Wnt-1-induced secreted protein 1 (WISP-1) is a member of the CCN (connective tissue growth factor, Cyr61, NOV) family of growth factors. Structural and experimental evidence suggests that CCN family member activities are modulated by their interaction with sulfated glycoconjugates. To elucidate the mechanism of action for WISP-1, we characterized the specificity of its tissue and cellular interaction and identified binding factors. WISP-1 binding was restricted to the stroma of colon tumors and to cells with a fibroblastic phenotype. By using a solid phase assay, we showed that human skin fibroblast conditioned media contained WISP-1 binding factors. Competitive inhibition with different glycosaminoglycans and treatment with glycosaminoglycan lyases and proteases demonstrated that binding to the conditioned media was mediated by dermatan sulfate proteoglycans. Mass spectrometric analysis identified the isolated binding factors as decorin and biglycan. Decorin and biglycan interacted directly with WISP-1 and inhibited its binding to components in the conditioned media. Similarly, WISP-1 interaction with human skin fibroblasts was inhibited by dermatan sulfate, decorin, and biglycan or by treatment of the cell surface with dermatan sulfate-specific lyases. Together these results demonstrate that decorin and biglycan are WISP-1 binding factors that can mediate and modulate its interaction with the surface of fibroblasts. We propose that this specific interaction plays a role in the regulation of WISP-1 function.

WISP-1\(^1\) is a member of the CCN family of growth factors (1–3) composed of WISP-1/Elm1, WISP-2/Cop1, WISP-3, connective tissue growth factor (CTGF), cysteine-rich 61 (Cyr61), and nephroblastoma overexpressed (NOV). WISP-1 was originally cloned based on its induced expression in the C57MG mouse mammary epithelial cell line transformed by the oncogene Wnt-1 (4). Although Wnt-1 is not present in the normal mammary gland, its expression in transgenic mice induces mammary tumors (5, 6). Because its induction is associated with the expression of Wnt-1, WISP-1 is a putative downstream effector of the Wnt/Frizzled pathway (4). Recently, WISP-1 was identified as a β-catenin-regulated gene that might contribute to Wnt-1-mediated tumorigenesis (7). Its link to tumorigenesis was also demonstrated by amplification of its genomic DNA and overexpression of its RNA in human colon tumors compared with patient-matched normal mucosa (4). WISP-1 is thought to act in an autocrine fashion because its overexpression in normal fibroblasts induces morphological transformation, accelerates cell growth, enhances saturation density, and induces tumor formation in a xenograft mouse model (7). The high level of expression in peritumoral stroma suggests that WISP-1 might also act in a paracrine fashion to support tumorigenesis (4, 7). Although WISP-1 appears to be implicated in tumor development, its mechanism of action remains uncertain.

Members of the CCN family demonstrate high structural homology (2, 3) sharing four conserved cysteine-rich modular domains with sequence similarity to insulin-like growth factor binding proteins, von Willebrand factor, thrombospondin, and growth factor cysteine knots (1). The thrombospondin domain contains a conserved WsXCSXXXG motif thought to be involved in binding to both soluble and matrix-associated macromolecules, in particular to sulfated glycoconjugates (1, 8). In addition, CTGF, NOV, and Cyr61 contain amino acid sequences that conform to the proposed heparin-binding consensus sequence XBBXBX, where B represents an amino acid with a basic charge and X an uncharged or hydrophobic amino acid (9). The binding of CTGF, NOV, and Cyr61 to heparin was demonstrated by affinity chromatography (10–12), and this consensus sequence was shown to participate directly in CTGF and Cyr61 interaction (13, 14). In addition, this consensus sequence is involved in the heparan sulfate proteoglycan interaction required for Cyr61-mediated human skin fibroblast adhesion (14, 15). This heparin binding consensus sequence is absent in WISP-1 (4). Because the CCN family members can be modulated by glycoconjugates, it is important to determine their glycosaminoglycan specificity and proteoglycan binding counterparts to better understand their mechanism of action.

The proteoglycan superfamily contains more than 30 members demonstrating a variety of biological functions including tissue organization, cell growth, maturation of specialized tissues, and tumor cell growth and invasion (16). Proteoglycans show a wide range of structures and are related only by the ability of the proteins to serve as acceptors for the xylosyltransferase, responsible for the synthesis of most types of glycosaminoglycans. Proteoglycans interact with the extracellular matrix, cell surface, growth factors, and cytokines (17). Extracellular matrix-associated proteoglycans play a key role in the recruitment, storage, and modulation of growth factors and cytokines (18).

In this report we investigate the tissue, cell surface, and glycosaminoglycan binding specificity of WISP-1. We show that WISP-1 binding is restricted to tumor stroma and to cells with a fibroblastic phenotype. This binding is mediated by the soluble and matrix-associated dermatan sulfate proteoglycans, decorin and biglycan.
EXPERIMENTAL PROCEDURES

Materials—Chondroitin sulfate A from bovine trachea, chondroitin sulfate C from shark cartilage, hyaluronide (EC 3.2.1.35) from bovine testes, and chondroitinase AC II (EC 4.2.2.5) from Arthrobacter aureus were purchased from Calbiochem. Chondroitin sulfate B, heparin, and heparan sulfate from porcine intestinal mucosa, decorin and biglycan from bovine articular cartilage, chondroitinase C, chondroitinase B, and heparinase I (EC 4.2.2.7) from Flavobacterium heparinum were obtained from Sigma. Chondroitin sulfate D from shark cartilage, chondroitin sulfate E from squid cartilage, and sheep anti-human decorin (proteoglycan II) antibody were purchased from U. S. Biological Corp. (Swampscott, MA). Neuraminidase (EC 3.2.1.18) from Vibrio cholerae, chondroitinase ABC (EC 4.2.2.4), protease-free from Proteus vulgaris, fatty acid ultra-free bovine serum albumin (BSA) fraction V, and the complete EDTA-free protease inhibitor mixture tablets were from Roche Molecular Biochemicals. Chondroitin-4-sulfatase (EC 3.1.6.9) was obtained from ICN Biomedicals (Aurora, OH). The horseradish peroxidase-conjugated, the biotinated goat anti-human IgG, Fc fragment-specific, and the biotinylated anti-sheep IgG were purchased from Jackson ImmunoResearch (Costa Mesa, CA). Proteinase K (EC 3.4.21.14) ready-to-use, Texas Red-conjugated streptavidin, and anti-vimentin monoclonal antibody (clone Vim 3B4) were from Dako (Carpinteria, CA). 5-Chloromethylfluorescein diacetate and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR). The Renaissance TSA indirect amplification kit was bought from PerkinElmer Life Sciences. Vectashield mounting media and biotinylated horse anti-mouse IgG were obtained from Vector (Burlingame, CA).

Full-length mouse WISP-1 (GenBank accession number NM_018865) was cloned into an expression vector encoding the human IgG Fc region. The WISP-1-Fc was expressed previously for tumor necrosis factor receptor 1 (19). The resulting recombinant fusion protein (WISP-1-Fc) was synthesized in a baculovirus expression system using Sf9 insect cells and purified to homogeneity from serum-free conditioned medium by affinity chromatography on a protein A-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) column. Unadsorbed proteins were washed out with 50 mM sodium phosphate buffer containing 150 mM NaCl and 0.05% Tween 20, and the pH was neutralized with 0.1 volume of 3 M Tris-HCl, pH 8. After dialysis (20 mM Tris-HCl, pH 7.5, 150 mM) the purified protein was concentrated by ultrafiltration using Centriprep-30 (Millipore Corp., Bedford, MA) and the purity estimated by SDS-PAGE and silver staining. Monoclonal anti-WISP-1 antibodies were generated using the WISP-1-Fc fusion protein as antigen. The antibody was raised in mice and purified by affinity chromatography on a protein A-agarose column. The antibody was biotinylated at room temperature. The biotinylated proteins were washed out with 50 mM sodium phosphate buffer containing 150 mM NaCl and the pH was neutralized with 0.1 volume of 3 M Tris-HCl, pH 8. After dialysis (20 mM Tris-HCl, pH 7.5, 150 mM) the purified protein was concentrated by ultrafiltration using Centriprep-30 (Millipore Corp., Bedford, MA) and the purity estimated by SDS-PAGE and silver staining.

In Situ Ligand Binding—Binding of WISP-1 to human colon tumor sections was evaluated using the in situ ligand binding procedure previously described with modifications (20). Slide-mounted human colon tumor sections were brought to room temperature and immediately incubated for 4 min in 35 mM acetic acid, pH 3.5, containing 3 mM CaCl2, 3 mM MgSO4, 5 mM KCl, and 1 mM NaCl. The slides were then washed in HBS-C (25 mM Hepes, pH 7.2, 150 NaCl, 3 mM CaCl2, 3 mM MgSO4, 5 mM KCl, Complete Protease Inhibitor mixture) containing 32 mM sucrose and the nonspecific binding sites were blocked for 1 h at room temperature with PBS, 0.3% BSA, and the nonspecific binding sites were blocked for 1 h at room temperature with PBS, 0.3% BSA. The buffer was incubated for 1 h at room temperature. The slides were washed and incubated for 1 h with 50 μl of WISP-1-Fc in HBS-C, 3% BSA was incubated for 2 h at room temperature. The slides were washed and incubated for 1 h with 50 μl of 2 μg/ml horseradish peroxidase-conjugated goat anti-human IgG Fc’ in HBS-C, 3% BSA. At the end of the incubation, the wells were washed 6 times with 200 μl of PBS containing 0.05% Tween 20, and the signal was visualized using 100 μl of 4°C horseradish peroxidase chromogenic substrate TMB (Kirkegaard & Perry Laboratories). The reaction was stopped with 100 μl of 1 M sodium phosphoric acid, and the absorbance at 450 nm was measured. Nonspecific WISP-1-Fc binding was determined in parallel incubations by omitting microtiter well coating. No signal was generated when WISP-1-Fc was omitted or replaced by an irrelevant Fc fusion protein, tumor necrosis factor receptor 1-Fc.

Detection of WISP-1 Binding Factors—Human skin fibroblasts were cycled between serum-containing and serum-free culture media every 3 days. The serum-free conditioned medium was concentrated using a Centriprep-30 (Millipore, Bedford MA). The buffer was then changed by sequentially adding 20 mM Tris-HCl, pH 7.4, 300 mM NaCl and rebuffering. The concentrated (150 μg of protein/ml) was snap-frozen and stored at −80°C until used. The concentrated conditioned medium was thawed, filtered, and applied on a Mono Q anion exchange column equilibrated in 20 mM Tris-HCl, pH 7.4, containing 300 mM NaCl. The column was washed, and the adsorbed proteins were eluted using a linear gradient of NaCl (300 mM to 2 M) in the same buffer. Fractions of 500 μl were analyzed for WISP-1 binding activity.

Protein Identification by Mass Spectrometry—The fractions containing the WISP-1 binding activity were pooled, desalted, reduced, and applied to a 4–15% gradient SDS-polyacrylamide gel without a previous incubation for 2 h at 37°C with 0.1 unit of chondroitinase ABC. The gels were silver-stained, and the protein bands demonstrating a mobility change upon chondroitinase ABC digestion were excised and digested in situ with trypsin as described previously (22). Tryptic peptides were extracted and analyzed by microcapillary reverse-phase liquid chromatography-mass spectrometry. Peptide mixtures were
FIG. 1. WISP-1 binds to human colon tumor stroma. Slide-mounted human colon tumor sections were brought to room temperature and immediately washed, saturated, and incubated for 1 h in HBS-C, 3% BSA and 1 mM WISP-1-Fc (A and B). The slides were washed, fixed, and further incubated with 1 μg/ml anti-WISP-1 monoclonal antibody for 1 h. The sections were washed, fixed, and incubated with biotinylated horse anti-mouse IgG, Fcγ for 30 min. The signal was amplified using the TSA indirect amplification kit and streptavidin-conjugated FITC (green). The sections were mounted using a Hoechst 33342 containing mounting media. In parallel, the immunofluorescent detection of vimentin (red) was performed on adjacent sections as described under “Experimental Procedures” (C and D).

WISP-1 Binds to Decorin and Biglycan—A model in which the autocrine and paracrine signals of WISP-1 contribute to tumor development was recently proposed (7). To better understand the role of WISP-1 in tumorigenesis, we generated a WISP-1-Fc fusion protein and analyzed its in situ binding to sections of human colon adenocarcinoma. Although vimentin staining revealed the presence of mesenchymal cells in both the tumor and the normal mucosa (Fig. 1, C and D), in situ WISP-1 binding was restricted to the peritumoral stroma (Fig. 1A). No binding was found to the tumor epithelial cells or to the normal mucosa (Fig. 1, A and B).

WISP-1 Binds to Fibroblasts—To verify the specificity of WISP-1 interaction with fibroblasts, we examined its binding to various cell types. The binding revealed an irregular pattern associated with patches and fibrillar structures and was restricted to the surface of cells with fibroblastic character (Fig. 2). WISP-1 bound to normal rat kidney fibroblasts (Fig. 2A), normal human skin fibroblasts (Hs 597.Sk), and human skin melanoma fibroblasts (Hs 839.T and Hs 908.Sk) but did not bind to human colon adenocarcinoma cells (COLO 320DM), mouse renal adenocarcinoma cells (RAG), human kidney epithelial cells (293), human umbilical vein endothelial cells, or human skin melanoma epithelial cells (WM-266-4) (data not shown). No signal was detected when WISP-1-Fc was omitted or replaced by human IgG or when an unrelated biotinylated antibody was used (Fig. 2B).

WISP-1 Binds to Human Skin Fibroblast Conditioned Media—The pattern of WISP-1 binding suggested that it might...
interact with extracellular components. To assess this possibility, we investigated the presence of WISP-1 binding factors in culture media from human skin fibroblasts. Microtiter plates were coated with concentrated conditioned medium, and the specific WISP-1 interaction was measured. Binding was proportional to the amount of media coated and to the concentration of WISP-1 added (Fig. 3, A and B) indicating that human skin fibroblasts produce soluble WISP-1 binding factors.

To characterize the nature of the interaction, the assay was performed under various conditions. The binding was abolished by 1 mM NaCl, reduced by 25% with 100 mM EDTA, whereas 0.05% Tween 20 had no effect (Fig. 4A). These results suggest a cation-independent ionic interaction between WISP-1 and human skin fibroblast secreted factors.

The nature of the secreted factors was investigated by treating coated wells with various lysases before assessing the binding. Treatment with chondroitinase A, chondroitin-6-sulfatase, heparinase, or neuraminidase did not alter WISP-1 interaction, whereas digestion with chondroitinase AC II and hyaluronidase resulted in a 40% and 35% reduction in binding, respectively (Fig. 4B). Treatment with chondroitinase ABC, chondroitinase B, chondroitin-4-sulfatase, or proteinase K abolished WISP-1 binding. The specificity of chondroitinase B and chondroitin-4-sulfatase indicates that dermatan sulfate glycosaminoglycan is required for WISP-1 interaction. Moreover, proteinase K sensitivity suggests that the binding factors also contain a protein component. Taken together these results indicate that WISP-1 binds to a secreted dermatan sulfate proteoglycan.

WISP-1 Binds Glycosaminoglycans—The specificity of WISP-1 interaction with skin fibroblast conditioned media was further analyzed by solid phase competition assay. The binding was proportionally reduced in the presence of increasing concentrations of various glycosaminoglycans (Fig. 5). IC_{50} was reached at 3 μg/ml dermatan sulfate, 10.5 μg/ml chondroitin sulfate D or heparin, 30 μg/ml chondroitin sulfate E, 75 μg/ml heparan sulfate, or 105 μg/ml chondroitin sulfate A. The presence of chondroitin sulfate C did not reduce WISP-1 binding.

Identification of WISP-1 Binding Factors—To identify WISP-1-binding proteins, serum-free human skin fibroblast conditioned medium was collected, concentrated, and fractionated using a Q-Sepharose anion exchange column. WISP-1 binding activity of all fractions was estimated by solid phase assay. As shown in Fig. 6A, 94% of the adsorbed WISP-1 binding activity was eluted at 0.8-2 mM NaCl, whereas only a minor fraction eluted earlier. Because the binding factors were putative dermatan sulfate proteoglycans, we analyzed fractions 14–16 containing the WISP-1 binding activity for electrophoretic mobility changes upon chondroitinase ABC digestion (Fig. 6A). Bands at 230, 70, and 60 kDa and a doublet at 44 kDa were revealed by lyase treatment (Fig. 6B). Mass spectroscopy analysis identified the 70-, 60-, and 46-kDa bands as containing decorin and the bands at 230 and 44 kDa as containing a mixture of decorin and biglycan (Table I). The identity of the chondroitinase ABC-sensitive protein migrating at 200 kDa is currently being analyzed. Apparent molecular weight heterogeneity may have resulted from an incomplete chondroitinase ABC digestion as well as protein aggregation. Taken together these results suggest that decorin and biglycan are WISP-1 binding factors.

WISP-1 Binds to Decorin and Biglycan—We assessed the interaction of WISP-1 with decorin and biglycan using a solid phase assay. WISP-1-specific binding was identical for both proteins and proportional to the amount of protein coated (Fig. 7A). Similarly, WISP-1 interaction with human skin fibroblast conditioned media was gradually decreased in the presence of increasing concentrations of decorin or biglycan (Fig. 7B). Competition curves gave an IC_{50} of 70 μg/ml for decorin and 105 μg/ml for biglycan. Dissociation constants were measured using saturation curves (Fig. 7, C and D). Scatchard analysis (25)
of the data, assuming a single class of binding site, demonstrated $K_d$ values of 1.8 nM for decorin (Fig. 7C) and 2.2 nM for biglycan (Fig. 7D). These results indicate that decorin and biglycan are WISP-1 binding factors secreted by human skin fibroblast conditioned media.

**WISP-1 Binding to Human Skin Fibroblasts Is Abolished by Dermatan Sulfate**—The role of glycosaminoglycans in WISP-1 cell surface interaction was investigated by monitoring binding to human skin fibroblasts in the presence (Fig. 8, B–E) or absence (Fig. 8A) of various competitors. Chondroitin sulfate C and chondroitin sulfate D reduced WISP-1 binding by 20% and 46%, respectively (Fig. 8E), whereas chondroitin sulfate A, chondroitin sulfate E (Fig. 8C), heparan sulfate, or heparin (Fig. 8D) diminished the interaction by 60–70% (Fig. 8E). On the other hand, the presence of dermatan sulfate completely abolished WISP-1 binding (Fig. 8, B and E) rendering the level of fluorescence comparable to a negative control executed in absence of WISP-1 (data not shown). The relative level at which the different glycosaminoglycans reduced WISP-1 interaction with the cell surface paralleled their efficiency at competing the binding to human skin fibroblast conditioned media (Fig. 6). These results suggest that WISP-1 dermatan sulfate binding domains may be responsible for its interaction with the cell surface.

**WISP-1 Binds to Cell Surface Dermatan Sulfate Proteoglycans**—To determine the role of glycosaminoglycans in the interaction of WISP-1 with the cell surface, we analyzed its binding to glycosaminoglycan lyase-treated human skin fibroblasts. Chondroitinase C or heparinase treatment marginally altered WISP-1 cell surface binding (Fig. 9, A and D). On the other hand, cells treated with chondroitinase ABC or chondroitinase B failed to bind WISP-1 (Fig. 9, B and C). Digestion with various glycolytic enzymes altered the interaction with the cell surface and the conditioned media in a similar fashion (Fig. 9 and also see Fig. 4). These results indicate that WISP-1 cell surface binding is mediated by dermatan sulfate proteoglycan.

**WISP-1 Binds to Decorin at the Surface of Human Skin Fibroblasts**—The presence of decorin at the surface of human skin fibroblasts was assessed by immunofluorescence. Although decorin is a secreted proteoglycan, the fluorescent staining demonstrated that it covered a large portion of the human skin fibroblast surface (Fig. 10A). This distribution paralleled the WISP-1 binding pattern (Fig. 10B). In addition, WISP-1 interaction was diminished by 88% and 94% in the presence of decorin (Fig. 10, C and D) or biglycan (Fig. 10D). These results show that decorin and biglycan act as WISP-1 binding factors that can mediate and modulate its interaction with the cell surface.

**DISCUSSION**

WISP-1 is a member of the CCN family of structurally related growth factors that also includes CTGF, Cyr61, NOV, WISP-2, and WISP-3. Although they participate in a variety of biological processes such as proliferation, differentiation, attachment, and migration, no specific function has been ascribed to the various CCN family members (2, 3). To help elucidate potential biological functions, it was of interest to characterize the tissue and cellular specificity of WISP-1 interaction and identify binding partners. We showed that *in situ* WISP-1 binding to sections of human colon adenocarcinoma is limited to the peritumoral stroma. By using a cell binding assay, we demonstrated that WISP-1 interaction was restricted to cell lines with a fibroblastic phenotype. WISP-1 is highly expressed by fibroblasts lying within the fibrovascular mammary gland tumor stroma of *Wnt-1* transgenic mice (4) and binds to fibroblasts and stroma of human colon tumors. The matching expression and binding pattern suggest that WISP-1 interacts with the surface of its expressing cells. Other members of the CCN family including WISP-2, CTGF, and Cyr61 are also expressed by fibroblasts (4, 26, 27). Moreover, CTGF and Cyr61 remain associated with the extracellular matrix and the cell surface upon secretion (26, 28, 29). Because it can not diffuse freely, it was hypothesized that Cyr61 plays a role in cell-cell communication involving the interaction of neighboring cells (29). Similarly, WISP-1 binding to the fibroblast cell surface suggests that it could act in an autocrine fashion. It is also possible that, while remaining associated with the cell surface, it exerts its action on neighboring cells. An autocrine/paracrine mode of action has been suggested for WISP-1 (7).

By using a solid phase assay, we showed that human skin fibroblast conditioned media contained WISP-1 binding factors. To determine the identity of these factors, we treated the conditioned media with glycosaminoglycan lyases of different specificity prior to assessing WISP-1 interaction. Chondroitinase ABC and chondroitinase B treatment completely abolished the binding, whereas treatment with chondroitinase C had no effect. Whereas chondroitinase ABC acts on a wider range of substrates, chondroitinase B only cleaves dermatan sulfate at the β-d-galactosamine-β-iduronic acid linkage. The specificity of this enzyme demonstrates an iduronic acid requirement for WISP-1 binding. Treatment with chondroitinase AC II or hyaluronidase partially reduced the binding. This could indicate that the glycosaminoglycan chain responsible for WISP-1 interaction consists of a dermatan sulfate-chondroitin sulfate co-polymer. By cleaving the susceptible galactosaminidic bonds, enzymes of various specificity could remove iduronic acid containing domains of the chain and reduce WISP-1 binding. Treatment with chondroitin-4-sulfatase abolished the binding, whereas chondroitin-6-sulfatase
and heparinase had no effect. This indicates that a sulfate group is only required at position 4 of the N-acetylgalactosamine and that the iduronic acid does not need to be sulfated at position 2 for WISP-1 binding. Proteinase K digestion abolished the interaction suggesting that the glycosaminoglycan is linked to a protein core detached from the wells by proteolytic degradation. Collectively, these results support the conclusion that WISP-1 binding is mediated by iduronic acid containing motifs of a human skin fibroblast proteoglycan.

By using a solid phase assay, we showed that WISP-1 binding to human skin fibroblast conditioned media was greatly reduced by dermatan sulfate, whereas other glycosaminoglycans competed the interaction to a lesser extent. This further demonstrates the higher specificity of WISP-1 binding for dermatan sulfate.

WISP-1 binding factors from human skin fibroblast conditioned media were identified by sequencing proteins whose electrophoretic mobility was modified upon chondroitinase ABC treatment. Analysis of six bands revealed the identity of decorin and biglycan, two members of a family of small leucine-rich proteoglycans present in the extracellular matrix of connective tissue. Decorin has a core protein of 36,319 Da and a single dermatan sulfate chain attached to a serine at position 456-57.

FIG. 5. WISP-1 binds to glycosaminoglycans. Serum-free conditioned medium from human skin fibroblasts was prepared as described under “Experimental Procedures.” Conditioned medium (50 µl) was coated in wells of microtiter plates overnight at 4 °C, and the nonspecific binding sites were saturated, and the wells were incubated for 2 h at room temperature with 0.5 nM WISP-1-Fc in the presence of various concentrations of different glycosaminoglycans. The wells were washed, and a signal was developed using a chromogenic substrate, and the absorbance at 450 nm was measured. Chondroitin sulfate A, filled circles; dermatan sulfate, open circles; chondroitin sulfate C, filled triangles; chondroitin sulfate D, open triangles; chondroitin sulfate E, filled squares; heparin, ×; and heparan sulfate, open squares.

FIG. 6. Purification of WISP-1 binding factors from human skin fibroblast conditioned media. A, the serum-free conditioned medium from human skin fibroblasts was collected after 3 days of culture, concentrated, transferred to a buffer containing 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, and applied on a Q-Sepharose anion exchange chromatography column. The column was washed, and the retained proteins were desorbed with an increasing concentration of NaCl. The presence of a WISP-1 binding factor was analyzed in each fraction using a solid phase binding assay. B, fraction 15 (indicated by an asterisk in A) was incubated at 37 °C for 2 h in the presence (+) or the absence (−) of 0.1 unit of chondroitinase ABC. The samples were separated by SDS-PAGE under reducing conditions, and the gels were silver-stained. The indicated bands were identified by mass spectroscopy.

TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue</th>
<th>Sequence</th>
<th>Band Mass</th>
</tr>
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<tr>
<td>Decorin</td>
<td>45–57</td>
<td>DFEPSLGPVCPRF</td>
<td>44</td>
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<tr>
<td>64–74</td>
<td>VQCSDLGLDK</td>
<td>44, 46, 60</td>
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<td>78–92</td>
<td>DLFPDTTLDDLQNNK</td>
<td>44, 46, 60, 230</td>
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<td>106–116</td>
<td>NHALILVNNK</td>
<td>46, 60</td>
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<tr>
<td>120–130</td>
<td>VSPGATPLVK</td>
<td>44, 46, 60, 70, 230</td>
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<tr>
<td>157–164</td>
<td>AHENEITK</td>
<td>44, 46</td>
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<td>284–292</td>
<td>VPGGLAEHK</td>
<td>44, 46, 230</td>
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<tr>
<td>351–359</td>
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<td>46</td>
<td></td>
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<tr>
<td>Biglycan</td>
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<td>44</td>
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Human skin fibroblast conditioned media was fractionated by anion exchange affinity chromatography. Fractions containing WISP-1 binding activity were digested with chondroitinase ABC and analyzed by SDS-PAGE. Protein bands demonstrating mobility change upon treatment were excised and digested in situ with trypsin, and the resulting peptides were analyzed by tandem mass spectrometry as described under “Experimental Procedures.”

By using a solid phase assay, we showed that WISP-1 binding to human skin fibroblast conditioned media was greatly reduced by dermatan sulfate, whereas other glycosaminoglycans competed the interaction to a lesser extent. This further demonstrates the higher specificity of WISP-1 binding for dermatan sulfate. WISP-1 binding factors from human skin fibroblast conditioned media were identified by sequencing proteins whose electrophoretic mobility was modified upon chondroitinase ABC treatment. Analysis of six bands revealed the identity of decorin and biglycan, two members of a family of small leucine-rich proteoglycans present in the extracellular matrix of connective tissue. Decorin has a core protein of 36,319 Da and a single dermatan sulfate chain attached to a serine at position 456-57.
Biglycan has a core protein of 37,983 Da substituted with one chain of dermatan sulfate and one of chondroitin sulfate (32). The molecular weights of the core protein of decorin and biglycan correspond to the predicted molecular weight of the two fastest migrating bands after chondroitinase ABC treatment. Slower bands corresponded to proteins bearing partially digested glycosaminoglycan chains. We subsequently confirmed WISP-1 interaction with the purified proteins using a solid phase assay, and we demonstrated that decorin and biglycan inhibited WISP-1 binding to decorin and biglycan.

**Fig. 7.** WISP-1 binds to decorin and biglycan. A, microtiter wells were coated with serial dilutions of decorin (filled circles) or biglycan (open circles). Nonspecific binding sites were saturated, and 0.25 nM WISP-1-Fc was incubated for 2 h. The wells were washed and incubated with horseradish peroxidase-conjugated anti-human IgG Fc’ (2 μg/ml) for 1 h. After six washes with PBS containing 0.05% Tween 20, a signal was developed by incubation with a chromogenic substrate. Color development was stopped by the addition of 1 M phosphoric acid, and the absorbance at 450 nm was measured. B, human skin fibroblast conditioned media (50 μl) was coated in wells of a microtiter plate. Nonspecific binding sites were saturated, and 0.25 nM WISP-1-Fc was incubated in the presence of various concentrations of decorin (filled circles) or biglycan (open circles) for 2 h. The binding of WISP-1-Fc was evaluated as described in A. Dissociation constants were determined by measuring the binding of various concentrations of WISP-1-Fc to microtiter wells coated with 5 μg of decorin (C) or biglycan (D).

**Fig. 8.** WISP-1 binding to human skin fibroblasts is competed by glycosaminoglycans. Human skin fibroblasts were seeded in chamber slides. The nonspecific binding sites were saturated, and 1 nM WISP-1-Fc was incubated for 1 h at room temperature in the absence (A) or the presence of 50 μg/ml dermatan sulfate (B), chondroitin sulfate E (C), or heparin (D). The cells were washed and fixed, and the binding of WISP-1-Fc was detected by immunofluorescence and measured (E) as described under “Experimental Procedures.”

4 (31). Biglycan has a core protein of 37,983 Da substituted with one chain of dermatan sulfate and one of chondroitin sulfate (32). The molecular weights of the core protein of decorin and biglycan correspond to the predicted molecular weight of the two fastest migrating bands after chondroitinase ABC treatment. Slower bands corresponded to proteins bearing partially digested glycosaminoglycan chains. We subsequently confirmed WISP-1 interaction with the purified proteins using a solid phase assay, and we demonstrated that decorin and biglycan inhibited WISP-1 binding to human skin fibroblasts.

**Fig. 9.** WISP-1 binding to human skin fibroblasts is abolished by digestion of the cell surface with chondroitinase B. Human skin fibroblasts were incubated for 2 h at 37 °C in the absence (A) or the presence of 0.1 unit of chondroitinase ABC (B), chondroitinase B (C), chondroitinase C, or heparinase (D). The cells were washed, and the nonspecific binding sites were saturated, and 1 nM WISP-1-Fc was incubated for 1 h at room temperature. The cells were washed and fixed, and the binding of WISP-1-Fc was detected by immunofluorescence and measured (E) as described under “Experimental Procedures.”
WISP-1 binds to decorin and biglycan. Human skin fibroblasts were seeded in chamber slides, and the nonspecific binding sites were saturated. Immunofluorescent detection of decorin was performed as described under “Experimental Procedures” (A). In adjacent wells, WISP-1-Fc (1 nM) was incubated for 1 h at room temperature in the absence (B) or the presence (C) of 1 mg/ml decorin or biglycan. The cells were washed and fixed, and the binding of WISP-1-Fc was detected by immunofluorescence and measured (D) as described under “Experimental Procedures.”

WISP-1 binding to human skin fibroblast conditioned media. Scatchard analysis predicted relatively high affinity interactions with apparent dissociation constants of 1.8 nM for decorin and 2.2 nM for biglycan. These values are similar to the dissociation constants for the interaction of the thrombospondin domain containing proteins, midkine, and pleiotropin/heparin binding growth-associated molecule with the proteoglycans versican and phosphacan (33, 34). Taken together, these results demonstrate that decorin and biglycan represent high affinity WISP-1 binding factors from human skin fibroblasts.

The role of glycosaminoglycans in the interaction of WISP-1 with human skin fibroblasts was assessed using a cell-binding assay. Whereas various glycosaminoglycans revealed a partial reduction, only dermatan sulfate was able to abolish cell surface interaction. In addition, the binding was prevented by treating the cell surface with chondroitinase ABC or chondroitinase B but only partially reduced by chondroitinase C or heparinase. Taken together these results suggest that WISP-1 cell surface binding is mediated by a dermatan sulfate proteoglycan.

By using immunofluorescence, we confirmed the presence of decorin at the surface of human skin fibroblasts. This is consistent with the predominant expression of decorin in mesenchymal cells (35, 36). We also demonstrated that decorin and biglycan significantly diminished WISP-1 interaction. These results suggest that decorin and biglycan act as WISP-1 binding factors that can mediate and modulate its interaction with the cell surface. Although decorin and biglycan appear to be the major binding factors, we cannot exclude WISP-1 interaction with other proteoglycans.

Other members of the CCN family including Cyr61 (11), CTGF (26), and NOV (12) have been shown to interact with glycosaminoglycans. Although their glycosaminoglycan specificity is not known, their elution from heparin-agarose affinity media requires 0.6–1 M NaCl reflecting their high affinity for heparin (29). Exogenous dermatan sulfate and decorin prevent Cyr61-mediated human skin fibroblast adhesion demonstrating that it can interact with dermatan sulfate proteoglycans (14). On the other hand, glycosaminoglycan lyase treatment of the cell surface showed that only heparan sulfate proteoglycan is required for the α6β1 integrin-mediated cell adhesion to Cyr61 (14). It is not possible to speculate on the uniqueness of the preferential interaction of WISP-1 with dermatan sulfate because the glycosaminoglycan binding specificity has not been established for the other members of the CCN family.

The specific WISP-1 sequence responsible for dermatan sulfate interaction is currently unknown. The heparin binding domain of CTGF is contained in the carboxyl-terminal 102 amino acids (13). In this region, CTGF, NOV, and Cyr61 contain amino acid segments that conform to the proposed heparin binding consensus sequence XBBXBX, where B represents an amino acid with a basic charge and X an uncharged or hydrophobic amino acid (9). The cooperative contribution of adjacent heparin binding sequences to the overall net heparin binding of CTGF and Cyr61 was also suggested (13, 14). Moreover, alanine replacement of eight basic amino acids extending over two heparin binding regions of Cyr61 was necessary to abolish heparin binding. Mutations in either of the two motifs marginally reduced Cyr61 binding affinity for heparin (14). The putative heparin-binding motif of the carboxyl-terminal region is well conserved in CTGF, NOV, and Cyr61 but not in the WISP proteins (2). Whereas this carboxyl-terminal domain is absent in WISP-2, WISP-1 and WISP-3 do not contain this heparin-binding consensus motif. Moreover, five of the eight basic amino acids required for Cyr61 heparin binding are replaced by non-basic amino acids in WISP-1 and WISP-3. Only 10 residues of the WISP-1 carboxyl-terminal 102 amino acids containing the heparin binding region are basic compared with 19–20 for CTGF, NOV, and Cyr61. The glycosaminoglycan binding specificity of WISP-1 may differ from the other CCN family members because it lacks the heparin binding consensus motifs. Variability in the number, the spacing, and the
takes place around developing tumors (4, 44). It is possible that connective tissue cells to the invading neoplastic cells (50, 51).

The growth of several carcinomas, its expression in the peritumoral stroma may reflect a regional response of the host con-"