/Wnt binding and in Wnt signaling has been established [Binari et al., 1997; Haerry et al., 1997]. Moreover, the positively charged hyaluronan-binding region of sFRP-1 may play an important role in binding to negatively charged matrix proteins like HSPG that results in increased local concentration of sFRP-1 and thus has a significant local antagonist effect on Wnt signaling. The matrix binding property of sFRP-1 may also result in the tissue-selective effect seen with sFRP. It should be noted that sFRP-1 is one of the key players of Wnt signaling in bone as deletion of this gene in mice lead to an increased bone mass without any apparent non-skeletal phenotypic changes [Bodine et al., 2004].

In the present study, functional analysis of the sFRP-1 mutants and sFRP-1/sFRP-2 chimeras has clearly demonstrated that both the CRD and the netrin domains are necessary for optimal Wnt antagonist function. Recently, a new role has been assigned to sFRP-1, based on the interaction of the sFRP-1 with a peptide sequence that is present in RANK ligand [Chuman et al., 2004; Hausler et al., 2004]. In biochemical interaction studies with sFRP-1 mutants, the peptide sequence, and RANKL, it has been shown that neither the CRD nor the netrin domain alone is sufficient for the binding of sFRP-1 to the peptide, however the last 71 amino acids of sFRP-1 are not necessary for its interaction with the peptide. Apparently, the contact points in both the CRD and netrin domains of sFRP-1 participated in the interaction with the peptide sequence [Chuman et al., 2004]. In contrast to the above findings, the Wnt antagonist function of sFRP-1 requires the CRD domain and also the carboxyl-terminal sequence, and therefore it is tempting to speculate that the functional domains for Wnt antagonist function and peptide interaction may be different or overlapping. Thus, the sFRP-1 mutants described in the present study will also be valuable tools to identify the critical motifs of sFRP-1 in its functions other than Wnt antagonism.

REFERENCES

Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA. 1999. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. J Biol Chem 274:16180–16187.

Banyai L, Patthy L. 1999. The NTR module: Domains of netrins, secreted frizzled related proteins, and type I procollagen C-proteinase enhancer protein are homologous with tissue inhibitors of metalloproteases. Protein Sci 8:1636–1642.

Bhat RA, Stauffer B, Komm BS, Bodine PV. 2004. Regulated expression of sFRP-1 protein by the GeneSwitch system. Protein Expr Purif 37:327–335.

Binari RC, Staveley BE, Johnson WA, Godavarti R, Sasisekharan R, Manoukian AS. 1997. Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. Development 124:2623–2632.

Bodine PV, Zhao W, Kharode YP, Bex FJ, Lambert AJ, Goad MB, Gaur T, Stein GS, Lian JB, Komm BS. 2004. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol 18:1222–1237.

Bodine PV, Billiard J, Moran RA, Ponce-de-Leon H, McLarney S, Mangine A, Scrimo MJ, Bhat RA, Stauffer B, Green J, Stein GS, Lian JB, Komm BS. 2005. The Wnt antagonist secreted frizzled-related protein-1 controls osteoblast and osteocyte apoptosis. J Cell Biochem 96: 1212–1230.

Chong JM, Uren A, Rubin JS, Speicher DW. 2002. Disulfide bond assignments of secreted frizzled-related protein-1 provide insights about frizzled homology and netrin modules. J Biol Chem 277:5134–5144.

Chuman Y, Uren A, Cahill J, Regan C, Wolf V, Kay BK, Rubin JS. 2004. Identification of a peptide binding motif for secreted frizzled-related protein-1. Peptides 25:1831–1838.

Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ. 2001. Insights into Wnt binding and signalling from the structures of two frizzled cysteine-rich domains. Nature 412:86–90.

Finch PW, He X, Kelley MJ, Uren A, Schaudies RP, Popescu NC, Rudikoff S, Aaronson SA, Varmus HE, Rubin JS. 1997. Purification and molecular cloning of a secreted, frizzled-related antagonist of Wnt action. Proc Natl Acad Sci USA 94:6770–6775.

Haerry TE, Heslip TR, Marsh JL, O'Connor MB. 1997. Defects in glucuronate biosynthesis disrupt wingless signaling in Drosophila. Development 124:3055–3064.

Han X, Amar S. 2004. Secreted frizzled-related protein 1 (SFRP1) protects fibroblasts from ceramide-induced apoptosis. J Biol Chem 279:2832–2840.

Hausler KD, Horwood NJ, Chuman Y, Fisher JL, Ellis J, Martin TJ, Rubin JS, Gillespie MT. 2004. Secreted frizzled-related protein-1 inhibits RANKL-dependent osteoclast formation. J Bone Miner Res 19:1873–1881.

Kawano Y, Kypta R. 2003. Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116:2627–2634.

Lin K, Wang S, Julius MA, Kitajewski J, Moos M, Jr., Luyten FP. 1997. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. Proc Natl Acad Sci USA 94:11196–11200.

Logan CY, Nusse R. 2004. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781–810.

Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei LD, Umansky SR. 1997. SARPs: A family of secreted apoptosis-related proteins. Proc Natl Acad Sci USA 94:13636–13641.

Miller JR. 2002. The Wnts. Genome Biol 3: REVIEWS3001.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. 2004. WNT and beta-catenin signalling: Diseases and therapies. Nat Rev Genet 5:691–701.

Nusse R. 2007. The Wnt home page http://www.stanford.edu/~rnusse/wntwindow.html.

Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. 1997. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc Natl Acad Sci USA 94:2859–2863.

Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell 78:409–424.

Uren A, Reichsman F, Anest V, Taylor WG, Muraiso K, Bottaro DP, Cumberledge S, Rubin JS. 2000. Secreted frizzled-related protein-1 binds directly to wingless and is a biphasic modulator of Wnt signaling. J Biol Chem 275: 4374–4382.

Wodarz A, Nusse R. 1998. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14:59–88.