Syndecans are cell surface proteoglycans involved in cell adhesion and motility. Syndecan-4 is an important component of focal adhesions and is involved in cytoskeletal reorganization. Previous work has shown that the syndecan-4 ectodomain can support cell attachment. Here, three vertebrate syndecan-4 ectodomains were compared, including that of the zebrafish, and we have demonstrated that the cell binding activity of the syndecan-4 ectodomain is conserved. Cell adhesion to the syndecan-4 ectodomain appears to be a characteristic of mesenchymal cells. Comparison of syndecan-4 ectodomain sequences led to the identification of three conserved regions of sequence, of which the NXIP motif is important for cell binding activity. We have shown that cell adhesion to the syndecan-4 ectodomain involves β1 integrins in several cell types.

Syndecans are type 1 transmembrane heparan sulfate proteoglycans involved in cell adhesion, spreading, and cell migration (for reviews, see Refs. 1–4). They consist of a short, highly conserved cytoplasmic domain, a single transmembrane domain, and a longer ectodomain that bears heparan sulfate side chains. Syndecan-4 is a focal adhesion component and is involved in focal adhesion formation in conjunction with the α5β1 integrin. Fibroblasts form focal adhesions in response to fibronectin fragments containing the integrin binding (RGD-containing) and heparin binding domains. Integrin engagement is not sufficient to stimulate this process, and the heparan sulfate binding occurs through syndecan-4 (5–7). The cytoplasmic domain of syndecan-4 can interact with a range of proteins. As with all syndecans, the C2 region of the cytoplasmic domain of syndecan-4 contains a PDZ binding site, and PDZ proteins, such as GIPC (GAIP-interacting protein-C terminus) and syndecan-4 contains a PDZ binding site, and PDZ proteins, such as GIPC (GAIP-interacting protein-C terminus) and syndecan-1-mediated spreading and binding was unaffected by this mutation (22). Recently, recombinant syndecan-2 ectodomain has been shown to promote capillary tube formation in microvascular endothelial cells when incorporated into matrigel (23).

Here we have shown that the cell adhesion activity of the syndecan-4 ectodomain is conserved across vertebrate evolution and that adhesion activity is restricted to mesenchymal and leukocytic cells. Epithelial cell lines appear not to interact with this protein. We have also identified a conserved NXIP motif within the syndecan-4 ectodomain that is important for cell adhesion. Finally, we have also shown evidence that the cellular response to the syndecan-4 ectodomain involves β1 integrins.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat embryo fibroblasts (REFs)2 and syndecan-4 null fibroblasts (24) were routinely grown and main-

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2 The abbreviations used are: REF, rat embryo fibroblast; FCS, fetal calf serum; RACE, rapid amplification of cDNA ends; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate.
tained in α minimal essential medium (Cambrex) in the presence of 5% fetal calf serum (FCS). Swiss 3T3, Madin-Darby canine kidney cells, T47D, Ovcar3, mouse skin endothelial cells, MCF7, and COS7 cells were grown in Dulbecco’s modified Eagle’s medium (Cambrex) supplemented with 10% FCS. HEK293 and Jurkat cells were cultured in Dulbecco’s modified Eagle’s/F-12 (Invitrogen) media with 10% FCS, whereas K562 were grown in RPMI 1640 medium (Invitrogen) with 5% FCS.

Cloning of Zebrafish Syndecan-4—Plasmids and primers are listed in supplemental Table S1. The expressed sequence tag clone IMAGp998K241082Q3 corresponding to the zebrafish homologue of syndecan-4 was obtained from Deutsches Resourcenzentrum für Genomschung GmbH (Berlin, Germany) and sequenced by conventional methods. The 5′-end of zebrafish syndecan-4 cDNA was obtained using 5′-RACE (Invitrogen). Briefly, zebrafish syndecan-4 cDNA was prepared by reverse transcription using primer ZebSDC4for and ZebSDC4rev, and this was used as a template in sequential PCR reactions, using primer Zeb4gsp1 in the primary PCR and Zeb4gsp2 in the secondary PCR. The blunt-ended PCR products were phosphorylated using T4 polynucleotide kinase (Invitrogen) and ligated into the EcoRV site of pBluescript II KS(+). The resultant PCR product was digested with BamHI and PstI and ligated into the corresponding sites of pBluescript II KS(+) to construct the pBSZebSDC4pr plasmid.

Glutathione S-Transferase (GST) Ectodomain Fusion Proteins; Construct Generation, Protein Expression, and Purification—Plasmids containing the full-length syndecan-4 cDNAs from human, mouse, and zebrafish were used as templates for PCR. The human, mouse, and zebrafish ectodomain sequences were amplified using the primer pairs S4petA and S4petB, MouEDfor and MouEDrev, and ZebEDfor and ZebEDrev, respectively. PCR products were kinned and digested with BamHI prior to ligation into the PshAI and BamHI sites of the bacterial expression vector pET41(a+) (Novagen). Human syndecan-4 ectodomain mutants were made using a PCR-based approach. Primer pairs SNKVSfor and SNKVSrev, NEVfor and NEVrev, or NHIPfor and NHIPrev were used to amplify linear pGSTHS4ED. The primer positions were designed such that the PCR product from each respective primer pair would lack the coding sequence for the amino acid sequences

\[\text{Glu}^{24} \text{– Gly}^{75}\]

of the syndecan-4 ectodomain were made as follows. Human syndecan-4 sequence coding from Pro\(^{76}\)–Ser\(^{152}\) was PCR-amplified using primers CBDa and CBDb and cloned into the PshAI and BamHI sites of pET41(a+) to make the plasmid pGSTHS4EDcbd. Using the same PCR mutagenesis protocol as above, a stop codon was introduced into the human syndecan-4 ectodomain coding sequence after Gly\(^{75}\). pGSTHS4ED was used as the template in conjunction with primers S4ectfrfor and S4ectfrrev, and the resultant PCR product was digested and ligated as above to produce pGSTHS4EDfr.

Plasmids were transformed into the Escherichia coli BL21 strain (Promega), and the bacteria were grown to an \(A_{600}\) of 0.6 at 37 °C prior to the addition of 0.25 ml/liter 1 mM isopropyl-\(\beta\)-\(D\)-galactopyranoside (Calbiochem) followed by a further 3-h incubation. Bacteria were resuspended in phosphate-buffered saline (PBS), lysed in a sonicator, and GST syndecan-4 ectodomain fusion proteins were purified on columns of glutathione–Sepharose 4B (GE Healthcare) as described in the manufacturer’s protocol.

Cell Adhesion Assay—Adhesion assays were performed using 24-well plates. The wells were coated either with 0.25 ml of the various syndecan-4 ectodomain fusion proteins at concentrations up to 10 \(\mu\)g/ml or with 5% bovine serum albumin (BSA) overnight at 4 °C, and human plasma fibronectin (10 \(\mu\)g/ml) was purified according the method described in Ref. 25. The wells were washed once with PBS and blocked using 1% w/v BSA for 1 h at 37 °C followed by two washes with PBS. Cells were detached using trypsin/EDTA and suspended in serum-containing medium. The cells were sedimented and resuspended in serum-free medium to a concentration of 2.5 \(\times\) 10\(^5\) cells/ml, and 0.2 ml of cell suspension/well was used in the assay. The non-adherent cell lines K562 and Jurkat were incubated for 15 min in serum-free medium containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA) prior to seeding on substrates. The blocking antibodies Alib2, Ha2/5 (Chemicon), and LM609 (Chemicon) were used at a concentration of 10 \(\mu\)g/ml; REF, Swiss 3T3, COS7, and Jurkat cells (PMA-treated as above) were incubated at 4 °C for 30 min in serum-free medium in the presence or absence of antibody and then seeded on substrate at 37 °C. Unless stated otherwise, the cells were allowed to adhere for 1 h at 37 °C, after which the medium was removed and replaced with serum-free medium containing 15 \(\mu\)M calcium (Invitrogen) and incubated for a further 20 min. The wells were washed twice with PBS, and cell-bound fluorescence was measured using a Fluostar Galaxy Plate reader (BMG Lab Technologies) at 485 nm excitation and 520 nm emission.

Immunofluorescence Microscopy—Coverslips were coated overnight with the appropriate substrate, and cells were seeded in serum-free conditions and allowed to spread for 1 h. The cells were then fixed for 20 min in 3% paraformaldehyde in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min. Thereafter, the cells were stained using conventional procedures with AlexaFluor 568-conjugated phalloidin (Molecular Probes) for F-actin and to detect focal adhesions, anti-vinculin (Sigma) followed by AlexaFluor 488-conjugated goat anti-mouse IgG (Molecular Probes) was used. The samples were analyzed on a Provis AX module fluorescence microscope (Olympus; objectives were UPlanApo 40 \(\times\) 1.0 oil iris and UPlanApo

**Syndecan-4 Ectodomain and Cell Adhesion**

**Immunofluorescence Microscopy**—Coverslips were coated overnight with the appropriate substrate, and cells were seeded in serum-free conditions and allowed to spread for 1 h. The cells were then fixed for 20 min in 4% paraformaldehyde in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 10 min. Thereafter, the cells were stained using conventional procedures with AlexaFluor 568-conjugated phalloidin (Molecular Probes) for F-actin and to detect focal adhesions, anti-vinculin (Sigma) followed by AlexaFluor 488-conjugated goat anti-mouse IgG (Molecular Probes) was used. The samples were analyzed on a Provis AX module fluorescence microscope (Olympus; objectives were UPlanApo 40 \(\times\) 1.0 oil iris and UPlanApo
FIGURE 1. A, nucleotide and putative protein sequence of the zebrafish syndecan-4 coding region. The putative signal peptide sequence was determined using the Signal P 3.0 program (29) and is shaded in dark gray. The transmembrane domain is shaded in light gray and was predicted using the DASTMfilter software (30). The probable sites of glycosaminoglycan substitutions are shown in white text on a black background. The sequence is deposited in the EMBL data base under accession number AM260521.

B, the zebrafish syndecan-4 ectodomain shares little homology with avian, mammalian, and amphibian syndecan sequences. The syndecan-4 ectodomain sequences between the GAG attachment sites and transmembrane domain from the *Xenopus laevis* (*xenopusl*), *Xenopus tropicalis* (*xenopust*), chick, pigeon, human, pig, mouse, rat, *Tetraodon nigroviridis* (*tetraodon*), and *Danio rerio* (*zeb*) were aligned using the ClustalW algorithm. The predicted conserved cell binding domain is shown in dark text on a light gray background. Areas of homology are shown in white text on a dark gray background.

C, the zebrafish syndecan-4 cytoplasmic domain shares homology with avian and mammalian syndecan sequences. The cytoplasmic domains from the syndecan-4 sequences indicated were aligned as described in B. The C1 and C2 regions are shown in white text on a dark gray background, and the V region is shown in dark text on a light gray background.
60 × 1.4 oil. Images were collected using a SPOT Insight Mono digital camera and processed in Adobe Photoshop.

RESULTS

Zebrafish Gene Cloning and Sequencing—To compare ectodomain properties across a spectrum of vertebrates, the zebrafish syndecan-4 was identified from a BLAST search of the I.M.A.G.E. Consortium (LLNL) zebrafish cDNA clone collection with the human syndecan-4 sequence. Clone IMAGp998K2410802Q3 contained a partial sequence of the zebrafish syndecan-4 homologue. The clone was truncated at the 5'-end, and 5'-RACE was used to obtain the entire zebrafish SDC4 (zSDC4) mRNA sequence (Fig. 1A), which is deposited in the EMBL database under accession number AM260521. zSDC4 has a predicted 17-amino-acid signal sequence principally comprising hydrophobic residues. The N terminus of the mature protein is well conserved with other syndecan-4 sequences, as is the frequency and distribution of the three potential GAG substitution sites. Consistent with other syndecan sequences, there is little sequence homology between the ectodomains of syndecan-4 proteins across the vertebrates (Fig. 1B); however, the transmembrane and cytoplasmic domains of zSDC4 share considerable homology with syndecan-4 sequences from other species (Fig. 1C).

Divergent Vertebrate Syndecan-4 Ectodomains Support Mesenchymal Cell Adhesion—Bacterially expressed mouse syndecan-4 ectodomain can support cell adhesion when used as a substrate for attachment assays (18, 19). GST fusion proteins containing the ectodomains of human (GSThS4ED), mouse (GSTmS4ED), and zebrafish (GSTzS4ED) syndecan-4 were prepared (Fig. 2, A and B). In each case, the ectodomain protein did not include either the signal sequence or the four amino acids immediately proximal to the transmembrane domain (Fig. 2A). Cleavage of the GST from the fusion proteins was unsuccessful, because syndecan-4 ectodomains aggregate to form insoluble complexes. The GST fusion proteins, however, remained dimeric and soluble (Fig. 2B). Cell attachment assays were performed using the three GST ectodomains, human plasma fibronectin, GST, and BSA, as substrates. REFs attached well to fibronectin and failed to adhere to either BSA or GST. As reported previously (18), the GSTmS4ED also supported cell attachment and equivalent activity was observed for the human and zebrafish ectodomains (Fig. 2C). This effect was not restricted to REFs, because Swiss 3T3 and mouse skin endothelial cells also exhibited similar levels of attachment to each syndecan-4 ectodomain. Further analysis revealed that, although a number of mesenchymal cell lines adhered to syndecan-4 ectodomain, all of the epithelial cell lines (except endothelial cells) failed to do so (Fig. 2C and Table 1). Leukocytic cell lines were variable; Jurkat cells adhered to the syndecan-4 ectodomains after PMA stimulation, whereas K562 cells were unresponsive.

Cell Spreading in Response to the Syndecan-4 Ectodomain—It was evident from the attachment assays that cells not only attached to the different syndecan-4 ectodomains but could also spread in response to these substrates. Fibroblasts, including REFs...
and Swiss 3T3 cells, all showed a similar spread morphology after 1 h of adhesion to the three forms of the syndecan-4 ectodomain (Fig. 3A). Spreading was not as extensive as that observed when REFs were seeded on fibronectin, whereas cells failed to spread on either BSA or GST (Fig. 3A). REFs did not organize actin stress fibers when spread on the ectodomain species. The actin was more cortical in organization, in contrast to fibronectin-mediated adhesion (Fig. 3B). In addition, REFs did not form focal adhesions during spreading on any form of the syndecan-4 ectodomain (Fig. 3C and data not shown). Vinculin-containing focal adhesions were observed in cells spread under equivalent conditions on fibronectin. In further experiments, it was demonstrated that allowing increased time for adhesion did not result in focal adhesion acquisition on syndecan-4 ectodomains (data not shown).

**The NXIP Motif Is Important for Syndecan-4 Ectodomain Activity**—Syndecan-4 ectodomain from mammals and fish elicited similar responses from cells when used as substrata in adhesion assays. In turn, this suggested the presence of conserved features within the syndecan-4 ectodomain sequence supporting cell adhesion. Previous work had mapped the cell binding activity of the murine S4ED to a 54-amino-acid region between the GAG attachment sites and transmembrane domain. Consistent with this, a GST fusion protein containing the equivalent sequence of human syndecan-4 supported REF cell attachment. A second fusion protein containing the remaining N-terminal portion of the ectodomain did not support cell adhesion (supplemental Fig. S2, A and B). A multiple alignment of the equivalent regions from all syndecan-4 sequences (Fig.

### Table 1

| Cell line     | Morphology | Adhesion on fibronectin | Adhesion on GSThS4ED | Adhesion on GSTmS4ED | Adhesion on GSTzS4ED |
|---------------|------------|-------------------------|----------------------|----------------------|----------------------|
| REF           | Fibroblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| COS7          | Fibroblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| MEF           | Fibroblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| 54KOMef (−/−) | Fibroblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| Swiss3T3      | Fibroblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| Send          | Endothelial | ✓                       | ✓                    | ✓                    | ✓                    |
| K562          | Lymphoblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| Jurkat        | Lymphoblastic | ✓                       | ✓                    | ✓                    | ✓                    |
| CHOKI         | Epithelial  | ✓                       | ✓                    | ✓                    | ✓                    |
| MDCK          | Epithelial  | ✓                       | ✓                    | ✓                    | ✓                    |
| T47D          | Epithelial  | ✓                       | ✓                    | ✓                    | ✓                    |
| Ovar          | Epithelial  | ✓                       | ✓                    | ✓                    | ✓                    |
| HEK293        | Epithelial  | ✓                       | ✓                    | ✓                    | ✓                    |
| MCF7          | Epithelial  | ✓                       | ✓                    | ✓                    | ✓                    |

*After PMA stimulation.*
revealed little sequence homology between species. Three small regions were identifiable as conserved, NXIP, NEV, and SNKVSM. Using the human syndecan-4 ectodomain (GSThS4ED) as the template, mutants with in-frame deletions of these three putative motifs were prepared (Fig. 4A). REF cell attachment at substrate-coating concentrations were essentially identical (Fig. 4B). However, at lower concentrations, cell attachment and spreading were much reduced in response to the mutant protein in which the NXIP motif had been deleted (Fig. 4,B and C). GSThS4ED lacking either the NEV or SNKVSM motif supported normal levels of REF cell attachment. To ensure that the wild-type and deletion mutant fusion proteins coated the plates with equal efficiency, enzyme-linked immunosorbent assay (using an antibody to GST) showed that all of the proteins coated the tissue culture plastic.

FIGURE 4. The NXIP motif is an important site for cell adhesion of syndecan-4 ectodomain. A, SDS-PAGE of affinity-purified and mutant forms of GSThS4ED in which the three conserved motifs had been separately deleted. B, attachment assays performed on wild-type GSThS4ED and GSThS4ED deletion mutants. GSThS4ED (○), GSThS4EDΔNEV (□), GSThS4EDΔNXIP (■), GSThS4EDΔSNKVSM (●), GSThS4EDΔNXIP (▲) were coated on wells at various concentrations. REFS were plated in serum-free conditions and allowed to attach for 1 h. Maximal attachment to GSThS4ED at 10 μg/ml was set at 100%. Error bars represent S.D. of triplicate wells from which six absorbance measurements were determined. C, REF exhibited reduced spreading on GSThS4EDΔNXIP. Shown are phase contrast micrographs of REFS seeded on coverslips coated with 4 μg/ml of either GSThS4ED or GSThS4EDΔNXIP in serum-free conditions. Cells were allowed to spread for 1 h prior to fixation. Scale bar, 50 μm.

FIGURE 5. The isoleucine in NXIP is critical for cell attachment and spreading on GSThS4ED. A, alanine scanning was performed sequentially on the NXIP motif in GSThS4ED as shown, and the mutant proteins were expressed and purified from E. coli (B). C, REF cell attachment is reduced for GSThS4EDI-A. Cell attachment assays were performed as described using GSThS4ED (○), GSThS4EDN-A (□), GSThS4EDH-A (■), GSThS4EDI-A (▲), GSThS4EDP-A (●), and GSThS4EDΔNXIP (▲) as substrata at the concentrations indicated. Attachment to (10 μg/ml) GSThS4ED was set at 100%. Error bars were calculated as described in the legend to Fig. 4, B and D. Phase contrast micrographs showing REFS seeded on 4 μg/ml of either GSThS4ED or GSThS4EDI-A for 1 h in serum-free conditions. Scale bar, 50 μm.
Syndecan-4 Ectodomain and Cell Adhesion

Figure 6. Cell attachment to GSThS4ED requires divalent cations and β1 integrins. A, REFs were seeded onto plates coated with fibronectin (FN) or GSThS4ED in the presence (white bars) or absence (gray bars) of 1 mM EDTA and allowed to adhere for 1 h. B, Jurkat adhesion to GSThS4ED required β1 integrins. Jurkat cells were seeded on either fibronectin or GSThS4ED in serum-free medium (black bars) in the presence of 10 ng/ml PMA (gray bars) or with 10 ng/ml β1 integrin-blocking antibody AIIb2 (white bars). C, fibroblast adhesion to GSThS4ED also required β1 integrins. REFs (gray bars) and Swiss 3T3 cells (white bars) were seeded in serum-free medium on either fibronectin or GSThS4ED in the presence or absence of 10 μg/ml β1 integrin-blocking antibody Hα2/5 as indicated. D, blockade of αυβ3 integrin in COS7 cells had no effect on adhesion to GSThS4ED. COS7 cells were seeded on either fetal calf serum or GSThS4ED in serum-free medium in either the absence (white bars) or presence (gray bars) of 10 μg/ml of the αυβ3 integrin-blocking antibody LM609. In A–C, attachment to fibronectin was set at 100% in the absence of EDTA or blocking antibodies. In D, attachment to FCS is set at 100%. Error bars represent the S.D. of triplicate wells from which six absorbance measurements were made. Data are representative of three experiments.

Equivalently (supplemental Fig. S3A). There was also no evidence to suggest that GSThS4EDΔNHIP was any more unstable than the wild-type fusion protein (Fig. 4A). No further compromise of cell attachment or spreading on the GSThS4EDΔNHIP was obtained from additional deletions of the NEV保守 motif or the 4-amino-acid proline-rich region immediately preceding the NHIP sequence. Neither substrate showed any reduction in cell attachment properties (supplemental Fig. S3B). Identical results to REFs in terms of adhesion were seen with Swiss 3T3 and Jurkat cells to each of these deletion mutants (data not shown).

To further characterize the NXIP motif, mutants of GSThS4ED were prepared in which each residue of the motif were substituted with an alanine residue (Fig. 5A). Of these single amino acid substitutions, the Ile—Ala mutant alone showed compromised cell attachment and spreading properties (Fig. 5, C and D). As described previously, all of the alanine scanning mutants had similar coating properties to wild-type GST syndecan-4 ectodomain fusion protein (supplemental Fig. S3C), and these mutants were as stable as the wild-type protein (Fig. 5B).

Syndecan-4 Ectodomain Is Integrin-dependent—

Cell adhesion and spreading responses to the syndecan ectodomain could have resulted from association between the exogenous recombinant syndecan-4 protein and endogenously expressed syndecan-4. However, because syndecan-4 null fibroblasts adhered to all three forms of syndecan-4 ectodomain fusion protein, this was effectively ruled out. As shown in Fig. 3C, cells seeded on the syndecan-4 ectodomain spread with actin organized in a cortical distribution. This was suggestive of integrin involvement and was further supported by the use of EDTA, where REF failed to attach to the human form of the syndecan-4 ectodomain (Fig. 6A).

Because Jurkat cell attachment to syndecan-4 ectodomains required phorbol ester pretreatment (Fig. 6B and Table 1), this also suggested a role for integrins. Integrin involvement was confirmed with the β1 integrin-blocking antibodies AIIb2, which inhibited PMA-induced adhesion of Jurkat cells to GSThS4ED (Fig. 6B) and Hα2/5 and which compromised REF and Swiss 3T3 fibroblast attachment (Fig. 6C). The Hα2/5 antibody also compromised REF attachment to the GST fusion protein containing only the cell binding domain of syndecan-4 (supplemental Fig. S2C). The αυβ3 integrin-blocking antibody LM609 had no effect on the adhesion of COS7 cells to GSThS4ED but did block adhesion to serum-coated substrate (Fig. 6D).

Discussion

Syndecan ectodomains are highly divergent in primary sequence, although there are small regions of homology among syndecan-4 sequences, including sites of glycosaminoglycan substitution. One of the most obvious roles for the syndecan ectodomain is to support and orient heparan sulfate and, where present, chondroitin sulfate chains. Syndecan-4 ectodomain is the only syndecan family member where cell binding activity has been observed when used as a substrate in adhesion assays (18, 19). We have shown here that it is a conserved feature of the syndecan-4 ectodomain being shared almost identically in terms of adhesion and spreading characteristics between zebrafish, mouse, and human versions. Although the actin cytoskeleton of fibroblasts adherent to the syndecan-4 ectodomain is well organized, this type of adhesion does not culminate in the
formation of focal adhesions. Analysis of the syndecan-4 ectodomain sequences revealed three small motifs common across the vertebrates. Of these, only one (the NXIP motif) appears to be important, because its deletion compromises both attachment and spreading of fibroblasts. However, this may be a complex situation, because deletion of the NXIP motif from the human syndecan-4 ectodomain did not ablate cell attachment but merely reduced it. Consistent with this was the finding from alanine-scanning mutations that the isoleucine, although critical, was the only residue identified by single amino acid substitutions to affect cell attachment to the syndecan-4 ectodomain. Quite likely, there are surrounding sequences that may represent a secondary site for adhesion, or there may be a more distant site not yet identified. Future structural work should provide information on the orientation of this NXIP motif and its relationships to other features of syndecan structure. Although the syndecan-1 and -2 ectodomains also carry cell adhesion sites, the NXIP motif is not common with these and, as such, may have distinct roles. In this regard, the finding that only fibroblast and some leukocytic cells are able to adhere to the syndecan-4 ectodomain is of interest. Syndecan-4, although abundant in these cell types, is not restricted to them and is also found in epithelial cells. However, the ectodomain does not promote epithelial cell adhesion, with the exception of vascular endothelial cells. In contrast, syndecan-1 ectodomain promotes epithelial cell adhesion mediated by integrins (20).

It has been shown here that cell adhesion to the syndecan-4 ectodomain is sensitive to the blockade of the β1 integrin but not αVβ3 integrin. This is consistent with experiments showing the requirement for divalent cations as well as the finding that Jurkat adhesion to the syndecan-4 ectodomain requires activation of protein kinase C through phorbol ester treatment. This is a known property of Jurkat integrins (26). Once again, however, it reveals that adhesion to syndecan-4 ectodomain through integrins may be complex, because many cell types, including epithelial cells, possess β1 integrins. Altogether this suggests that mesenchymal cells and Jurkat cells possess an intermediate that is responsible for the bridging between syndecan-4 and the β1 integrin. The identity of this intermediate remains unknown but could be predicted to be absent in epithelial cells.

In the case of syndecan-1 ectodomain, MDA-MB-231 cell adhesion is dependent on the αVβ3 integrin, whereas in B82L fibroblasts, adhesion is dependent on the αVβ5 integrin (21, 27). Because the adhesion of fibroblasts to syndecan-4 ectodomains is a well conserved function, demonstrable in lower vertebrates, this appears to be an important function of syndecan-4 worthy of further exploration. It may, in part, explain the special relationship that syndecan has with integrins in adhesion to extracellular molecules such as fibronectin. Of the four vertebrate syndecan family members, only syndecan-4 has the ability to become incorporated into focal adhesions, but it is not yet understood whether this involves the ectodomain site identified here. The present experiments report on the activity of syndecan-4 in trans, a feature that has also been suggested to occur in *Xenopus* development with respect to syndecan-2 (28). So far, syndecan-4 regulation of focal adhesion formation has been suggested to be in cis but should now be further investigated.

REFERENCES

1. Oh, E. S., and Couchman, J. R. (2004) Mol. Cells 17, 181–187
2. Couchman, J. R. (2003) Nat. Rev. Mol. Cell Biol. 4, 926–937
3. Tkachenko, E., Rhodes, J. M., and Simons, M. (2005) Circ. Res. 96, 492–500
4. Couchman, J. R., Chen, L., and Woods, A. (2001) Int. Rev. Cytol. 207, 113–150
5. Woods, A., Couchman, J. R., Johansson, S., and Höök, M. (1986) EMBO J. 5, 665–670
6. Woods, A., and Couchman, J. R. (1994) Mol. Biol. Cell 5, 183–192
7. Saoncella, S., Echtermeyer, F., Denhze, F., Nowlen, J. K., Mosher, D. F., Robinson, S. D., Hynes, R. O., and Goetinck, P. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2805–2810
8. Grootjans, J. J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Durr, I., and David, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13683–13688
9. Gao, Y. H., Li, M., Chen, W. Z., and Simons, M. (2000) J. Cell. Physiol. 184, 375–379
10. Lee, D., Oh, E. S., Woods, A., Couchman, J. R., and Lee, W. (1998) J. Biol. Chem. 273, 13022–13029
11. Oh, E. S., and Couchman, J. R. (1997) J. Biol. Chem. 272, 11805–11811
12. Couchman, J. R., Vogt, S., Lim, S. T., Lim, Y., Oh, E. S., Prestwich, G. D., Theibert, A., Lee, W., and Woods, A. (2002) J. Biol. Chem. 277, 49296–49303
13. Keum, E., Kim, Y., Kim, J., Kwon, S., Lim, Y., Han, I., and Oh, E. S. (2004) Biochem. J. 378, 1007–1014
14. Lim, S. T., Longley, R. L., Couchman, J. R., and Woods, A. (2003) J. Biol. Chem. 278, 13795–13802
15. Greene, D. K., Tumova, S., Couchman, J. R., and Woods, A. (2003) J. Biol. Chem. 278, 7617–7623
16. Baci, P. C., Saoncella, S., Lee, S. H., Denhze, F., Leuthardt, D., and Goetinck, P. F. (2000) J. Cell Sci. 113, 315–324
17. Longley, R. L., Woods, A., Fleetwood, A., Cowling, G. J., Gallagher, J. T., and Couchman, J. R. (1999) J. Cell Sci. 112, 3421–3431
18. McFall, A. J., and Rapraeger, A. C. (1997) J. Biol. Chem. 272, 12901–12904
19. McFall, A. J., and Rapraeger, A. C. (1998) J. Biol. Chem. 273, 28270–28276
20. Beauvais, D. M., and Rapraeger, A. C. (2003) Exp. Cell Res. 286, 219–232
21. Beauvais, D. M., Burbach, B. J., and Rapraeger, A. C. (2004) J. Cell Biol. 167, 171–181
22. Langford, J. K., Yang, Y., Kieber-Emmons, T., and Sanderson, R. D. (2005) J. Biol. Chem. 280, 3467–3473
23. Fears, C. Y., Gladson, C. L., and Woods, A. (2006) J. Biol. Chem. 281, 14533–14536
24. Ishiguro, K., Kadomatsu, K., Kojima, T., Muramatsu, H., Tsuzuki, S., Nakamura, E., Kusugami, K., Saito, H., and Muramatsu, T. (2000) J. Biol. Chem. 275, 5249–5252
25. Miekka, S. I., Ingham, K. C., and Menache, D. (1982) Thromb. Res. 27, 1–14
26. Faull, R. J., Kovach, N. L., Harlan, J. M., and Ginsberg, M. H. (1994) J. Exp. Med. 179, 1307–1316
27. McQuade, K. J., Beauvais, D. M., Burbach, B. J., and Rapraeger, A. C. (2006) J. Cell Sci. 15, 1453–1456
28. Kramer, K. L., and Yost, H. J. (2002) Dev. Cell 2, 115–124
29. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
30. Cserzo, M., Eisenhaber, F., Eisenhaber, B., and Simon, I. (2002) Protein Eng. 15, 745–752