Characterization of the High Affinity Cell-binding Domain in the Cell Surface Proteoglycan Syndecan-4*

(Received for publication, May 7, 1998, and in revised form, July 22, 1998)

Aidan J. McFall and Alan C. Rapraeger‡

From the Program in Cellular and Molecular Biology and Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706-1532

The syndecan family of cell surface proteoglycans regulates cell adhesion via their glycosaminoglycan chains and discrete domains of their core proteins. Core protein domains that are variable between syndecan family members may regulate syndecan-specific associations, thereby endowing individual syndecans with unique functions. A syndecan-4-specific domain has been identified in the extracellular syndecan-4 protein. This region mediates cell adhesion when provided as an artificial substratum and is localized within amino acids 56–109 of the recombinant extracellular protein domain of mouse syndecan-4 (mS4ED) (McFall, A. J., and Rapraeger, A. C. (1997) J. Biol. Chem. 272, 12901–12904). To characterize its interaction with the cell surface, radiolabeled ligand binding studies were performed. A single high affinity interaction, with a dissociation constant of $2 \times 10^{-9}$ M, was observed between mS4ED and both human and mouse cells. Both chicken S4ED and mS4ED compete for this interaction, although they are only 34% identical within the cell-binding domain sequence. The extracellular protein domains of syndecan-1, -2, and -3, however, fail to compete. The interaction is also observed with native syndecan-4 shed from cell surfaces. Interestingly, the extracellular protein domain of syndecan-1 also mediates cell adhesion, suggesting a similar but discrete interaction for this family member.

The syndecan family of cell surface proteoglycans contains four vertebrate members, syndecans 1–4, that regulate cell adhesion, growth factor signaling, and maintenance of cell morphology (1–3). They do so via interactions with the glycosaminoglycan chains which decorate the syndecan core proteins and the core proteins themselves. Most cells express multiple syndecan family members, suggesting that the individual syndecan proteins, which are distinct gene products, are likely to have unique functions (4).

The syndecan proteins can be divided into three domains. These are (i) a short cytoplasmic domain about 30 amino acids long, (ii) a single transmembrane domain, and (iii) an extracellular domain that is modified by the addition of heparan sulfate glycosaminoglycan chains. Within these domains are both conserved and variable regions. Conserved regions have the potential to modulate functions shared by all syndecan family members. For example, the cytoplasmic domains of all known syndecans contain the C-terminal sequence EFYA which has recently been shown to interact with syntentin, a novel PDZ domain-containing protein (5). This association has the potential to link the syndecans to both structural and signaling pathways within the cell (6). The transmembrane domains of the syndecans are also homologous and are likely to contribute to the shared ability of all syndecan core proteins to homodimerize and potentially multimerize (1, 7, 8).

In contrast to conserved regions within the syndecan core proteins, variable regions are candidates for unique, or syndecan-specific, interactions. One variable region of the syndecans is found within the cytoplasmic domain. This region mediates a direct association between syndecan-4 and the catalytic domain of protein kinase C (PKC) (8, 9). This interaction appears to be specific for this syndecan family member and stimulates PKC activity, suggesting a syndecan-4-specific signaling function within cells. A second variable region of the syndecan core proteins is the extracellular domain. Previous work has shown that the recombinant syndecan-4 extracellular protein domain (S4ED) can function to mediate cell adhesion when supplied to cells as an artificial substratum (10). The adhesive interaction is specific for syndecan-4 since (i) the extracellular protein domain of syndecan-1 fails to compete for cell adhesion and (ii) the extracellular protein domains of both mouse and chicken syndecan-4 compete equally well for cell adhesion. This interaction is mediated by a discrete domain within mS4ED, namely the cell-binding domain.

To further explore the association between the extracellular protein domain of syndecan-4 and the cell surface, radiolabeled ligand binding studies were performed. These studies demonstrate that recombinant and native syndecan-4 shed from the cell surface specifically interact with a single class of cell surface-binding sites. Cell adhesion assays suggest that at least one other syndecan family member, syndecan-1, has a similar but discrete cell-surface association.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Neonatal human foreskin fibroblasts, kindly provided by Dr. Donna Peters (University of Wisconsin-Madison, Madison, WI), were cultivated in DMEM (Life Technologies, Inc.) supplemented with 7.5% fetal calf serum (Hyclone) and used prior to 60 days in culture. Mouse nortic endothelial cells, kindly provided by Dr. Robert Auerbach (University of Wisconsin-Madison, Madison, WI) and Swiss 3T3, 143 osteosarcoma, and NIH 3T3 cell lines were incubated in DMEM supplemented with 10% calf serum (Hyclone). Low passage NMuMG epithelial cells were incubated in the same medium supplemented with 10 µg/ml insulin. The ARH-77 lymphocyte cell lines, kindly provided by Dr. Ralph Sanderson (University of Arkansas, Little Rock, AR), were...

---

* This work was supported by Grant HD21881 from the National Institutes of Health. 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.

‡ To whom correspondence should be addressed: Dept. of Pathology and Laboratory Medicine, University of Wisconsin-Madison, 1300 University Ave., Madison, WI 53706-1532. Tel.: 608-262-7577; Fax: 608-265-3301; E-mail: acraprae@facstaff.wisc.edu.

1 The abbreviations used are: PKC, protein kinase C; mS1ED-mS4ED, mouse syndecan-1 through -4 extracellular protein domains; cS4ED, chicken syndecan-4 extracellular domain; GST, glutathione S-transferase; HEPES/DMEM, HEPES-buffered Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
incubated in RPMI (Life Technologies, Inc.) and supplemented with 10% calf serum.

**Production of Recombinant Syndecan Extracellular Protein Domains**—Mouse syndecan-2 cDNA was obtained from Dr. Guido David (University of Leuven, Leuven, Belgium) (11). Mouse syndecan-3 cDNA was a gift of Dr. Virginia Dixit (Salk Institute, La Jolla, CA). Nucleotides 578 through 952 of the mouse syndecan-2 sequence (which corresponds to 247 through 1260 of the rat sequence) were amplified with 10X of the pGEX-2T vector and a (5' XbaI restriction enzyme sites and (ii) nucleotides 325 of mS4ED through 1020 of the pGEX-2T vector and a (5') NheI restriction enzyme site.

**Cell Adhesion Assays**—Two cell adhesion assays were used. In the first assay, cells were trypsinized (except for the ARH-77 cell lines which are cultured in suspension) and plated in U-shaped microtiter wells (Costar) coated with protein ligands as described previously (10). After an adhesion period of 1 or 2 h at 37 °C, unbound cells were washed from the wells and the remaining cells fixed and quantified by colorimetric detection with 1% bromphenol blue. Unless otherwise noted, cells were incubated with 25 μg/ml cycloheximide for 2 h prior to and during this type of adhesion assay.

The second cell adhesion assay is identical to the first, with the following exceptions. Cells were not exposed to cycloheximide and after an adhesion period of 1 h, the wells were centrifuged at 150 × g for 1.5 min. Both bound and unbound cells, which pellet, remain in the wells and were fixed by the addition of 0.8% glutaraldehyde. The cells in the wells were stained with 1% bromphenol blue and photographed.

**Radioabeled mS4ED Binding Assays—Thrombin-released recombinant mS4ED peptide was labeled with 125I to a specific activity of 200–250 cpm/nmol using a modified chloramine T procedure. Reactions typically contained 100 μg of mS4ED, 1 mCi of Na[125]I (NEN Life Science Products), and 40 μg of chloramine T. Unconjugated iodine was removed by G-10 Sephadex chromatography.

Cell monolayers were prepared by plating cells on fibronectin-coated tissue culture plastic. Fibronectin facilitates monolayer formation but it is not required for specific binding of mS4ED to cells (Fig. 1A and data not shown). Adherent cells were washed with buffer containing 25 mM HEPES, 5 mM KCl, 138 mM NaCl, 5.6 mM glucose, 0.25 mM EDTA, pH 7.4, prior to trypsinization with 0.1% trypsin dissolved in the same. Cells were subsequently washed with HEPES/DMEM (HEPES-buffered medium containing 0.05% trypsin inhibitor (Sigma). Cells cultured in suspension, such as the ARH-77 cell lines, were washed once with buffer containing 25 mM HEPES, 5 mM KCl, 138 mM NaCl, 5.6 mM glucose, 0.25 mM EDTA, pH 7.4. All cells were then suspended in HEPES/DMEM and added to 24-well tissue culture plates pre-coated with 0.5 mM NaOH to determine the amount of cell-associated radioactivity. To determine the amount of cell-surface bound mS4ED, cells were incubated with 25 μg/ml fibronectin. The ARH-77 and NMuMG cells were plated at 5 × 10⁵ cells per cm²; all other cells were plated at 2.5 × 10⁵ cells per cm². After incubating for 30 min at 37 °C, non-adherent cells were aspirated from the wells and the resulting monolayers were blocked with 1% diazoyl heat-denatured BSA for an additional 30 min.

The adherent monolayers of cells were subsequently used to perform radioabeled ligand binding studies. Binding buffer consisted of HEPES/DMEM or 25 mM HEPES, 5 mM KCl, 138 mM NaCl, 5.6 mM glucose, pH 7.4, if testing the contribution of divalent cations. After equilibration on an ice-water bath, iodinated mS4ED, competitor molecules, and 0.5% diazoyl heat-denatured BSA were added to cells and binding reactions allowed to proceed for 200 min at 4 °C (unless otherwise specified). Cell monolayers were then washed five times with ice-cold HEPES/DMEM containing 0.2% diazoyl heat-denatured BSA. The contents of the wells were solubilized with buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM Tris containing 0.5 mM NaOH to determine the amount of cell-associated radioactivity. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled ligand and subtracted from total binding to determine specific binding.

To examine the minimum of cell-surface bound mS4ED, cells were washed with low pH buffer containing 15 mM glycine and 150 mM NaCl, pH 2.0, or buffer containing 25 mM HEPES, 5 mM KCl, 138 mM NaCl, 5.6 mM glucose, pH 7.4, supplemented with 10 mM EDTA and 10 mM EGTA for 3.5 min at 4 °C. Alternatively, cells were treated with 5 mg/ml Pronase (Sigma) for 15 min at 4 °C.

**Production of Syndecan-4 Antibody**—The fusion protein glutathione S-transferase-mS4ED (GST-mS4ED) bound to glutathione-Sepharose beads was used as an antigen for injection into New Zealand White rabbits (University of Wisconsin Animal Resources). The crude serum recognizes both mouse and rat syndecan-4 via immunoblotting (data not shown). Affinity-purified antibodies were generated by applying serum sequentially to GST and GST-mS4ED columns. Bound antibodies were affinity-purified against the COOH terminus of mS4ED (12).

**Production of Shed Syndecan-4—Conditioned medium was collected from ARH-77 cells stably expressing rat syndecan-4 (14). Urea was added to 6 M, sodium acetate to 50 mM, and EDTA to 5 mM prior to adjusting the pH to 4.5. After application to a DEAEP Sephacel column, bound syndecan-4 was eluted with a 100-ml NaCl gradient ranging from 0 to 1000 mM NaCl. Fraction containing syndecan-4 were detected against HEPES/DMEM and concentrated with a Centricon 30 filter (Amicon). The resulting preparations were left untreated or digested with 1.2 milliunits/ml heparinase I and III and 50 milliunits/ml chondroitin ABC lyase (ICN). For immunoprecipitations, affinity-purified anti-syndecan-4 antibody and control rabbit immunoglobulin were covalently coupled to cyanogen bromide-activated Sepharose 4B. Precipitated material was released with 100 mM glycine, pH 2.5, and immediately neutralized with 1 mM HEPES, pH 8.0. A fraction of each of the released glycosaminoglycan-denuded solutions was biotinylated by incubation on ice for 2 h in the presence of 0.5 mg/ml NHS-LC-biotin (Pierce). Eluants were dialyzed against HEPES/DMEM prior to use.

**SDS-PAGE and Immunoblotting**—Samples were resolved by Laemml SDS (15% total acrylamide) or Laemml gradient SDS (4–15% total acrylamide step-PAGE). Gels were transferred to Immobilon-N (Millipore) and probed with anti-syndecan-4 serum or streptavidin-conjugated alkaline phosphatase. Signals generated with chemiluminescence were detected with a STORM 860 and quantified with ImageQuanNT™ (Molecular Dynamics).
suggests that syndecan-1, like syndecan-4, interacts with molecules at the cell surface. However, the interactions mediated by syndecan-1 and -4 appear to be distinct since (i) the extracellular protein domains of the two syndecans do not mediate binding of the same cells and (ii) each domain fails to compete for adhesion to the other (Fig. 1B).

Binding of Iodinated mS4ED to Cells Indicates a Single Class of mS4ED-Binding Sites—To further characterize the interaction between syndecan-4 and the cell surface, a radiolabeled mS4ED binding assay was developed. To test whether there exists a finite population of cell surface-binding sites for mS4ED, saturation binding experiments were performed with cells capable of adhering to an mS4ED substratum. Two cell types were chosen for the analyses to assess whether different cells adhere to mS4ED via a single class of binding sites or multiple classes of binding sites.

Human dermal fibroblasts and mouse aortic endothelial cells, both of which adhere to an mS4ED substratum (Fig. 1B), were radiolabeled with iodine-125 for binding assays. Human fibroblasts were also tested and near-maximal binding was observed with 1 μM added iodinated mS4ED (Fig. 2A). Specific binding, calculated as described under "Experimental Procedures," reached a plateau at approximately 25 ng added mS4ED, indicating a finite number of mS4ED cell surface-binding sites present on cells which adhere to an mS4ED substratum (Figs. 2 and 3).

The saturation binding data obtained with these two cell types have been analyzed by both nonlinear and linear regression. Both nonlinear curve-fitting (GraphPad Prism® software) to a single site occupancy model and Scatchard analysis indicate a single high affinity interaction between mS4ED and the cell surface (Fig. 3) (18). The Kd of the interaction is approximately 2 × 10⁻⁹ M and is the same for both cell types, suggesting that different cell types express the same class of mS4ED-binding sites. Both cell types also have comparable values for Bmax, with the estimated number of binding sites at approximately 10,000 per cell. These sites were demonstrated to be at the cell surface by releasing 90% of the cell-associated mS4ED with a low pH wash or by treatment with Pronase™ (Fig. 4).

Divalent Cations Are Required for the mS4ED-Cell Surface Interaction—To test whether the interaction between mS4ED and the cell surface requires divalent cations, cells with bound iodinated mS4ED were briefly incubated with EDTA. EDTA, which chelates divalent cations, removed 68% of the cell-associated mS4ED (Fig. 4). To determine if specific divalent cations are capable of facilitating the interaction between mS4ED and the cell surface, calcium, magnesium, or manganese were added during iodinated mS4ED binding assays. Calcium and manganese supported near-maximal binding of iodinated mS4ED by 330 μM added cation, whereas magnesium failed to support maximal binding even at the highest concentration tested, namely 9 mM (Fig. 5A). Since calcium, magnesium, and manganese are present in the blood at approximately 1.3 mM, 1.5 mM, and 0.5 μM, respectively, calcium is the only divalent cation of the three likely to facilitate the syndecan-4 extracellular domain interaction in vivo (19, 20, 21). The ability of calcium to support cell adhesion to an mS4ED substratum was also tested and near-maximal adhesion was observed with 1 mM added cation. This confirms that adhesion, like binding of soluble ligand, occurs with physiological concentrations of calcium (Fig. 5B).

Binding of Iodinated mS4ED to Cells Is Specific for Syndecan-4 Core Protein.
can-4 and Is Mediated by the Cell-binding Domain of mS4ED—
The specificity of the interaction between iodinated mS4ED and the cell surface was tested by assessing the ability of the different syndecan extracellular protein domains to compete for binding. Previous data has demonstrated that mS1ED failed to compete for adhesion of human dermal fibroblasts to an mS4ED substratum (10). The extracellular domains of syndecan-2 and -3 also fail to effectively compete for adhesion to an mS4ED substratum at a concentration which exceeds the IC50 of mS4ED by 100-fold (Fig. 1A). All three syndecans likewise fail to compete for binding of iodinated mS4ED to cells at concentrations which exceed the Kd by 100-fold (Fig. 6A). As previously observed in the cell adhesion assay, both mS4ED and cS4ED compete equally well for binding of iodinated mS4ED to the cell surface, with IC50 values of 7.8 and 8.1 × 10^-9 M, respectively (Fig. 6B). These data confirm that a minimal cell-binding domain has been identified and that deletion of this domain from intact mS4ED renders it nonfunctional. The cell-binding domain peptide, mS4ED56–109, also competes for binding of iodinated mS4ED to cells, with an IC50 of 3.9 × 10^-9 M (Fig. 6C). As anticipated, the cell-binding domain deletion mutant, mS4EDΔ56–108, fails to compete for binding of iodinated mS4ED at concentrations which exceed the Kd by 100-fold (Fig. 6D). Thus, both the adhesion and radiolabeled ligand binding assays measure interactions mediated by the cell-binding domain of syndecan-4.

FIG. 3. Iodinated mS4ED interacts with a single class of binding sites. Specific binding of iodinated mS4ED to cells was quantified as a function of ligand concentration. A, data collected using dermal fibroblasts were curve-fit to a single site binding model with the computer program GraphPad Prism® to determine the affinity (Kd) and number of binding sites (Bmax). These same data were subjected to Scatchard analysis. C and D, data using aortic endothelial cells were analyzed as in A and B, respectively. Data from duplicate samples are shown (mean ± S.E.).

FIG. 4. Iodinated mS4ED binds cell surface sites. Aortic endothelial cells, with bound iodinated mS4ED, were subjected to a low pH wash, an EDTA wash, and Pronase™ digestion as described under “Experimental Procedures.” Cell-associated mS4ED is expressed relative to the untreated control, which is arbitrarily set to 100%. Data from duplicate samples are shown (mean ± S.E.).

FIG. 5. Binding of iodinated mS4ED to cells requires divalent cations. A, aortic endothelial cells were incubated with 3 × 10^-9 M iodinated mS4ED and increasing amounts of divalent cations. Specific binding is expressed as a percentage of the binding obtained in HEPES/DMEM. Data from duplicate samples are shown (mean ± S.E.). Calcium (•), manganese (○), and magnesium (■). B, dermal fibroblasts were incubated with GST-mS4ED, GST, and dialyzed heat-denatured BSA substrata in the presence of increasing amounts of calcium. Cell adhesion was quantified after 2 h as described under “Experimental Procedures.” Data from triplicate samples are shown (mean ± S.E.). GST-mS4ED (•), GST (○), and dialyzed heat-denatured BSA (■).
Aortic endothelial cells were incubated by the cell-binding domain. Specific binding is expressed as a percentage of the binding obtained in the absence of competitor. Data from duplicate samples are shown (mean ± S.E.). A, competing mS1ED (●), mS2ED (○), and mS3ED (▲). B, competing mS4ED (●) and cS4ED (▲). C, competing mS4ED56–109 (●). D, competing mS4EDΔ56–108 (▲).

**FIG. 6.** Binding of iodinated mS4ED is specific and mediated by the cell-binding domain. Aortic endothelial cells were incubated with increasing amounts of unlabeled competitor proteins in the presence of $3 \times 10^{-9}$ M iodinated mS4ED. Specific binding is expressed as a relative percentage, with 100% arbitrarily set at the amount of binding observed with aortic endothelial cells. Data from duplicate samples are shown (mean ± S.E.). E, aortic endothelial cells; F, dermal fibroblasts; S, the Swiss 3T3 fibroblast cell line; N, NMuMG epithelial cell line; A, the ARH-77 lymphocyte cell line; AS4, the ARH-77 lymphocyte cell line stably expressing syndecan-4; and O, the 143 osteosarcoma cell line.

**FIG. 7.** Cell adhesion to an mS4ED substratum and binding of iodinated mS4ED are correlated. A, cell adhesion to a GST-mS4ED substratum was quantified after 1 h as described under “Experimental Procedures.” Cells were not treated with cycloheximide. B, specific binding of $3 \times 10^{-9}$ M iodinated mS4ED was determined after incubation at 16°C for 90 min. Increased cell-associated mS4ED is seen at 16°C compared with 4°C. This temperature is used here to demonstrate the low but measurable specific binding to NMuMG cells. Results have been normalized to the number of adherent cells and expressed as a relative percentage, with 100% arbitrarily set at the amount of binding observed with aortic endothelial cells. Data from duplicate samples are shown (mean ± S.E.). E, aortic endothelial cells; F, dermal fibroblasts; S, the Swiss 3T3 fibroblast cell line; N, NMuMG epithelial cell line; A, the ARH-77 lymphocyte cell line; AS4, the ARH-77 lymphocyte cell line stably expressing syndecan-4; and O, the 143 osteosarcoma cell line.

**Cells which Fail to Adhere to an mS4ED Substratum Fail to Express Cell Surface-binding Sites for mS4ED**—Not all cells adhere to a mS4ED substratum (Fig. 7A). While all adhesion competent cells might be expected to bind iodinated mS4ED, an inability to adhere to an mS4ED substratum could result from a variety of possibilities. Adhesion deficient cells may express less binding sites than adhesion competent cells or alternatively, express abundant sites but be unable to transmit occupancy of these sites into a productive adhesion event. Cells positive for mS4ED adhesion, for example, dermal fibroblasts, aortic endothelial cells, and the Swiss 3T3 cell line, were also found to be positive for specific binding of iodinated mS4ED (Fig. 7). Cells negative for adhesion, for example, the ARH-77 lymphocyte cell line, the ARH-77 cell line stably expressing syndecan-4, and the 143 osteosarcoma cell line, did not detectably bind iodinated mS4ED. This observation is especially noteworthy in regards to the ARH-77 cell line expressing syndecan-4. Since most cultured cells express syndecan-4 and all syndecans homodimerize, it is formally possible that syndecan-4 mediates binding of mS4ED (1, 4, 7, 8). These data, however, suggest that other cell surface molecules are responsible for mediating binding. The correlation between binding of iodinated mS4ED and adhesion to mS4ED also extends to cells which adhere weakly, for example, NMuMG epithelial cells. These cells bind low levels of iodinated mS4ED (Fig. 7).

**Shed Syndecan-4 Contains a Fully Active Cell-binding Domain**—To test whether native syndecan-4 shed from the cell surface contains an active cell-binding domain, glycosaminoglycan-denuded shed syndecan-4 was prepared as described under “Experimental Procedures” and subjected to immunoprecipitation with either control rabbit or affinity-purified anti-mS4ED antibodies. Immunoblotting and biotinylation were used to confirm that only one protein, namely syndecan-4, was specifically precipitated (Fig. 8, A and B). To quantify the amount of syndecan-4 present in the specific eluant, immunoblotting was performed with recombinant mS4ED protein as a standard (Fig. 8B).

Eluants were tested for their ability to compete for binding of $1 \times 10^{-9}$ M iodinated mS4ED to cells. The syndecan-4 eluant competes for binding of iodinated mS4ED with an IC$_{50}$ of $1 \times 10^{-7}$ M shed syndecan-4 (Fig. 8C). Although the IC$_{50}$ value of native syndecan-4 appears to be lower than that of recombinant mS4ED, which is $8 \times 10^{-9}$ M, this apparent difference in activity can be attributed to the use of less iodinated mS4ED in the binding assay. An equal volume of control eluant was tested for each volume of syndecan-4 eluant and failed to affect the binding of iodinated mS4ED to cells (data not shown). The syndecan-4 eluant was further subjected to protein A precipitation to confirm that the activity of the eluant was not attributable to contaminating antibody. This treatment had no effect on the activity of the syndecan-4 eluant, further confirming that shed syndecan-4 protein is the active component of the eluant (data not shown).
action between the cell-binding domain and the cell surface (10). To confirm this finding, glycosaminoglycan-bearing syndecan-4 was also immunopurified and tested in the iodinated mS4ED binding assay. The resulting eluant competed for binding of iodinated mS4ED to cells with an IC50 of 6 × 10^{-10} M shed glycosaminoglycan-bearing syndecan-4. This value is comparable to the IC50 obtained with glycosaminoglycan-denuded syndecan-4, strongly suggesting that shed syndecan-4 contains a fully active cell-binding domain. Furthermore, the activity of the cell-binding domain does not appear to be directly regulated by the glycosaminoglycan content of syndecan-4.

**DISCUSSION**

In this study, the extracellular protein domain of syndecan-4 has been shown to interact with a single class of binding sites present at the cell surface. This interaction is of high affinity and functionally conserved between species. The association is also specific for syndecan-4; none of the other vertebrate syndecan family members contain this domain. Furthermore, native syndecan-4 shed from the cell surface contains a fully active cell-binding domain, suggesting an unanticipated role for shed syndecan-4 in vivo.

The **Cell-binding Domain of Syndecan-4 Interacts with a Single Class of Binding Sites**—Analyses of saturation binding data collected with two cell types, namely fibroblast and endothelial cells, indicate that the mS4ED ligand interacts with a single class of high affinity cell surface-binding sites. This association is identical by several criteria to the interaction which mediates adhesion to an artificial substratum of immobilized mS4ED. First, the ability to bind iodinated ligand and adhere to an mS4ED substratum is strictly correlated for all cells tested. Cells which fail to adhere to an mS4ED substratum also fail to express detectable binding sites for iodinated mS4ED. Apparently, the molecules which bind mS4ED are expressed or regulated in a cell-specific manner. Second, the interaction in both assays depends on the presence of divalent cations and can be facilitated by physiological concentrations of calcium. Several cell surface molecules depend on the presence of divalent cations to adopt an appropriate three-dimensional structure, including the well characterized integrin and cadherin families of adhesion receptors (19, 22, 23). Potentially either the cell surface molecules which interact with mS4ED, or the mS4ED ligand itself, may be conformationally regulated by the divalent cation calcium. Although calcium binding sequences are not well conserved and hence not easy to predict, the cell-binding domain of syndecan-4 contains both aspartic and glutamic acid, two amino acids commonly involved in calcium coordination (10, 19). Third, all specificity tests yielded qualitatively identical results. None of the other vertebrate syndecan family members, namely syndecan-1, -2, and -3, can compete for the interaction between mS4ED and the cell surface. Thus, the association between syndecan-4 and its cell surface-binding sites is specific to this family member and reflects a syndecan-4 specific function. Furthermore, the cell-binding domain of mS4ED competes as effectively as intact mS4ED. This domain is contained within amino acids 56–109 of mS4ED. Removing 13 amino acids from the C terminus or 20 amino acids from the N terminus abrogates the ability of this domain to function. A deletion mutant, lacking amino acids 56–108 of mS4ED, fails to compete for binding of mS4ED to cells in both assays, further confirming that the cell-binding domain is discrete and represented once in the extracellular protein domain of syndecan-4.

The **Cell-binding Domain of Recombinant mS4ED Is Present and Active in Native Syndecan-4 Shed from the Cell Surface**—The identification and characterization of the cell-binding domain of syndecan-4 has relied on the use of syndecan-4 derivatives expressed by bacteria. Bacterially expressed syndecan-4 is free of glycosaminoglycan modifications, ensuring that any interactions observed are mediated solely by syndecan-4 protein sequences. It is important, however, to confirm that the binding sites present on the cell surface interact with syndecan-4 produced and post-translationally modified by mammalian cells. The glycosaminoglycan content of syndecan-4, for example, could regulate the cell-binding domain interaction, potentially inhibiting it.

Mammalian cells express syndecan-4 as a transmembrane molecule and as a shed product, which is cleaved at the cell surface by an undefined proteolytic activity (4, 17, 24). While full-length syndecan-4 contains the cell-binding domain sequence, it was unknown whether it is present in shed syndecan-4. Data from this report demonstrate that syndecan-4 shed from the cell surface contains a fully active cell-binding domain. Mutagenesis of recombinant mS4ED indicates that one
or more amino acids C-terminal to residue 96 are critical for the cell-binding domain to function, suggesting that proteolytic cleavage of syndecan-4 occurs within the 26 amino acids that immediately precede the transmembrane domain. Furthermore, shed syndecan-4 competes equally well for binding of mS4ED to cells with or without its glycosaminoglycan chains. Thus, the glycosaminoglycan content of syndecan-4 does not appear to directly regulate the activity of the cell-binding domain. These data indicate that shed syndecan-4 may represent a protein ligand for cells in vivo.

The Extracellular Protein Domain of Syndecan-1 May Also Engage Specific Cell Surface Molecules—The extracellular protein domains of the individual syndecans are logical sites for mediating syndecan-specific interactions because they are divergent, both in size and sequence. However, for a given family member, there is little conservation between species (1). For example, a comparison of the cell-binding domain of mouse to the same region within chicken syndecan-4 indicates that the two regions are only 34% identical (10). Therefore, it is of particular significance that on a functional basis, the interaction between syndecan-4 and the cell surface is highly conserved. Syndecan-4 from mouse and chicken compete equally well for the interaction between mouse syndecan-4 and the cell surface of mouse aortic endothelial cells. Furthermore, both mouse and human cells interact with the cell-binding domain of mouse syndecan-4 with the same affinity.

This high degree of functional conservation within the syndecan-4 protein suggests that the extracellular protein domains of the other syndecan family members may also harbor binding activities despite their apparent divergence. Preliminary evidence indicates that syndecan-1 engages cell surface molecules present on at least one cell type via its extracellular protein domain. This interaction also has the potential to be syndecan-specific since mS4ED fails to compete for cell binding to mS1ED.

Potential Functions for the Cell-Binding Domain of Syndecan-4 in Vivo—Both the shed and membrane-anchored forms of syndecan-4 are candidates for mediating cell-binding domain interactions in vivo. By extrapolation from the cell adhesion assay, shed syndecan-4 may function as a matrix-embedded adhesive ligand for cells, possibly anchored to extracellular matrix molecules via its heparan sulfate chains. Alternatively, shed syndecan-4 may interact with cells as a soluble ligand. As a recently identified component of wound fluid, shed syndecan-4 has the potential to regulate the activity of heparan-binding molecules, such as growth factors, involved in the wounding response (25). The cell-binding domain could potentially serve to facilitate or down-regulate the activity of such ligands by either bringing them in close proximity of their cell surface receptors or accelerating their endocytosis and subsequent degradation. Equally plausible, the cell-binding domain may also regulate the assembly of cell surface complexes containing membrane-anchored syndecan-4.

REFERENCES
1. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L., and Lose, E. J. (1992) Annu. Rev. Cell Biol. 8, 365-393
2. Carey, D. J. (1997) Biochem. J. 327, 1-16
3. Couchman, J. R., and Woods, A. (1996) J. Cell. Biochem. 61, 578-584
4. Kim, C. W., Goldberger, O. A., Gallo, R. L., and Bernfield, M. (1994) Mol. Biol. Cell 5, 797-805
5. Grootejan, J. J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Durr, J., and David, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13683-13688
6. Saras, J., and Heldin, C. H. (1996) Trends Biochem. Sci. 21, 455-458
7. Asundi, V. K., and Carey, D. J. (1995) J. Biol. Chem. 270, 26404-26410
8. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 11805-11811
9. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 8133-8136
10. McFall, A. J., and Rapraeger, A. C. (1997) J. Biol. Chem. 272, 12901-12904
11. David, G., Bai, X. M., Van der Schueren, B., Marynen, P., Cassiman, J. J., and Van den Berge, H. (1995) Development 119, 841-854
12. Carey, D. J., Conner, K., Asundi, V. K., O’Mahony, D. J., Stahl, R. C., Showalter, I. J., Cizmeci-Smith, G., Hartman, J., and Rothblum, L. I. (1997) J. Biol. Chem. 272, 2873-2879
13. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Stanley, M. J., Liebersbach, B. F., Liu, W., Anhalt, D. J., and Sanderson, R. D. (1995) J. Biol. Chem. 270, 5077-5083
15. Laemmli, U. K. (1970) Nature 227, 680-685
16. Saunders, S., Jalkanen, M., O’Farrell, S., and Bernfield, M. (1989) J. Cell Biol. 108, 1547-1556
17. Kojima, T., Shworak, N. W., and Rosenberg, R. D. (1992) J. Biol. Chem. 267, 4870-4877
18. Limbird, L. E. (1986) Cell Surface Receptors: A Short Course on Theory and Methods, Martinus Nijhoff Publishing, Boston
19. Maurer, P., Hohenester, E., and Engel, J. (1996) Curr. Opin. Cell Biol. 8, 609-617
20. Darnell, J., Lodish, H., and Baltimore, D. (1990) in Molecular Cell Biology, 2nd ed (Darnell, J., Lodish, H., and Baltimore, D., eds) Scientific American Books, Inc., New York
21. Saito, K. (1994) in Dynamics of Trace Elements in Human Body and Diseases (Saito, K., ed) Vol. 31, Hokkaido University School of Medicine, Sapporo, Japan
22. Amagai, M. (1995) J. Invest. Dermatol. 104, 146-152
23. Chereh, D. A. (1993) in Advances in Molecular and Cell Biology, Vol. 6, pp. 225-252, JAI Press, Inc., Greenwich, CT
24. David, G., Van der Schueren, B., Marynen, P., Cassiman, J. J., and Van den Berghe, H. (1992) J. Cell Biol. 118, 961-969
25. Subramaniam, S. V., Fitzgerald, M. L., and Bernfield, M. (1997) J. Biol. Chem. 272, 14713-14720