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Journal

Journal of Biological Chemistry, 273(35)

ISSN

0021-9258

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Publication Date

1998-08-01

DOI

10.1074/jbc.273.35.22825

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 35, Issue of August 28, pp. 22825–22832, 1998

Printed in U.S.A.

Heparan Sulfate Proteoglycans as Adhesive and Anti-invasive Molecules

SYNDECANS AND GLYPICAN HAVE DISTINCT FUNCTIONS*

(Received for publication, March 3, 1998, and in revised form, May 18, 1998)

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ARH-77 cells do not adhere to type I collagen and readily invade into collagen gels, but following expression of the transmembrane heparan sulfate proteoglycan syndecan-1, they bind collagen and fail to invade. We now show that cells transfected with syndecan-2 or syndecan-4 also bind collagen and are non-invasive. In contrast, cells transfected with the glycosylphosphatidylinositol-anchored proteoglycan glypican-1 do not bind to collagen and remain invasive, even though glypican- and syndecan-expressing cells have similar surface levels of heparan sulfate, and their proteoglycans have similar affinities for collagen. Analysis of cells expressing syndecan-1-glypican-1 chimeric proteoglycans reveals that inhibition of invasion requires the extracellular domain of syndecan but not its transmembrane or cytoplasmic domain. Surprisingly, cells bearing a chimera composed of the glypican extracellular domain fused to the syndecan transmembrane and cytoplasmic domains bind to collagen but remain invasive, implying that adhesion to collagen is not by itself sufficient to inhibit invasion. Apparently, the extracellular domain of syndecan-1, presumably by interacting with cell-surface signal transducing molecules, directly regulates complex cell behaviors such as motility and invasiveness. These results also show for the first time that syndecans and glypicans can have distinct functions, even when expressed by the same cell type.

Receptors for the extracellular matrix are critical participants in regulating both embryonic development and the maintenance of tissue homeostasis. In addition to mediating physical attachment of cells, such receptors can promote intracellular signaling that directs specific alterations in gene expression that are coupled with changes in cell shape, growth, and motility (1). Changes in the expression and/or activation state of receptors for the extracellular matrix often accompany the progression of pathological states, particularly where cell migration and tissue invasion occur (*e.g.* inflammatory diseases and cancer) (2, 3). The role of integrins in extracellular matrix-associated adhesion and signaling is now well established (4). However, the role of another class of cell-surface molecules, the heparan sulfate proteoglycans, is less well understood, even though it is clear that these molecules also participate in signaling processes (5–8).

The cell-surface heparan sulfate proteoglycans belong primarily to two families of molecules, the syndecans and the glypicans, that differ significantly in core protein domain structure (9–11). The syndecans have a transmembrane and cytoplasmic domain, whereas the glypicans are anchored to the extracytoplasmic face of the plasma membrane via glycosylphosphatidylinositol (GPI).[1] Furthermore, the core proteins of syndecans are thought to adopt a highly extended conformation, due to a relatively high proline content (12), and to have most or all of their heparan sulfate chains attached at positions distal to the plasma membrane, near the N terminus of the core protein (13). In contrast, the core proteins of glypicans appear to have a largely globular structure and to possess heparan sulfate chains that are attached close to the plasma membrane (10, 14, 15). Because syndecans and glypicans often coexist on the same cells, it is important to determine whether they perform functions that are similar or distinct. Also unknown is whether individual members of the syndecan family perform different functions. The four family members (designated syndecans 1–4) all have highly related transmembrane and cytoplasmic domains, but their extracellular domains show little homology except with regard to the placement and number of some of the heparan sulfate attachment sites. Different members of the syndecan family exhibit tissue-specific expression in adult mice and undergo large changes in expression during development (16). Most murine cells express at least one member of the syndecan family, and many cells and tissues express multiple syndecans (17). Syndecans-1, -2, and -4 all bind to basic FGF and support FGF receptor-1 signaling in K562 cells (7). In contrast, syndecan-4, but not other syndecans, localizes to focal adhesions (18, 19). Furthermore, plates coated with the core protein of syndecan-4, but not syndecan-1, are able to mediate adhesion of fibroblasts (20). Whereas syndecan-1 binds to collagen and fibronectin, purified syndecan-3, which is found primarily in neural tissues, apparently does not (21). Thus, there is at least some evidence for functional differences among syndecans.

Given that cells can use syndecan-1 to attach to the extra-

* This work was supported by National Institutes of Health Grants CA 55879, CA 68494 (to R. D. S), and NS 26862 (to A. D. L.) and National Research Service Award CA 71145 (to J. K. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol phospholipase C; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; FGF, fibroblast growth factor.

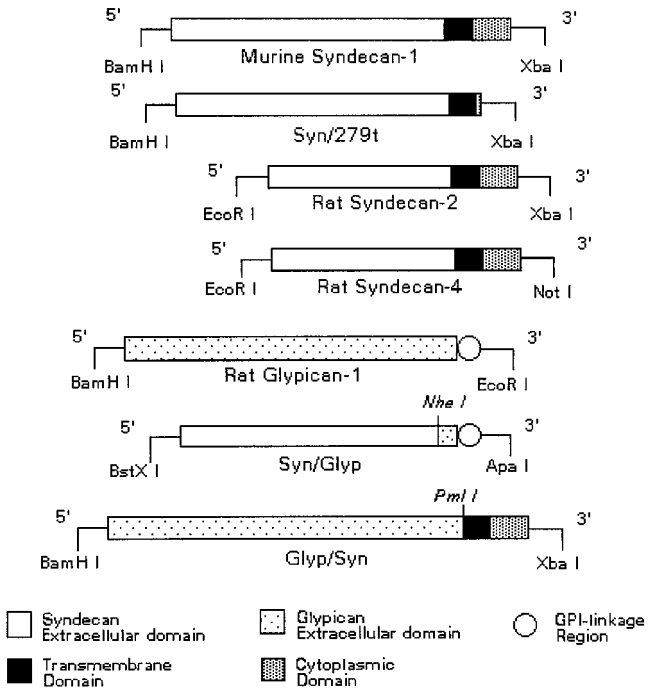

FIG. 1. **Constructs prepared for transfection into ARH-77 cells.**

cellular matrix, and given the regulated pattern of expression of syndecans during both development and the progression of tumor cells to the metastatic phenotype, it has been proposed that syndecans are important regulators of the migratory and invasive behaviors of cells, both normal and transformed (22, 23). For example, syndecan-1 expression is lost on epithelial cells undergoing changes in shape and position during development (24–27) and on migrating keratinocytes during wound healing (28, 29). Normal murine mammary gland epithelial cells made deficient in syndecan-1 lose their epithelial morphology, become fibroblastic in appearance, and exhibit characteristics of neoplastically transformed cells including anchorage-independent growth and increased invasiveness (30). Transformed epithelial cells having low levels of syndecan-1 expression regain their epithelial morphology and growth characteristics following transfection with the cDNA for syndecan-1 (31). Furthermore, syndecan-1 expression is reduced during malignant transformation, and loss of syndecan-1 expression is associated with a poor prognosis in carcinomas of the head and neck (32, 33).

We have previously used ARH-77 B-lymphoid cells as a model for studying the role of syndecan-1 in regulating adhesion, migration, and growth of tumor cells (34–36). This cell line was derived from a human plasma cell leukemia and expresses low amounts of heparan sulfate on the cell surface. ARH-77 cells do not bind to type I collagen and will readily invade into native type I collagen gels, but following their transfection with the cDNA for syndecan-1 they both bind to collagen and are rendered non-invasive on collagen gels (34). In the present work we have used the ARH-77 cell invasion model to explore structure-function relationships among heparan sulfate proteoglycans, including relationships among syndecans and between syndecans and glypican-1.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The DNA fragments containing the full coding sequences of different proteoglycans were cloned into the pcDNA3 vector (Invitrogen, San Diego) (Fig. 1), and orientations were confirmed by restriction mapping. The murine syndecan-1 full coding sequence was cleaved from pGEM-3Z-murine syndecan-1 (12), subcloned into pBK-CMV phagemid vector (Stratagene, La Jolla) at the *Eco*RI (5′) and *Hin*dIII (3′) sites, and then cloned into the pcDNA3 vector. A tail-less murine syndecan-1 construct designated syn/279t (lacking the coding region for the syndecan-1 cytoplasmic domain) was generated via oligonucleotide-directed mutagenesis (Transformer Site-directed Mutagenesis Kit, CLONTECH, Palo Alto, CA) by creating a stop codon at the position of amino acid 279, leading to truncation of all but one amino acid of the cytoplasmic domain. The pcDNA3 vectors containing the full coding sequence for either rat syndecan-2 or rat syndecan-4 were provided by Dr. John Gallagher (37, 38). The rat glypican-1 full coding sequence was cleaved from pBluescript-glypican-1 (39) and cloned into the pcDNA3 vector. Two constructs encoding chimeric proteoglycans were prepared, one designated syn/glyp, coding for the syndecan-1 extracellular domain and the portion of glypican-1 coding for the GPI-linkage region, the other glyp/syn, containing the glypican-1 extracellular domain and the syndecan-1 transmembrane and cytoplasmic domains. For the syn/glyp chimera, the cDNAs for both murine syndecan-1 and rat glypican-1 were cloned into pBluescript KS (+) (Stratagene). Using oligonucleotide-directed mutagenesis, a unique restriction site, *Nhe*I, was inserted between the extracellular domain and the transmembrane domain of the syndecan-1 cDNA (between amino acids 252 and 253). Similarly, an *Nhe*I site was also inserted between the extracellular domain and the GPI linkage region of the glypican-1 cDNA (between amino acids 523 and 524). The DNA fragments encoding syndecan-1 and glypican-1 extracellular domains were cleaved from their intact constructs with *Not*I (5′) and *Nhe*I (3′). The syndecan-1 extracellular domain coding sequence was then ligated to the vector, which retained the coding sequence of the glypican-1 GPI linkage region. The sequence of the syn/glyp chimeric construct was confirmed using dideoxy sequencing with Sequenase version 2.0 (U. S. Biochemical Corp.), and the construct was cloned into the pcDNA3 vector. For the glyp/syn-1 chimera, an *Eco*RI DNA fragment containing the full coding region of rat glypican-1 was inserted into pGEM3Z-murine syndecan-1 at the *Eco*RI site, upstream of the syndecan-1 coding region. The vector DNA containing both glypican-1 and syndecan-1 coding sequences in tandem was digested with *Bbs*I (cut at 1636 base pairs of glypican-1 cDNA) and *Sfi*I (cut at 1027 base pairs of syndecan-1 cDNA) to remove a DNA fragment that contained the 3′ end of glypican-1 (including the GPI anchorage signal) and the 5′ end of syndecan-1 (including extracellular domain and part of transmembrane domain). Two oligonucleotides (the + strand, 5′-cggccgccactcgcccagagcctcacgtgctgggaggtgtcattgccggaggcctagt-3′; the −strand, 5′- aggcctccggcaatgacacctcccagcacgtgaggctctgggcgagtggcg-3′) were annealed to form a short DNA fragment with cohesive ends of *Bbs*I (5′) and *Sfi*I (3′). This fragment, containing the GPI anchorage signal of glypican-1 and part of the transmembrane domain of syndecan-1, was then ligated into the digested vector to link the glypican-1 extracellular domain in frame with the syndecan-1 transmembrane and cytoplasmic domains. The resulting glyp/syn-1 chimera was confirmed by nucleotide sequencing, excised, and cloned into the *Eco*RI/*Hin*dIII sites of the pMV7 vector. In order to clone the glyp/syn chimeric construct into pcDNA3 in the sense orientation, the construct was excised from pMV7, subcloned into pBK-CMV phagemid vector (Stratagene) at *Eco*RI (5′) and *Hin*dIII (3′) sites, and then cloned into pcDNA3. Although the sequence coding for GPI anchorage remains in this construct, the presence of the syndecan-1 transmembrane and cytoplasmic domains prevents addition of a GPI anchor and results in a transmembrane proteoglycan.

Transfection—ARH-77 cells were maintained and transfected by electroporation as described previously (34) with the plasmid DNAs containing the full coding sequence for either syndecan-2 or syndecan-4 or by liposome-mediated transfection with the plasmid DNAs containing full-length syndecan-1, syn/279t, glypican-1, or chimeras. For controls, ARH-77 cells were transfected with pcDNA3 plasmid DNA not containing inserted DNA. Liposome-mediated transfection was performed as described in the manufacturer's protocol (Life Technologies, Inc.). All transfected cells were maintained in RPMI 1640 containing 5% fetal bovine serum and geneticin (0.3 mg/ml). Clones of syndecan-2- or syndecan 4-transfected cells were isolated by two limiting dilutions (34) and were analyzed for syndecan-2 or -4 expression by Northern blotting. The transfected cells expressing syndecan-1, syn/279t, glypican-1, or chimeric proteoglycans were isolated by cell sorting using either monoclonal antibody 281.2 against the murine syndecan-1 extracellular domain (40) or a polyclonal antibody against the rat glypican-1 extracellular domain (41).

Dot Blot Assay—To demonstrate that the syn/glyp chimeric proteoglycan is linked to the cell membrane via a GPI linkage, a dot blot assay was used to detect the release of the syndecan-1 extracellular domain following treatment of the cell surface with phosphatidylinositol-specific phospholipase C (PI-PLC). Briefly, cells expressing the chimera

were harvested by centrifugation and resuspended in RPMI 1640 containing 0.1% BSA (Sigma) and 2.5 units/ml PI-PLC (Boehringer Mannheim) and incubated for 30 min at 37 °C. Following centrifugation, the supernatant was brought to 6 M urea and 50 mM sodium acetate, pH 4.5. Equal fractions of the supernatants were loaded onto Gene-Trans membrane (Plasco, Woburn, MA) using an immunoblot apparatus (Milliblot D; Millipore, Bedford, MA). The membrane was probed with monoclonal antibody 281.2 (10 μg/ml) in Blotto (3% powdered milk, 0.5% BSA, 10 mM Tris, pH 8.0, 0.15 M NaCl, 0.3% Tween 20, and 0.025% NaN_3) followed by a biotinylated rabbit anti-rat IgG (5 μg/ml) (Vector Laboratories, Burlingame, CA) in PBS. Bound antibody was detected using avidin-horseradish peroxidase and color developed with diaminobenzidine (Vector Laboratories). To confirm that the glyp/syn chimera contained the syndecan-1 cytoplasmic domain, cells bearing the chimeric proteoglycan were extracted with buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 0.15 M NaCl, 5 mM *N*-ethylmaleimide, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. The extracts were clarified by centrifugation, and the supernatants were brought to 6 M urea and 50 mM sodium acetate, pH 4.5, and subjected to dot blotting as described above. The membrane was probed with an antiserum generated against a 7-amino acid peptide corresponding to the 7 C-terminal amino acids that are present within the syndecan-1 cytoplasmic domain (12) followed by a biotinylated goat anti-rabbit IgG (5 μg/ml) (Vector Laboratories) and color-generated as described above. To compare the levels of core protein present on the glyp/syn and syndecan-1 transfectants, equal numbers of cells were extracted and dot-blotted as described above. After probing blots with the antibody against the syndecan-1 cytoplasmic domain, blots were scanned, and the relative amounts of core present were determined by analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Western Blotting—Cells were extracted as described above and brought to 6 M urea and 50 mM sodium acetate, pH 4.5. The samples were boiled for 10 min and centrifuged to remove cell debris. DEAE-Sepharose beads (Pharmacia, Uppsala, Sweden) were added to the supernatants, and the mixtures were rotated overnight at 4 °C. The DEAE beads were washed with PBS containing 0.1% Triton X-100, the bound proteoglycans eluted from the beads with 1 M NaCl in PBS containing 0.1% Triton X-100, and were then diluted with 20 mM Tris, pH 7.0, containing 5 mM CaCl to a final concentration of 0.1 M NaCl. For analysis of core protein size of the syndecan-1 and the syn/glyp chimera, half of each sample was sequentially treated twice with heparitinase (1 milliunits/ml; Seikagaku, Rockville, MD) at 42 °C for 30 min and twice with chondroitinase ABC (50 milliunits/ml) (Seikagaku) at 37 °C for 30 min. Because glypican-1 does not have chondroitin sulfate chains, half of the glypican-1 or glyp/syn sample was treated with heparitinase only. The other half of each sample was left untreated to analyze the intact proteoglycans. All samples were then desalted by passing over excellulose columns (Pierce) and eluted with distilled H_2O containing 0.1% SDS. The samples were dried, dissolved in sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol (v/v), 0.005% bromphenol blue), boiled for 5 min, and loaded onto a 4–12% Tris glycine SDS-PAGE gel (Novex, San Diego). The proteins were transferred to the Gene-Trans membrane in Towbin's buffer (25 mM Tris, pH 8.3, containing 0.19 M glycine and 10–20% methanol) using a Semi-Phor semi-dry transfer unit (Hoeffer Scientific, San Francisco). For syndecan-1 and syn/glyp samples, the membrane was probed with the ^{125}I-labeled monoclonal antibody 281.2 (40) (10 μg/ml) in Blotto and exposed to Kodak X-Omat XAR-5 film. For glypican-1 and glyp/syn samples, the membrane was probed with an antisera to glypican-1 (41) (2.5 μg/ml) in Blotto followed by a biotinylated goat anti-rabbit IgG (5 μg/ml) and avidin- horseradish peroxidase (Vector Laboratories). Bound horseradish peroxidase was detected by chemiluminescence (Amersham Pharmacia Biotech).

Flow Cytometry—Relative amounts of heparan sulfate present on the various transfectants were determined by flow cytometry of cells stained with 10E4, a monoclonal antibody against heparan sulfate (42). 2×10^6 cells were harvested by centrifugation and incubated with 10 μg/ml 10E4 (Seikagaku) in RPMI 1640 containing 5% fetal bovine serum for 30 min on ice. Cells were washed with medium and then incubated with 5.5 μg/ml biotinylated goat anti-mouse IgM (Vector Laboratories) for 30 min followed by avidin-fluorescein isothiocyanate (Vector Laboratories) for 10 min then analyzed by flow cytometry.

For comparison of relative core protein amounts, cells expressing the syndecan-1 ectodomain were harvested by centrifugation, incubated with 10 μg/ml fluorescein isothiocyanate-labeled monoclonal antibody 281.2, washed, and analyzed by flow cytometry. For cells expressing the glypican-1 extracellular domain, cells were incubated with the antisera to glypican-1 (2.5 μg/ml) followed by a biotinylated goat anti-rabbit IgG (5 μg/ml) and avidin-fluorescein isothiocyanate (Vector Laboratories) and analyzed by flow cytometry.

TABLE I
Expression of syndecan-2 inhibits cell invasion

Clones of transfected cells were isolated and analyzed for syndecan-2 (syn-2) expression by Northern blotting. Isolation of clone A5P3, which expresses syndecan-1, was previously described (34). Cells were plated on the surface of type I collagen gels, returned to the culture incubator for 48 h, and the percent cells invading and the depth of invasion were quantified (34). Data shown are from a minimum of three separate experiments, and duplicate wells of each clone were included in each experiment. Values represent means ± S.E.

Transfectant clone	Syndecan expression	Percent cells invading	Depth of invasion
			μm
A5P3	Syn-1	3.3 ± 0.7	509 ± 8.2
2–2-1	Syn-2	3.1 ± 0.3	459 ± 19.3
2–2-3	Syn-2	3.4 ± 0.1	477 ± 17.9
2–2-5	Syn-2	3.1 ± 0.2	482 ± 11.1
Neo	Negative	18.4 ± 0.3	1345 ± 44.4

Affinity Co-electrophoresis—The affinities of proteoglycans to collagen were determined using affinity co-electrophoresis as described previously (43, 44). Briefly, $^{35}SO_4$-labeled cells that expressed syndecan-1 or glypican-1 were extracted and proteoglycans bound to DEAE beads as described above for Western blotting. The proteoglycans were then eluted with 1 M NaCl in PBS containing 0.1% Triton X-100 and diluted with 10 mM Tris, pH 8.0, to a final concentration of 0.15 M NaCl. Samples were digested with chondroitinase ABC (50 milliunits/ml) for 1 h at 37 °C, desalted by passing over excellulose columns, and eluted from the columns with electrophoresis buffer containing 0.1 M sodium acetate and 50 mM sodium MOPSO (Fluka Biochemika), pH 7.0. The samples (50,000 cpm per gel) were subjected to electrophoresis through 1% agarose gels containing lanes in which rat tail type I collagen had been incorporated at a range of concentrations. Following electrophoresis, the gel was soaked in 5% acetic acid for 4 h and dried with forced warm air. The labeled material was detected using a PhosphorImager (Molecular Dynamics), and plots of radioactivity *versus* distance from the origin were obtained for each collagen-containing lane using ImageQuant software (Molecular Dynamics). Because proteoglycan samples were heterogeneous in their affinities for type I collagen, and because it had previously been shown that proteoglycan binding results in an arrest of migration at the start of collagen-containing lanes (44), bound fraction was quantified as the percentage of radioactivity that had been shifted to within 100 pixels of the origin (representing a mobility less than one-third of that of free proteoglycan in the same gels). Bound fraction (θ) was plotted as a function of collagen concentration and fit to the equation $\theta = 1/(1 + (K_d/[\text{collagen}])^2)$ (44).

Cell Spreading Assay—A 24-well tissue culture plate was coated with 250 μl (per well) of either 0.1% BSA in PBS or 20 μg/ml monoclonal antibody 281.2 in PBS overnight at 4 °C. The coated wells were washed with PBS, blocked with 0.1% BSA in PBS for 1 h at room temperature, and washed again with PBS. Cells were then plated on the coated wells (5×10^4 cells per well) in 500 μl of RPMI 1640 containing 5% fetal bovine serum and incubated for 2 h at 37 °C. In some experiments, cells were preincubated for 1 h at 37 °C in medium either with or without 10 μM cytochalasin D (Calbiochem).

RESULTS

Cells Expressing Syndecans-1, -2, or -4 Fail to Invade Collagen Gels—Clones of syndecan-2- or syndecan-4-transfected cells were isolated by two limiting dilutions and analyzed for specific syndecan expression by Northern blotting (data not shown). All clones expressing syndecan-2 or syndecan-4 bound to type I collagen-coated microtiter wells, whereas the neo-transfected cells failed to bind (data not shown). Cells expressing the proteoglycans, or neo-transfected controls, were plated on the surface of native type I collagen gels, and the distance to the leading front of the invading cells and the percentage of cells invading the gel were quantified as described previously (34). Clones expressing syndecan-2 are inhibited from invading gels in a manner similar to syndecan-1 (Table I; relative to neo-transfected cells, expression of syndecan-2 results in an 83% inhibition of cell invasion). Clones expressing syndecan-4

TABLE II
Expression of syndecan-4 inhibits cell invasion

Clones of transfected cells were isolated and analyzed for syndecan-4 (Syn-4) expression by Northern blotting and analyzed for invasion into type I collagen gels. Data shown are from a minimum of three separate experiments, and duplicate wells of each clone were included in each experiment. Values represent means ± S.E.

Transfectant clone	Syndecan expression	Percent cells invading	Depth of invasion
			μm
A5P3	Syn-1	4.3 ± 0.2	335 ± 11.6
4–2-4	Syn-4	8.1 ± 0.4	638 ± 27.1
4–2-5	Syn-4	7.8 ± 0.6	624 ± 25.0
4–4-3	Syn-4	7.9 ± 0.4	565 ± 8.6
Neo	Negative	19.1 ± 0.5	1190 ± 17.5

also invade gels poorly but to a greater extent than cells expressing syndecan-1 or syndecan-2 (Table II; relative to neo-transfected cells expression of syndecan-4 results in an 59% inhibition of cell invasion). Thus, multiple members of the syndecan family can inhibit cell invasion.

Cells Expressing Glypican-1 Readily Invade Collagen Gels—Glypican-1-expressing cells (ARH-77^{glyp}) were isolated by flow cytometry and their ability to invade gels analyzed. In contrast to cells expressing syndecans, cells expressing glypican-1 can migrate through a collagen matrix as well as neo-transfected control cells (Table III).

The molecular characteristics of syndecan-1 and glypican-1 expressed by the ARH-77 cells were compared to determine if differences in proteoglycan size, relative amount of heparan sulfate, or affinity for collagen could account for the differences in their effects on cell behavior. First, cell extracts from the ARH-77^{syn} and ARH-77^{glyp} cells were subjected to Western blotting with antibodies specific for syndecan-1 and glypican-1. As shown in Fig. 2, both proteoglycans, when expressed in ARH-77 cells, give rise to broad smears of similar electrophoretic mobility. Treatment with heparitinase prior to SDS-PAGE analysis converted the glypican-1 smear to a tight core protein band and condensed the syndecan-1 smear to a band with some residual smearing, consistent with the presence of small chondroitin sulfate chains on some of the syndecan-1 molecules (34, 45). Thus, syndecan-1 and glypican-1 expressed by these cells are similar in apparent overall size and bear predominantly heparan sulfate chains. Moreover, because the syndecan-1 and glypican-1 core proteins migrate at similar positions on SDS-PAGE gels, the finding that the intact proteoglycans also migrate similarly suggests that, on a per molecule basis, they carry about the same amount of heparan sulfate.

Second, we compared the relative amount of heparan sulfate present at the cell surface using 10E4, a monoclonal antibody specific for native heparan sulfate chains (7, 42). We analyzed a series of syndecan-1-expressing clones and found that one of them, ARH-77$^{syn(B3P3)}$, which we have previously shown is non-invasive on collagen gels (34), had levels of heparan sulfate similar to that on the surface of ARH-77^{glyp} cells (Fig. 3). The mean fluorescence intensities of ARH-77^{glyp} and ARH-77$^{syn(B3P3)}$ cells were 177.89 and 208.47, respectively, a ratio of 0.85:1. Amounts of ^{35}S-labeled heparan sulfate proteoglycan from the ARH-77^{glyp} and ARH-77$^{syn(B3P3)}$ were also compared using cetylpyridinium chloride and trichloroacetic acid precipitation of cell extracts spotted on Whatman 3MM filter discs (46). This analysis revealed a ratio of approximately 0.8:1 (ARH-77^{glyp}:ARH-77$^{syn(B3P3)}$ (data not shown)). Thus, the amount of heparan sulfate and the amount of ^{35}S-labeled proteoglycan is about 20% greater on the ARH-77^{syn} than on ARH-77^{glyp} cells. To be sure that this difference in heparan sulfate is not responsible for the differences in cell behavior in the invasion assay, we used flow cytometry to isolate ARH-77$^{syn(B3P3)}$ low expressing (the 25% of cells expressing the lowest amount of syndecan-1) and ARH-77^{glyp} high expressing (the 25% of cells expressing the highest amount of glypican-1) cells. In these cells, heparan sulfate levels as determined by 10E4 staining were higher on the ARH-77^{glyp} cells than on the ARH-77$^{syn(B3P3)}$ cells (ratio 1:0.87; data not shown). When assayed for invasion, 9.6 ± 0.3% of the ARH-77$^{syn(B3P3)}$ low cells invaded and 17.9 ± 1.0% of the ARH-77^{glyp} high cells invaded. Thus, even transfectants expressing the highest levels of glypican-1 are not inhibited from invading into the gels. Taken together, these results suggest that differences in behavior of the ARH-77^{glyp} and ARH-77^{syn} cells are not due to differences

TABLE III
Glypican-1 does not inhibit cell invasion

Transfected cells expressing glypican-1 or syndecan-1 were isolated by flow cytometry and analyzed for invasion into type I collagen gels. Data shown are from a minimum of three separate experiments, and duplicate wells were included in each experiment. Values represent means ± S.E.

Proteoglycan expression	Percent cells invading	Depth of cell invasion
		μm
Syndecan-1	4.5 ± 0.2	502 ± 6
Glypican-1	16.1 ± 1.1	1040 ± 20
Neo	17.7 ± 0.7	1353 ± 77

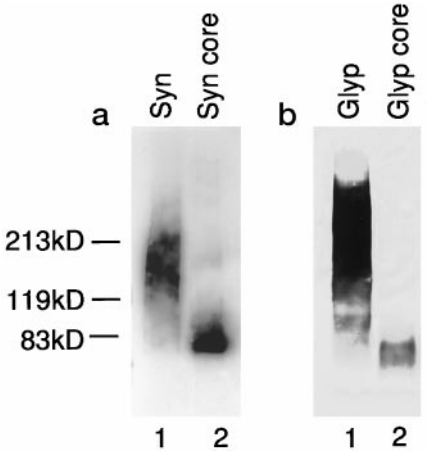

FIG. 2. **When expressed on ARH-77 cells, syndecan-1 and glypican-1 bear predominantly heparan sulfate chains and are similar in apparent molecular size.** Western blot of extracts from ARH-77 cells expressing syndecan-1 (*a*) or glypican-1 (*b*). To visualize core proteins, samples were treated with heparitinase prior to electrophoresis (*lane 2*).

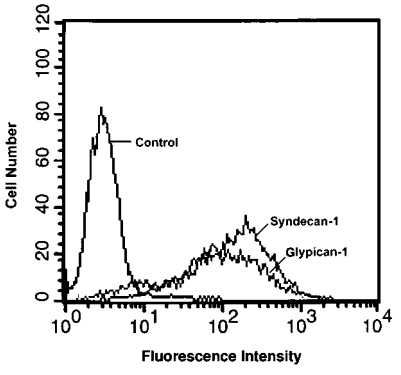

FIG. 3. **Syndecan-1 and glypican-1 expressing cells have similar levels of heparan sulfate on their cell surface.** ARH-77$^{syn(B3P3)}$ and ARH-77^{glyp} cells were stained with anti-heparan sulfate antibody 10E4 or isotype-matched control antibody and analyzed by flow cytometry.

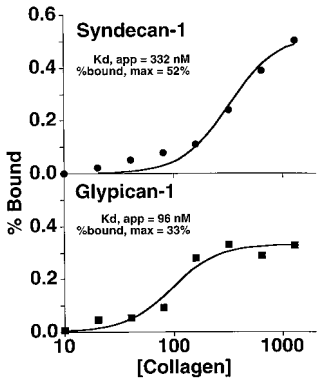

FIG. 4. **Heparan sulfate proteoglycans present on the syndecan-1 and glypican-1 expressing cells have similar affinities for type I collagen as assessed by affinity co-electrophoresis.** The fraction of each proteoglycan sample that bound and the apparent affinity constants were calculated from electrophoretic mobility at multiple collagen concentrations and are shown.

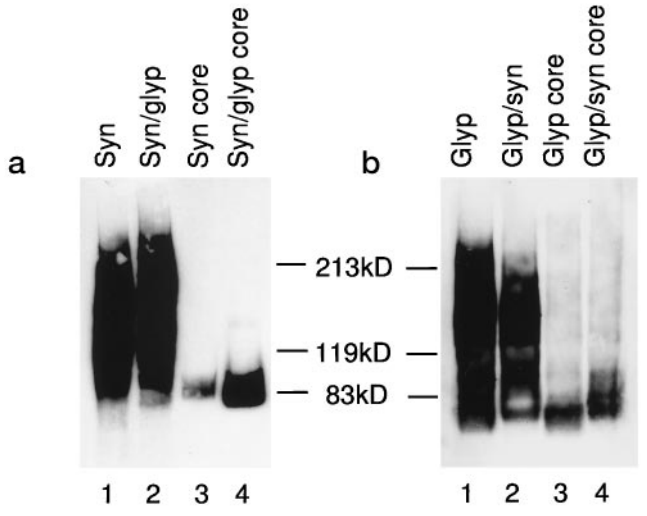

FIG. 5. **Western blot of wild-type and chimeric proteoglycans and their core proteins.** Extracted proteoglycans were isolated and probed with antibodies to the extracellular core protein domain of syndecan-1 (*a*) or glypican-1 (*b*). To visualize core proteins, samples were treated with heparitinase and chondroitinase ABC (for syn and syn/glyp) or with heparitinase only (for glyp and glyp/syn).

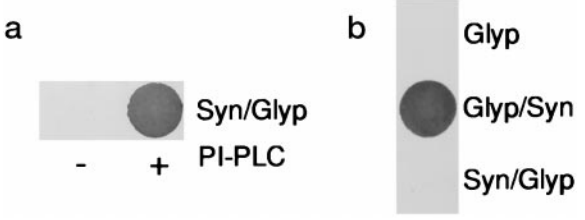

FIG. 6. **Syn/glyp and glyp/syn are chimeric proteoglycans.** *a,* PI-PLC releases the syndecan-1 ectodomain from cells expressing the syn/glyp chimeric proteoglycan. Syn/glyp cells were incubated in the presence or absence of PI-PLC, and the supernatants were harvested and analyzed for syndecan-1 by immuno-dot blotting using antibody 281.2. *b,* the syndecan-1 cytoplasmic domain is present in cells expressing the glyp/syn chimeric proteoglycan. Extracts from cells were blotted onto filters and probed with antisera to the syndecan-1 cytoplasmic domain.

in the amount of heparan sulfate at the cell surface. However, the data do not rule out the possibility that differences in heparan sulfate fine structure (*i.e.* patterns of epimerization/sulfation) between the glypican-1 and syndecan-1 proteoglycans contribute to the differences observed in cell behavior.

Third, the affinity for type I collagen of heparan sulfate proteoglycans extracted from the ARH-77^{syn} and ARH-77^{glyp} cells was analyzed (Fig. 4). As previously seen for syndecan-1 expressed in murine myeloma cells (44), collagen I binding by syndecan-1 and glypican-1 expressed in ARH-77 cells was heterogeneous, with 52% of syndecan-1 molecules and 33% of glypican-1 molecules showing measurable binding. Of those molecules, glypican-1 actually shows a somewhat higher apparent affinity for type I collagen (apparent K_d = 96 nM) than syndecan-1 (apparent K_d = 332 nM). Thus, the ability of ARH-77^{glyp} cells to invade collagen gels is not due to a low affinity of glypican-1 for type I collagen.

Characterization of Chimeric Proteoglycans Expressed in ARH-77 Cells—The results described above suggest that differences in the effects of syndecan-1 and glypican-1 on the behavior of ARH-77 cells are not due to differences in proteoglycan size, cell-surface heparan sulfate content, or proteoglycan affinity for collagen. We therefore speculated that the different effects of these proteoglycans depended on their different modes of anchorage to the cell membrane. To test this idea, constructs encoding chimeric proteoglycans were produced, one a syndecan-1 extracellular domain with the GPI-linkage region of glypican-1 (designated syn/glyp) and another a glypican-1 extracellular domain with a syndecan-1 transmembrane and cytoplasmic domain (designated glyp/syn). Following transfection into ARH-77 cells, chimeric proteoglycans were analyzed by Western blotting (Fig. 5). As expected, the core protein of the syn/glyp chimera is smaller than the wild-type syndecan-1 core, and the core protein of the glyp/syn chimera is larger than that of the wild-type glypican-1.

To confirm that the syn/glyp chimeric proteoglycan is attached to the cell surface via a GPI anchor, cells bearing this chimera were treated with PI-PLC that cleaves GPI-anchored proteins. Analysis of supernatants by immunoblotting following PI-PLC treatment indicates that the syndecan-1 extracellular domain is released (Fig. 6*a*). To demonstrate that the syndecan-1 transmembrane and cytoplasmic domain are present on the glyp/syn chimera, cell extracts were probed with an antisera specific for the 7 C-terminal amino acids present in the syndecan-1 cytoplasmic domain. As expected, this antibody detects the glyp/syn chimera but not the syn/glyp chimera or the glypican-1 proteoglycan (Fig. 6*b*).

The relative amounts of the core proteins of the chimeras were also determined by flow cytometry of cells stained with antibodies to either the syndecan-1 or glypican-1 ectodomain. Similar levels of syndecan-1 ectodomain core protein were present on the syndecan-1, syn/glyp, and 279t expressing cells (mean fluorescence intensities of 122.26, 130.68, and 123.87, respectively), and similar levels of the glypican-1 core protein were present on the glypican-1 and glyp/syn expressing cells (mean fluorescence intensities of 111.11 and 112.65, respectively). Although a direct comparison of the level of core proteins expressed by cells bearing the syndecan and glypican ectodomains cannot be made because a single antibody does not recognize them both, we were able to make an indirect comparison using the antibody to the syndecan cytoplasmic domain. Quantification of proteoglycan present in cell extracts using dot blots probed with the syndecan-1 anti-cytoplasmic domain antibody indicates that the amount of core protein present on the glyp/syn cells is 1.12 times that on the cells expressing wild-type syndecan-1 (data not shown). Together with the data shown above indicating that the glyp/syn and glypican-1-expressing cells have similar amounts of glypican core protein and that the syn/glyp and syndecan-1-expressing cells have similar levels of syndecan-1 core protein, we conclude that the levels of glypican-1 and syndecan-1 ectodomains present on all these transfected cells are similar. Thus, the finding that cells expressing high levels of glypican-1 invade collagen

TABLE IV
The mode of anchorage to the membrane does not regulate proteoglycan function as an anti-invasive molecule

Cells expressing chimeric proteoglycans were isolated by flow cytometry and analyzed for invasion into type I collagen gels as described in the legend to Table I. Data shown are from a minimum of three separate experiments, and duplicate wells of each clone were included in each experiment. Values represent means ± S.E.

ARH-77 transfectants	Percent cells invading	Depth of invasion
		μm
Neo	21.6 ± 1.0	1,906 ± 68
Glypican-1	20.8 ± 1.1	1,882 ± 40
Glyp/Syn	17.3 ± 1.5	1,420 ± 23
Syndecan-1	4.3 ± 0.3	670 ± 18
Syn/Glyp	7.5 ± 0.9	723 ± 12
Syn/279t	4.7 ± 1.6	700 ± 1

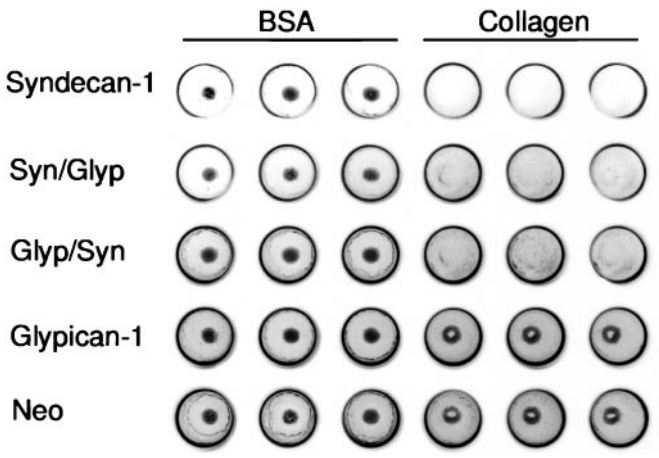

FIG. 7. **Adhesion to type I collagen of ARH-77 cells bearing wild-type or chimeric proteoglycans.** Unbound cells form a pellet in the center of the well, and bound cells form a uniform coating on the well surface.

gels (as described above for sorted cells) strongly suggests that the differences in the behavior of the cells bearing the syndecan-1 and glypican-1 ectodomain are not due differences in the relative levels of their core protein.

The Extracellular Domain Determines Invasive Phenotype—Analysis of chimeric proteoglycans in the collagen invasion assay demonstrates that cells expressing syndecan-1 with a GPI anchor (syn/glyp) are largely non-invasive, whereas those expressing glypican-1 with a syndecan-1 transmembrane and cytoplasmic domain (glyp/syn) remain invasive (Table IV). Thus, mode of anchorage to the membrane does *not* correlate with invasive phenotype. These results indicate that the highly conserved transmembrane and cytoplasmic domains of syndecans are not required for inhibition of invasion, a surprising result given that syndecan cytoplasmic domains are known to interact with the actin cytoskeleton (47) and may be involved in signaling (48). Therefore, to confirm that the syndecan-1 cytoplasmic domain is not required for inhibition of invasion, we prepared a syndecan-1 mutant that retains the transmembrane domain but lacks all but one amino acid of the cytoplasmic domain (ARH-77$^{syn/279t}$). Cells expressing this mutant invaded gels poorly, closely resembling the behavior of cells expressing wild-type syndecan-1 (Table IV). Taken together, these experiments demonstrate that the determining factor for the anti-invasive function of syndecan-1 is the extracellular domain.

Inhibition of Invasion Is Not Simply Due to Cell Adhesion to Collagen—The ability of syndecan-1 to mediate cell adhesion to type I collagen likely plays a role in its ability to inhibit invasion into collagen gels (34). Therefore, one possible explanation for the inability of glypican-1 to inhibit invasion is that glypican-1, even though it interacts with collagen (Fig. 4), it is not able to bind cells to collagen. To test this, we compared the ability of the various transfected cells to bind to collagen in a solid phase assay (49). As shown in Fig. 7, the non-invasive ARH-77^{syn} and the ARH-77$^{syn/glyp}$ cells bind collagen tightly, whereas both the highly invasive ARH-77^{glyp} and ARH-77^{neo} cells failed to bind. Yet the cells expressing the glyp/syn chimera, which are invasive, also bind collagen (Fig. 7). Binding via the glyp/syn chimera is lost following heparitinase treatment of cells (not shown) thereby demonstrating that the glypican-1 extracellular domain bears heparan sulfate chains that can bind cells to collagen. Moreover, because the glyp/syn expressing cells are invasive, it indicates that cell binding to collagen is not sufficient for inhibition of invasion. Interestingly, the data also suggest that for promotion of cell adhesion by some cell-surface heparan sulfate proteoglycans, the presence of a transmembrane or cytoplasmic domain (as opposed to a GPI anchor) is important.

The Syndecan-1 Extracellular Domain Also Mediates Cell Spreading—The finding that the extracellular domain of syndecan-1 is sufficient to inhibit invasion and that this effect is not just a consequence of cell binding to collagen suggests that the syndecan-1 extracellular domain interacts with molecules at the cell surface that control cell behavior, perhaps through effects on signaling pathways. Results from a recent study analyzing spreading of Raji cells on plates coated with antibody to syndecan-1 indicated that syndecan-1 mediates spreading through the formation of a multimolecular signaling complex at the cell surface (6). As shown in Fig. 8*b*, ARH-77 cells transfected with syndecan-1 also spread after 30 min at 37 °C on a surface coated with anti-syndecan-1 antibody 281.2, whose epitope is on the syndecan-1 extracellular domain. To determine if the syndecan-1 extracellular domain alone mediates this spreading response, ARH-77 cells expressing the syn/glyp chimera were plated onto wells coated with antibody 281.2. After 30 min at 37 °C, cell spreading has clearly taken place (Fig. 8*c*). When the same experiment was performed in the presence of cytochalasin D, an inhibitor of actin polymerization, the syn/glyp cells fail to spread (Fig. 8*d*). Thus, as with invasion of collagen gels, the determining factor for cells spreading appears to be the syndecan-1 extracellular domain.

DISCUSSION

The present study reveals several new findings relevant to understanding the biology of cell-surface heparan sulfate proteoglycans. By using assays that assess proteoglycan-mediated adhesion and inhibition of cell invasion, we find that (i) syndecans-1, -2, and -4 all mediate cell binding to collagen and inhibit cell invasion; (ii) glypican-1 fails to mediate cell adhesion to collagen and does not inhibit invasion; (iii) chimeric proteoglycans inhibit cell invasion if and only if they contain a syndecan extracellular domain; and (iv) a chimeric proteoglycan can mediate cell attachment to collagen yet fail to inhibit invasion of collagen gels. From these results, we conclude the following.

The ability to inhibit invasion is a specific property of the syndecans, and this property is shared among the different syndecan family members. This conclusion agrees with other studies suggesting functional redundancy among syndecans. For example, syndecans-1, -2, and -4 can all promote the interaction of basic FGF with FGF receptor-1 (7) and the binding and uptake of lipoproteins (50). Furthermore, we have also found that syndecans-2 and -4 can mediate cell-cell and cell-

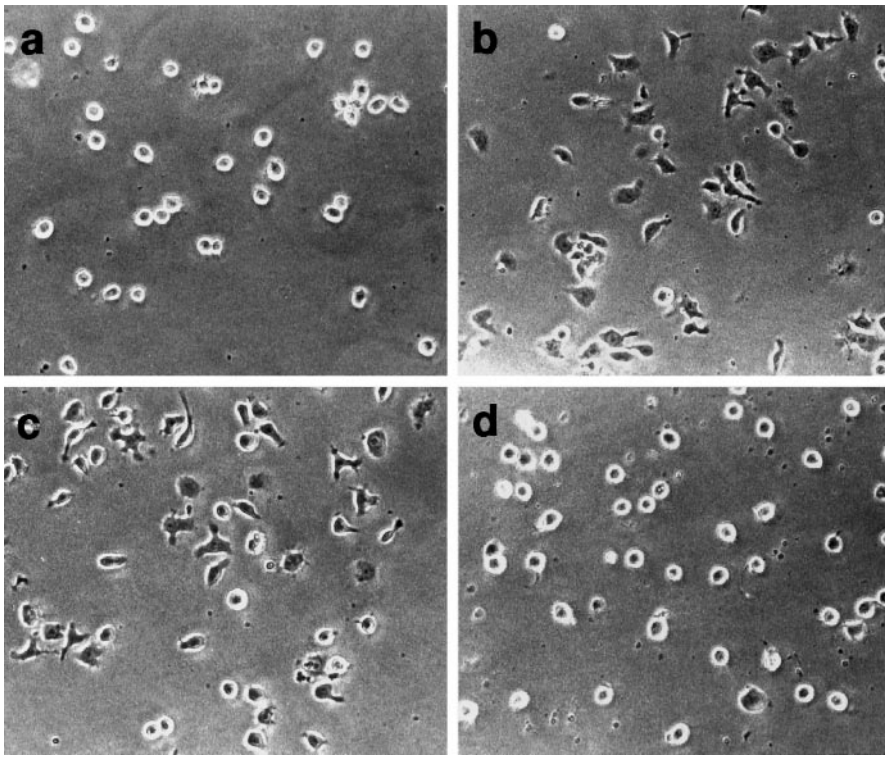

FIG. 8. **Cells expressing the syn/glyp chimeric proteoglycan spread on wells coated with antibody to the extracellular core protein domain of syndecan-1.** Neo-transfected control cells (*a*), cells expressing syndecan-1 (*b*), cells expressing the syn/glyp chimeric proteoglycan in the absence (*c*) or presence (*d*) of cytochalasin D are shown.

matrix (type I collagen) adhesion in a fashion indistinguishable from that of syndecan-1.[2]

Inhibition of invasion into collagen gels is not simply a consequence of cell adhesion to collagen (although such adhesion could be a prerequisite for inhibition of invasion). Interestingly, the ability of both the syn/glyp chimera and the glyp/syn chimera, but not native glypican-1, to mediate cell adhesion to type I collagen suggests that neither the syndecan extracellular core protein domain nor the cytoplasmic and transmembrane domains are absolutely required for promotion of adhesion. Yet the presence of heparan sulfate chains alone is clearly not sufficient for adhesion, since collagen-binding heparan sulfate chains are found on glypican-1 (Fig. 4). Apparently, amino acid sequences in either the extracellular domain of syndecans or in the transmembrane plus cytoplasmic domains are sufficient to enable syndecan-1 to participate in cell-extracellular matrix adhesion. Such a role for syndecan cytoplasmic domains fits with the mounting evidence that these highly evolutionarily conserved domains interact with cytoskeletal elements (47, 51) and with the widely held view that coupling of cell-surface receptors to the actin cytoskeleton is a prerequisite for strong cell adhesion (52). How the syndecan-1 extracellular domain can also participate in strong cell adhesion (even when lacking a transmembrane and cytoplasmic domain, as in the syn/glyp chimera) remains a mystery.

Unlike the ability to promote cell adhesion, the ability to inhibit invasion into collagen gels "maps" only to the syndecan extracellular domain. Yet, as with promotion of cell adhesion, the presence of heparan sulfate chains alone cannot account for this activity. We suspect, therefore, that the extracellular domains of syndecans engage in protein-protein interactions with macromolecules on the same cell surface and that these interactions lead to the transduction of a signal in a manner that depends on the binding of syndecan to extracellular ligands. In one version of this hypothesis, these interactions are between adjacent syndecan molecules. Indeed, the core proteins of syndecan-1 and syndecan-2 can spontaneously oligomerize into detergent-resistant complexes (53, 54), and syndecan-3 expressed in 293 cells can be cross-linked to itself (55). However, experiments with syndecan-3 have suggested that transmembrane domain sequences are required for such aggregation (55). The fact that transmembrane sequences are absent from syn/glyp chimeras (which inhibit invasion) argues against an essential role for syndecan aggregation in inhibition of invasion. Furthermore, even if aggregation is important in the inhibition of invasion, the absence of any cytoplasmic domain in the syn/glyp chimera suggests that other surface molecules would have to be involved as well.

Interestingly, Lebakken and Rapraeger (6) recently also proposed that signals may be transduced by the syndecan-1 core protein via interactions with other membrane proteins. They found that syndecan-1-transfected Raji cells underwent actin-dependent spreading on substrata containing either heparan sulfate-binding ligands or antibodies to the syndecan-1 core protein; that spreading occurred independent of the presence of a syndecan cytoplasmic domain; and that spreading on anti-syndecan antibodies did not require the presence of glycosaminoglycan chains on the syndecan molecules. If the mechanisms underlying Raji cell spreading and inhibition of myeloma cell invasion of collagen gels are similar, then our data extend those of Lebakken and Rapraeger (6) by showing that the syndecan-1 transmembrane domain is not required and cell-matrix adhesion alone is not sufficient for this activity.

Clearly, an important goal for the future will be the identification of plasma membrane signaling molecules with which syndecan extracellular domains interact. Although no such molecules are currently known, an increasing number of signaling pathways are being described in which ligand binding and signal transduction functions exist in separate cell-surface proteins; these include the transforming growth factor-β and the glial cell line-derived neurotrophic factor signaling systems (56, 57). The possibility that syndecans are involved in similar systems, although novel, fits with the overall view that cell-

[2] W. Liu, M. Stanley, and R. D. Sanderson, unpublished observations.

surface proteoglycans play co-receptor roles in the responses of cells to their environment (11).

In addition to addressing possible roles of cell-surface proteoglycans in signaling, the results of the present study also speak to the issue of why two distinct families of cell-surface heparan sulfate proteoglycans, the syndecans and the glypicans, exist. The glypicans, because they are unable to bind cells to matrix, may predominantly perform co-receptor functions involving the presentation or delivery of soluble, heparin-binding proteins (*e.g.* growth factors) to their receptors. To this end, the increased plasma membrane mobility associated with a GPI anchor may prove advantageous. In contrast, syndecans may be specialized to interact with the extracellular matrix in a manner that directly influences both cell adhesion and motility. This idea is supported by the observation that syndecan-1 and -4 (but not glypican-1) are sorted almost entirely to the basolateral surface of adherent, polarized cells (14) and that syndecan-4 specifically localizes to the focal contacts of mesenchymal cells (18, 19).

Acknowledgments—We are grateful to Drs. John Gallagher and Graham Cowling (University of Manchester, Manchester, UK) for the syndecan-2 and syndecan-4 cDNAs and to Dr. Merton Bernfield (Harvard Medical School, Boston) for the syndecan-1 cDNA and for antibodies to the syndecan-1 core protein. We thank Allison Theus and Debbie Anhalt for technical assistance and Jeff Woodliff and John Theus for assistance with flow cytometry.

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