

# Syndecan-1 Expressed in Schwann Cells Causes Morphological Transformation and Cytoskeletal Reorganization and Associates with Actin during Cell Spreading

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**Abstract.** To investigate the biological functions of transmembrane proteoglycans we have produced clonal cell lines of rat Schwann cells that express the hybrid proteoglycan syndecan-1. This was done by transfection of newborn rat Schwann cells with a plasmid vector bearing the rat syndecan-1 cDNA sequence under transcriptional control of the constitutively active cytomegalovirus promoter, and a neomycin resistance gene. Stably expressing cells were selected by growth in G418. Expression of syndecan-1 was verified by Northern and immunoblot analysis and immunoprecipitation of  $^{35}\text{SO}_4$ -labeled proteoglycans. The syndecan-1 expressing cells exhibited significantly enhanced spreading on several different substrata, including fibronectin and laminin, and an altered morphology. The enhanced spreading appeared to result from the

presence of syndecan-1, based on the observation that anti-syndecan-1 antibodies inhibited the enhanced substratum spreading. There was also a reorganization of cytoskeletal structures and formation of focal adhesions, visualized by anti-vinculin staining, which were absent from control Schwann cells. There was no apparent stable association of cell surface syndecan-1 with focal contact sites, as determined by dual staining with anti-syndecan-1 and anti-vinculin antibodies. Colocalization of patches of cell surface syndecan-1 with actin was observed, but only during cell spreading. These findings provide evidence for a role of transmembrane proteoglycans in cellular morphogenesis, and suggest that transient association of syndecans with microfilaments may be an important aspect of their biological function.

**W**ITHIN the last several years cDNAs coding for proteins that comprise a gene family of transmembrane proteoglycans have been cloned and sequenced. The proteoglycans of this gene family have been called syndecans, based on the name given to the first of these proteins to be sequenced (Bernfield et al., 1992). The known members of the syndecan gene family are syndecan (also called syndecan-1) (Saunders et al., 1989), fibroglycan (syndecan-2) (Marynen et al., 1989), N-syndecan (syndecan-3) (Carey et al., 1992; Gould et al., 1992), and ryuodocan (also called amphiglycan or syndecan-4) (David et al., 1992; Kojima et al., 1992). Each of these proteoglycans possesses an  $\text{NH}_2$ -terminal extracellular domain with attachment sites for 3-8 glycosaminoglycan chains, an internal hydrophobic membrane-spanning domain, and a short COOH-terminal cytoplasmic domain. The amino acid sequence homology of the extracellular domains of the syndecans is low, whereas the sequence homology of the transmembrane and cytoplasmic domains is high (>50% amino acid identity). Thus, the proteins appear to be modular in design, with structurally distinct extracellular domains but

structurally similar membrane attachment and cytoplasmic domains.

The precise functions of the syndecan family proteoglycans have not been elucidated. In general, they appear to provide cell surface binding sites for extracellular regulatory macromolecules, in particular adhesive proteins of the extracellular matrix and certain polypeptide growth factors. Syndecan-1, which has been studied in greatest detail, binds *in vitro* to the matrix proteins fibronectin (Saunders and Bernfield, 1988), collagen type I (Koda et al., 1985), thrombospondin (Sun et al., 1989) and tenascin (Salmivirta et al., 1991), and to bFGF (Elenius et al., 1992), a member of the heparin binding growth factor family.

Syndecan expression is highly regulated among tissues and as a function of development. Expression of specific syndecan types is induced at critical periods of development or tissue remodeling. Examples include syndecan-1 induction in skin during wound healing (Elenius et al., 1991) and syndecan-3 induction during limb bud morphogenesis (Gould et al., 1992). Based on these patterns of expression and their ability to bind to matrix adhesive proteins and growth factors the syndecans have been proposed to play important roles in the control of cell growth and differentiation and tissue morphogenesis.

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One difficulty in assigning specific functions to syndecans is that in most cells syndecans do not represent the sole, or even primary, cell surface binding sites for their potential ligands. For example, receptors of the integrin family are well characterized receptors for matrix proteins such as fibronectin (Ruoslahti, 1988). Both syndecans and integrins are expressed simultaneously by most cells. Another difficulty in studying syndecan function is that all of the known binding interactions use the glycosaminoglycan side chains. With the exception of syndecan-bFGF binding, these interactions are relatively weak, so that ligand specificity and functional activity under physiological conditions have been difficult to demonstrate. Moreover, most cell types synthesize more than one form of syndecan (Lories et al., 1989). The structural variability of the extracellular domains makes it unlikely that the syndecans all carry out the same function. At this point, however, functional specificity of different syndecan forms has not been demonstrated.

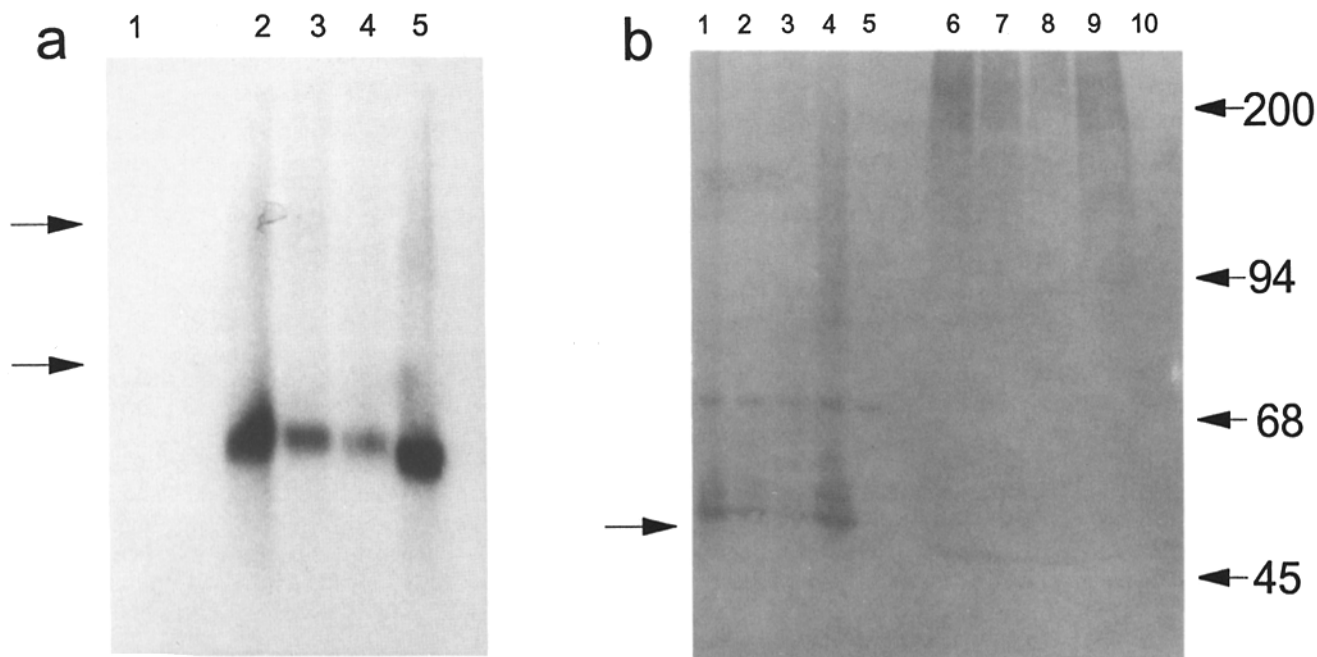
One approach to investigating the biological functions of the syndecans is to express a specific syndecan form in a cell that does not normally produce that syndecan and to identify the resulting alterations in the functional characteristics of the cell. We have begun to carry out such experiments. This paper presents our findings on the expression of syndecan-1 in neonatal rat Schwann cells.

## Materials and Methods

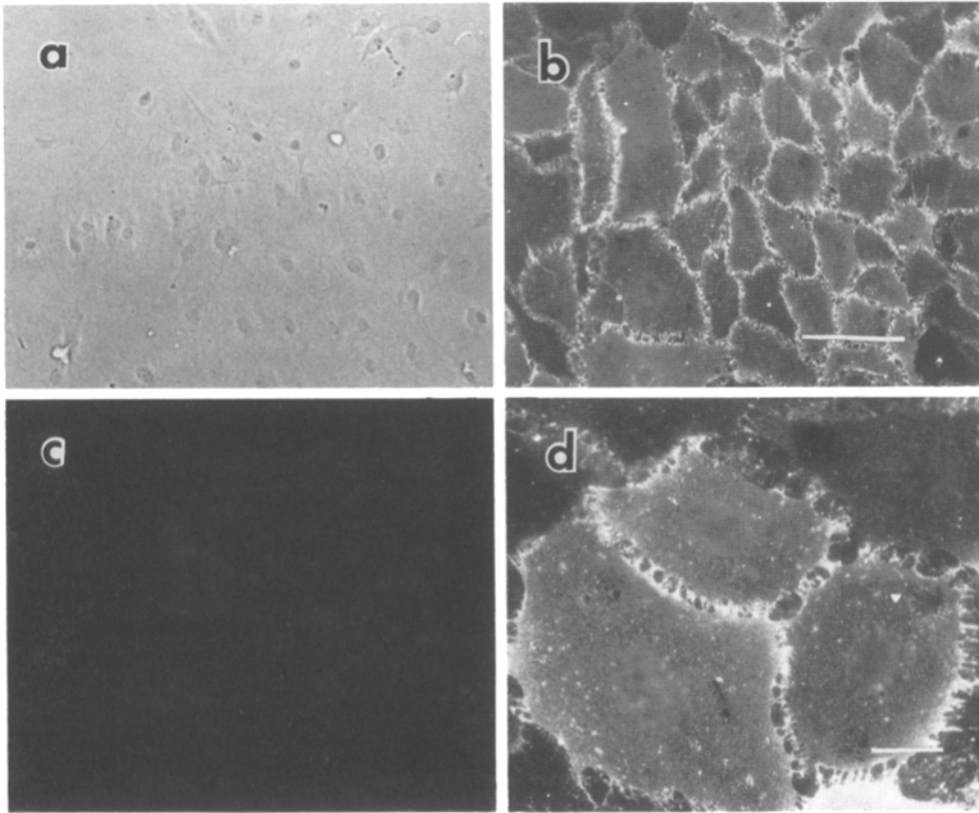
### Preparation of Syndecan-1 Expressing Schwann Cells

Cultures of Schwann cells from newborn rat sciatic nerve were grown on poly-L-lysine coated dishes in DME (GIBCO-BRL, Gaithersburg, MD) containing 10% FCS and 2  $\mu$ M forskolin. The cells were transfected with the mammalian expression plasmid pCMVneo containing a 1 kb insert coding for full length rat syndecan-1 (Cizmeci-Smith et al., 1992). Control cells were transfected with pCMVneo containing either no insert or a partial cDNA insert coding for a lipid anchored heparan sulfate proteoglycan (Asundi, V., and D. Carey, unpublished observations). For transfections, 40  $\mu$ g of purified plasmid and 30  $\mu$ g of Lipofectin (GIBCO-BRL) in a total volume of 200  $\mu$ l of water were mixed in a glass tube and allowed to sit for 15 min. The DNA mixture was added dropwise with swirling to cultures of Schwann cells ( $\sim$ 70% confluent) growing in 100-mm culture dishes (200  $\mu$ l of DNA solution) or 4 cm<sup>2</sup> glass slide chambers (50  $\mu$ l of DNA solution) in serum free OptiMEM culture medium (GIBCO-BRL). The cells were incubated at 37°C for 3 h, rinsed once with normal growth medium, and cultured in normal growth medium for 5 d. After 5 d the cells were switched to normal growth medium supplemented with G418 (200–300  $\mu$ g/ml). After  $\sim$ 3 wk viable colonies were subcultured using cloning rings. In one experiment the viable cells on 1 100-mm culture dish were passaged without cloning individual colonies.

For routine culturing the transfected cells were grown on plastic tissue culture dishes coated with poly-L-lysine. For some experiments the cells were grown on dishes coated with human plasma fibronectin (a gift of Dr. Michael Chernousov) or mouse tumor laminin (Collaborative Research, Bedford, MA). For the experiments reported in this paper Schwann cells between the fourth and eighth passage were used. It has been shown previ-



**Figure 1.** Analysis of syndecan-1 expression in transfected Schwann cells. Syndecan-1 expression was monitored by Northern blot analysis (a) and immunoblot analysis (b). (a) Total RNA was isolated from control Schwann cells (lane 1), three clonal lines of syndecan-1 expressing Schwann cells (clone 1A, lane 3; clone 2A, lane 4; clone 1B, lane 5) and an uncloned population of syndecan-1 expressing Schwann cells (lane 2). 20  $\mu$ g of RNA was electrophoresed on a formaldehyde-agarose gel, transferred onto a nylon filter and hybridized with <sup>32</sup>P-labeled rat syndecan cDNA probe. The filter was exposed to X-ray film for 18 h without intensifying screens. The arrows to the left indicate the positions of migration of the 28s and 18s rRNA bands, as visualized by ethidium bromide staining. (b) Equal amounts of Triton X-100 extracts (lanes 1–5) or culture medium (lanes 6–10) from syndecan-1 expressing Schwann cell clones (lanes 1–3 and 6–8), an uncloned population of syndecan-1 expressing Schwann cells (lanes 4 and 9) or control Schwann cells (lanes 5 and 10) were electrophoresed on a 7.5% acrylamide SDS gel, transferred to an Immobilon filter and stained with affinity purified anti-syndecan-1 antibodies. The arrow to the left indicates the position of migration of syndecan-1 core protein visible in the cell extracts. Numbers and arrows to the right indicate position of migration of molecular weight standards (in thousands).



**Figure 2.** Immunofluorescent staining of syndecan-1. Control Schwann cells (c) and a clonal line of syndecan-1 expressing Schwann cells (a, b, and d) were stained with anti-syndecan-1 antibodies. The cells were incubated with the primary antibody solution before fixation and were not permeabilized so that only cell surface proteoglycan was stained. a and b show the same field photographed with phase contrast and fluorescence optics. Bars (b) 100  $\mu$ m; (d) 20  $\mu$ m.

ously that Schwann cells of these passages retain essentially normal capacity for terminal differentiation (Porter et al., 1986).

### Biochemical Characterization of Syndecan-1

Expression of syndecan-1 in the transfected Schwann cells was verified by Northern blot and immunoblot analysis. For Northern blot analysis total RNA was isolated as described previously (Cizmeci-Smith et al., 1992). 15–20  $\mu$ g of RNA was electrophoresed on formaldehyde-agarose gels, transferred to Nylon membranes, and hybridized to  $^{32}$ P-labeled rat syndecan-1 cDNA.

For immunoblot analysis aliquots of conditioned culture media or 1% Triton X-100 extracts of cells were subjected to SDS gel electrophoresis in 7.5% polyacrylamide gels, electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA) and stained with affinity purified rabbit anti-syndecan-1 antibodies. These antibodies have been described previously, and were prepared by immunizing rabbits with recombinant rat syndecan-1 extracellular domain (Cizmeci-Smith et al., 1992, 1993). Bound antibodies were detected on immunoblots by incubation with goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison, WI) followed by incubation with phosphatase substrates as described previously (Carey et al., 1992). Proteoglycans were chemically deglycosylated by incubation in trifluoromethanesulfonic acid, as described previously (Cizmeci-Smith et al., 1993).

Metabolic labeling with  $^{35}$ SO<sub>4</sub> and immunoprecipitation of Schwann cell proteoglycans was carried out as described previously (Carey and Stahl, 1990). The immunoprecipitates were analyzed on a 0.75  $\times$  30 cm Ultraspherogel SEC 4000 column (Beckman Instruments, Palo Alto, CA) eluted with 0.1% SDS, 0.1 M Tris-HCl, pH 7.5 at a flow rate of 1 ml/min. The radioactivity in the eluates was quantitated with an on-line scintillation detector.

### Immunofluorescence Microscopy

Cells to be analyzed by immunofluorescence microscopy were grown in glass slide chambers (Lab-Tech, Thomas Scientific, Swedensboro, NJ). To visualize cell surface staining the culture medium was removed and the cells were incubated in the first antibody solution (1:20 dilution of affinity purified anti-syndecan-1 in 5% non-fat milk, 0.1 M sodium chloride, 0.02 M

sodium phosphate, pH 7.4) for 30 min on ice. After removing this solution and rinsing with 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4, the cells were fixed for 20 min at room temperature by incubation in 3% paraformaldehyde in the same buffer. This solution was removed and the bound antibodies were detected by incubating the cells with affinity purified fluorescein conjugated goat anti-rabbit IgG (1:20 dilution, Sigma Chem. Co., St. Louis, MO). For anti-vinculin staining the cells were fixed with 3% paraformaldehyde and permeabilized by incubation for 2 min in a solution of 0.05% Triton X-100 before the antibody incubation steps. Mouse monoclonal anti-vinculin antibodies were used at a dilution of 1:100 and were a gift of Drs. A.M. Belkin and V. Koteliensky, Cardiology Research Center, Moscow. For phalloidin staining the cells were fixed and permeabilized as for vinculin staining and then incubated for 15 min in a solution of rhodamine-phalloidin (Sigma Chem. Co.) or fluorescein-phalloidin (Molecular Probes, Inc., Eugene, OR) at a concentration of 0.5  $\mu$ g/ml in 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4. The stained cells were examined and photographed using a Zeiss Axiovert 35 inverted microscope equipped for epifluorescence.

### Quantitation of Cell Spreading

For quantitation of cell spreading, phase contrast images of individual cells in subconfluent cultures were recorded using a Newvicon tube video camera and digitized as described previously (Carey et al., 1990). The outlines of cell borders were traced manually with a mouse and the included areas were calculated using a morphometric analysis program (Technology Resources, Nashville, TN).

### Scanning Electron Microscopy

Cells to be analyzed by scanning electron microscopy were grown on glass coverslips. The cells were fixed with 2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M cacodylate, dehydrated through a graded ethanol series, impregnated with Peldri II (Ted Pella, Redding, CA), and dried under vacuum. Fragments of the coverslips were mounted on scanning electron microscopy specimen holders and shadowed with gold-palladium (60:40) at an angle of 30° under vacuum. The specimens were examined in a JEOL 1200EX scanning/transmission electron microscope operating at an accelerating voltage of 60 kV.

## Results

### Expression of Syndecan-1 in Schwann Cells by Transfection

We have shown previously that Schwann cells synthesize the transmembrane heparan sulfate proteoglycan N-syndecan (Carey et al., 1992). A variety of methods were used to demonstrate that Schwann cells do not synthesize the related hybrid transmembrane proteoglycan syndecan-1 (summarized below). We were interested in determining whether expression of syndecan-1 in Schwann cells would result in observable phenotypic changes in the cells. For this purpose a cDNA coding for full length rat syndecan-1 (Cizmeci-Smith et al., 1992) was subcloned into the selectable expression vector pCMVneo and used to transfect cultures of rat Schwann cells. Clones of Schwann cells that stably expressed syndecan-1 were selected by growth in medium containing G418. As controls Schwann cells were also transfected with the same vector but containing either an irrelevant insert or no insert. A total of 12 G418 resistant clones transfected with the syndecan-1 construct were isolated. Of these 10 expressed significant amounts of syndecan-1. Several of these were chosen for more detailed analysis.

Fig. 1 *A* shows the results of Northern blot analysis using a syndecan-1 cDNA probe that was hybridized to total RNA isolated from control Schwann cells (lane 1), 3 syndecan-1 transfected clones (lanes 3–5), and an uncloned population of stably transfected Schwann cells (lane 2). No detectable hybridization of the probe to RNA from the control cells was observed, whereas strong hybridization signals were detected with RNA isolated from all four stably transfected cell lines. The size of the syndecan-1 RNA was  $\sim 1.2$  kb, which is consistent with the expected size of the polyadenylated product of the syndecan-1 expression vector.

Fig. 1 *B* shows the results of immunoblot analysis using polyclonal anti-rat syndecan-1 antibodies of control cells and the same stably transfected syndecan-1 cell lines. Detergent lysates of the transfected cells (lanes 1–4) showed anti-syndecan-1 immunoreactivity at a  $M_r = 58,000$ , consistent with the migration of rat syndecan-1 core protein on SDS polyacrylamide gels (Cizmeci-Smith et al., 1992) as well as diffuse high molecular weight smears suggestive of processed syndecan-1. A lysate prepared from a clonal line of control cells transfected with a vector lacking the syndecan-1 insert (lane 5) did not show any specific anti-syndecan-1 immunostaining. Immunoblot analysis of cell surface syndecan-1 isolated by trypsinization of cells revealed only the processed (high molecular weight) form of the proteoglycan (not shown). Analysis of conditioned culture media from the syndecan-1 transfected cell lines (lanes 6–9) showed strong anti-syndecan-1 immunoreactivity in the high molecular weight region of the gel, suggesting that syndecan-1 was released from the cell surface. No anti-syndecan-1 staining of conditioned medium from control cultures was observed (lane 10).

$^{35}\text{SO}_4$ -labeled syndecan-1 could be immunoprecipitated from detergent extracts of metabolically labeled cultures of stably transfected Schwann cells. Results of nitrous acid and chondroitinase ABC digestions of the immunoprecipitated proteoglycan indicated the Schwann cell-expressed syndecan-1 was a hybrid proteoglycan with  $\sim 85\%$  heparan sulfate and 15% chondroitin sulfate (data not shown).

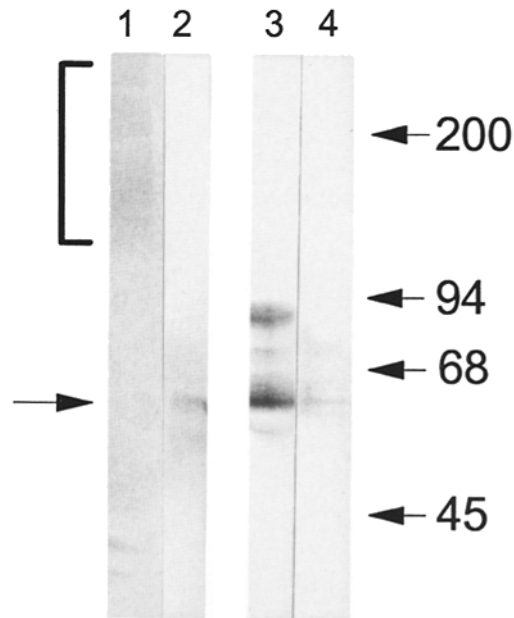
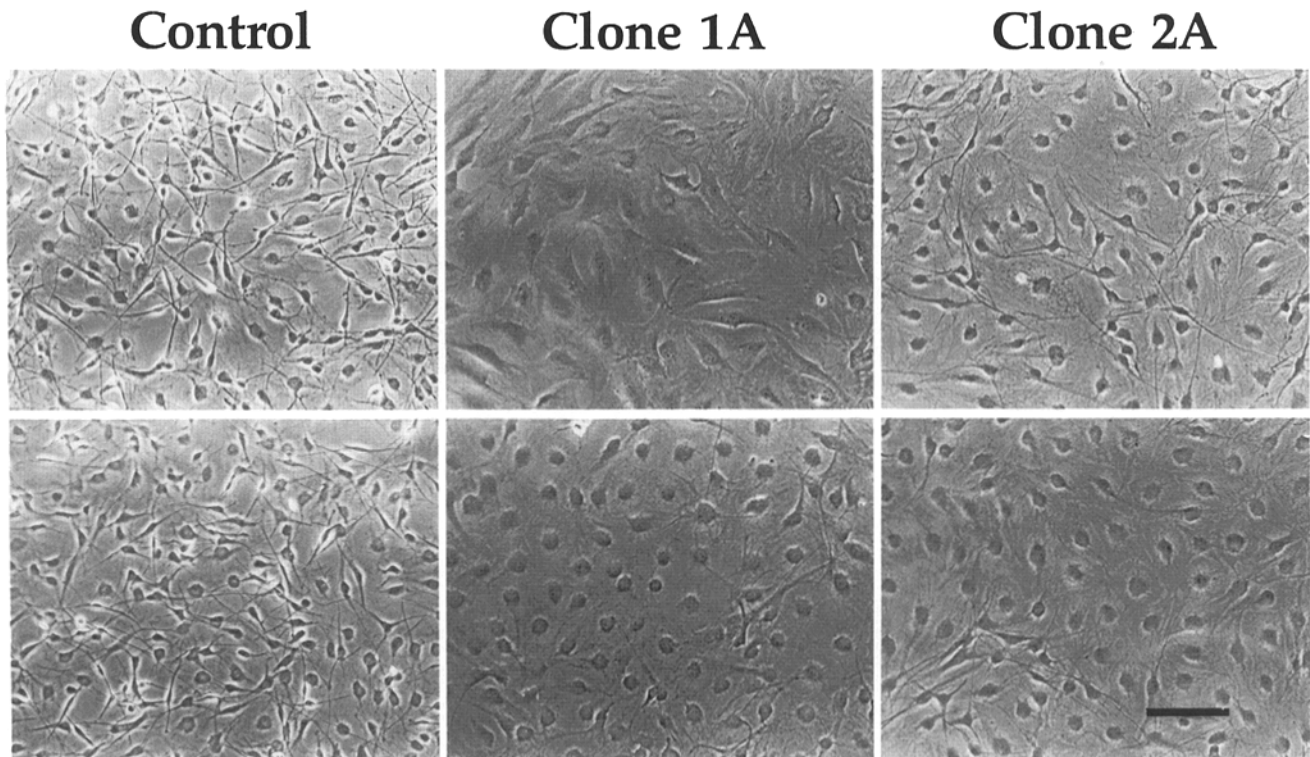


Figure 3. Immunoblot analysis of cell surface syndecan-1. Syndecan-1 expressing Schwann cells were incubated in calcium and magnesium free buffer containing 0.25% trypsin for 5 min at 37°C. The trypsinized cells were harvested by centrifugation and lysed in gel sample buffer, and aliquots of the trypsin-released (lanes 1 and 2) and cell lysate (lanes 3 and 4) material were subjected to immunoblot analysis and stained with anti-syndecan-1 antibodies, either without further treatment (lanes 1 and 3) or after deglycosylation by TFMS (lanes 2 and 4). The bracket to the left indicates the position of migration of the high molecular weight smear characteristic of processed syndecan-1; the arrow to the left indicates the position of migration of syndecan-1 core protein. Arrows and numbers to the right indicate the position of migration of molecular weight standards (in kD).

Immunofluorescent staining with anti-syndecan-1 antibodies of control and syndecan-1 transfected cells is shown in Fig. 2. Consistent with the results of Northern blot and immunoblot analyses no staining of control cells was observed (*c*). In contrast, bright cell surface staining of the transfected cells was observed (*b* and *d*). These cells were stained without prior fixation or permeabilization. These results indicate, therefore, that while much of the processed syndecan-1 was shed into the culture medium, a significant amount was also present on the cell surface.

To determine whether the cell surface syndecan-1 was the processed proteoglycan, syndecan-1 expressing cells were incubated in medium containing trypsin to cleave the ectodomain from the cell surface (Rapraeger and Bernfield, 1985). The trypsinized cells were lysed in detergent, and aliquots of the trypsin-released and cell lysate material were analyzed by immunoblot analysis and stained with anti-syndecan-1 antibodies. As shown in Fig. 3, in the trypsin-released material the antibodies stained only a diffuse high molecular weight smear (lane 1), characteristic of the migration of processed syndecan-1 proteoglycan. Deglycosylation of this material resulted in the appearance of a band migrating at an apparent  $M_r = 58,000$  (lane 2), which is the expected migration of rat syndecan-1 core protein (see Cizmeci-Smith et al., 1993). In contrast, the lysate of the trypsinized cells did not contain significant amounts of the



**Figure 4.** Morphology of syndecan-1 expressing Schwann cells. Confluent cultures of control Schwann cells and two clonal lines of syndecan-1 expressing Schwann cells growing on culture dishes coated with human plasma fibronectin (*top row*) or mouse tumor laminin (*bottom row*) were photographed using phase contrast optics. Bar, 100  $\mu\text{m}$ .

high molecular weight smear, but two major bands migrating at  $M_r = 58,000$  and  $90,000$  (lane 3). These were, presumably, the core protein and a processing intermediate form of syndecan-1. In support of this, deglycosylation of the cell lysate material resulted in the appearance of only the  $M_r = 58,000$  band (lane 4). These results indicated that the cell surface syndecan-1 is predominantly the processed proteoglycan form.

#### **Syndecan-1 Expression Alters Schwann Cell Spreading and Morphology**

The syndecan-1 expressing cells exhibited a morphology that differed from that of the control cells. As is typical of normal Schwann cells, the latter appeared mostly as networks of small, bipolar cells with little spreading onto the substrata except for thin neurite-like processes extending from the cell bodies (Fig. 4). The syndecan-1 expressing cells, in contrast, on both fibronectin- and laminin-coated dishes were spread more extensively onto the substrata, and had larger nuclei and fewer neurite-like processes (Fig. 4). Similar results were observed for cells grown on poly-L-lysine-coated dishes (not shown). These morphological characteristics were typical of all of the syndecan-1 expressing Schwann cell clones that were examined, and were not observed in the four control clones analyzed or wild-type Schwann cells.

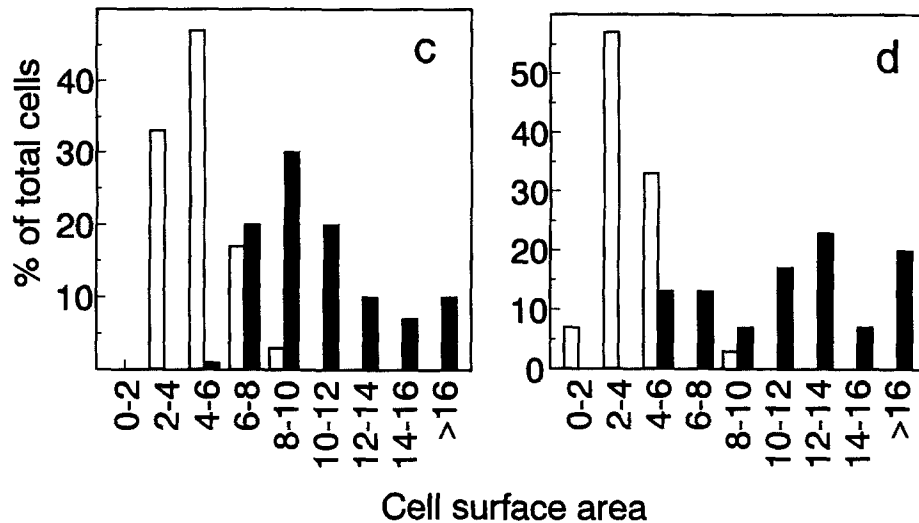
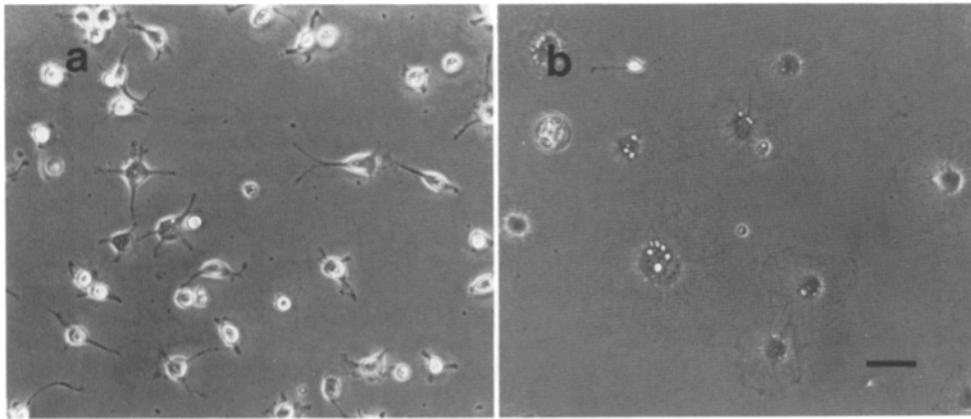
In confluent cultures syndecan-1 expressing Schwann cells formed a non-overlapping, continuous monolayer of flat polygonal cells with centrally located nuclei (Figs. 2 and 4). In these cultures the cell surface syndecan-1 staining was concentrated around the borders of individual cells where they were in contact with neighboring cells (Fig. 2). This pe-

ripheral staining coincided with the location and shape of the filopodial projections visible on the cells by scanning electron microscopy (not shown).

The difference between control and syndecan-1 expressing Schwann cells was especially apparent during active cell spreading after replating. Fig. 5 shows control cells (*a*) and syndecan-1 expressing cells (*b*) 4 h after replating. The control cells attached to the dishes and extended thin neurite-like processes. In contrast, the syndecan-1 expressing cells spread much more extensively in all directions, and produced large, flattened, essentially round cells. The extent of enhanced substratum spreading was quantified by measuring the total spread areas of phase contrast images of subconfluent control and syndecan-1 expressing cells. As shown in Fig. 5, syndecan-1 expressing cells exhibited an average increase in spread areas of 2.3- and 3.4-fold on poly-L-lysine- (*c*) and fibronectin- (*d*) coated plates. On both substrata nearly all of the syndecan-1 expressing cells were larger than the most spread control cells.

The effects of syndecan-1 expression on spreading and cell morphology were also clearly visible by scanning electron microscopy. Fig. 6 shows micrographs of control cells and one of the syndecan-1 expressing clones. The syndecan-1 expressing cells produced large, flat expanses of membrane rimmed by filopodial projections (*b*), in contrast to the more compact, elongated profiles of the control cells (*a*).

To establish that these effects on cell spreading were due to the presence of syndecan-1 on the cell surface, we examined the effects of anti-syndecan-1 antibodies on syndecan-1 expressing cells grown on fibronectin- and laminin-coated dishes. As shown in Fig. 7, the syndecan-1 expressing cells (*B* and *E*) were flatter and more spread than control cells (*A*



**Figure 5.** Attachment and spreading of syndecan-1 expressing Schwann cells. Clonal lines of control Schwann cells (transfected with vector alone, *a*) and syndecan-1 expressing Schwann cells (*b*) were trypsinized and plated onto plastic tissue culture dishes coated with human plasma fibronectin. After incubation at 37°C for 4 h the cells were photographed using phase contrast optics. For quantitation of cell spreading control Schwann cells (*open bars*) and syndecan-1 expressing Schwann cells (*solid bars*) growing on dishes coated with poly-L-lysine (*c*) or human plasma fibronectin (*d*) were imaged by phase contrast microscopy and areas of individual cells were measured as described in Materials and Methods. Areas are expressed as arbitrary units. The differences between control and expressing cells were statistically significant ( $p < 0.001$ ). Bar in *b*, 50  $\mu\text{m}$ .

and *D*). Addition of anti-syndecan-1 antibodies inhibited the spreading of the syndecan-1 expressing cells (*C* and *F*), so that these cells more closely resembled the control cells.

### Effects on Cytoskeletal Organization

In addition to enhanced spreading syndecan-1 expressing cells exhibited a reorganization of cytoskeletal filaments. As shown in Fig. 6 *a*, phalloidin labeling of control cells revealed intense staining of closely packed, fine microfilaments bundles oriented parallel to the long axes of the cells, as well as bright staining of the thin Schwann cell processes. Syndecan-1 expressing cells (Fig. 8 *b*) were larger and more spread and contained an extensive network of microfilaments throughout the cell cytoplasm. Many of the cells contained thick, short microfilament bundles around the periphery of the cells or oriented radially across smaller parallel arrays of microfilament bundles.

### Syndecan-1 Does Not Colocalize with Vinculin

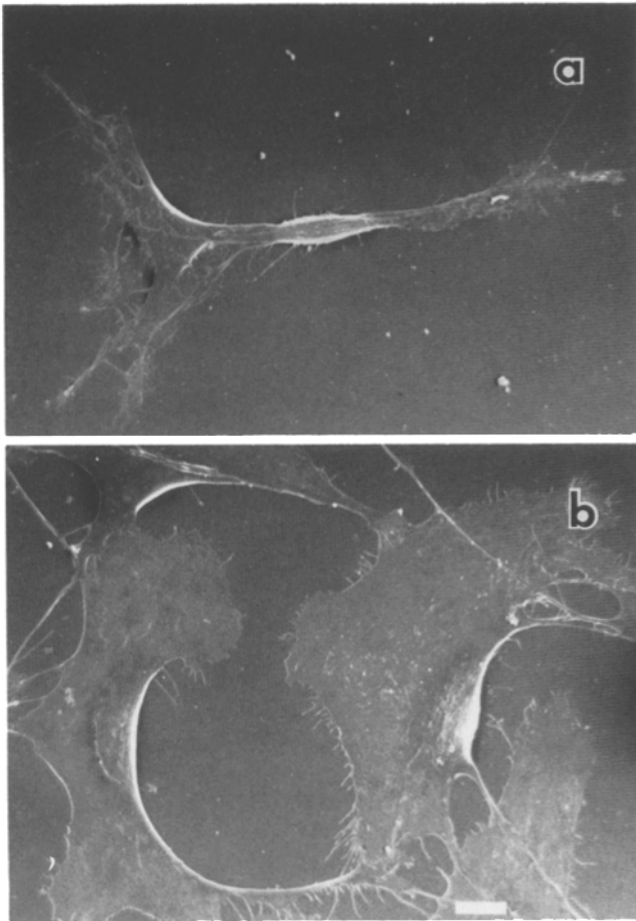
To determine whether there was a stable colocalization of syndecan-1 with focal contacts that could account for the enhanced spreading and cytoskeletal reorganization, we doubly stained the cells with anti-syndecan-1 and anti-vinculin antibodies (Fig. 9). Superimposition of these images revealed that the focal contacts (marked by anti-vinculin stain-

ing) did not overlap the regions of anti-syndecan-1 staining, but were internal to and adjacent to these sites. Thus, cell surface syndecan-1 did not appear to be a stable component of focal contacts in these cells.

### Colocalization of Cell Surface Syndecan-1 and Actin during Cell Spreading

We next wanted to determine whether association of syndecan-1 with cytoskeletal structures could be observed during active cell spreading. Syndecan-1 expressing Schwann cells were trypsinized and replated onto fibronectin-coated dishes and the distribution of cell surface syndecan-1 and filamentous actin were determined after several hours by fluorescence microscopy, at a time when the cells had attached to the substratum and were in the process of spreading. In these cells the cell surface anti-syndecan-1 immunostaining was visible in a punctate pattern on the entire cell surface, and as patches of staining at the lateral edges of the cells (Fig. 10 *A*, *arrowheads*). These lateral patches of syndecan-1 immunoreactivity corresponded to areas of the lateral cell edges where actin filaments had polymerized, as visualized by phalloidin staining (Fig. 10 *B*). These results indicated, therefore, that during cell spreading there was an apparent association of cell surface syndecan-1 and actin filaments.





**Figure 6.** Scanning electron microscopy of syndecan-1 expressing Schwann cells. Control Schwann cells (a) and a clonal line of syndecan-1 expressing Schwann cells (b) were fixed and processed for scanning electron microscopy as described in Materials and Methods. Bar, 10  $\mu\text{m}$ .

### Radiolabeling of Proteoglycans

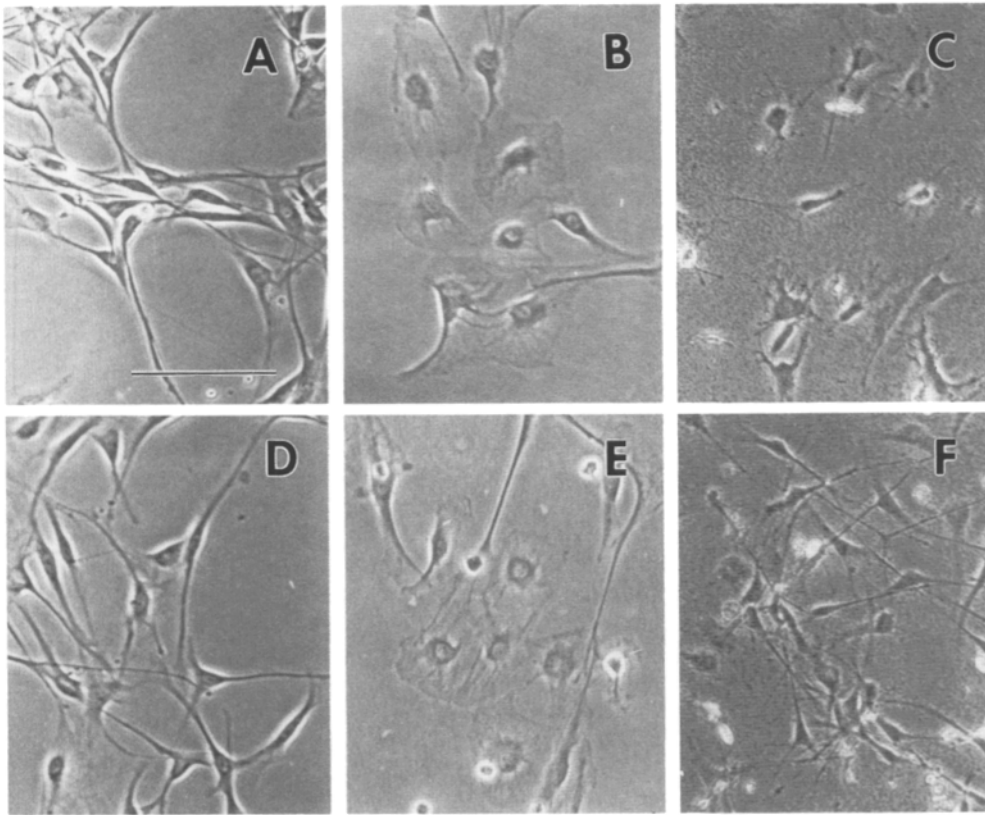
One potential explanation for the observed effects is that syndecan-1 expressing cells had vastly increased (non-physiological) levels of cell surface glycosaminoglycans that produced enhanced substratum adhesiveness through non-specific mechanisms. It was of interest, therefore, to estimate the relative amounts of syndecan-1 associated and endogenous glycosaminoglycans in the cells. To do this we radiolabeled the cells with  $^{35}\text{SO}_4$  for 24 h and measured the incorporation of radiolabel into syndecan-1 and a lipid anchored heparan sulfate proteoglycan (Carey and Stahl, 1990) that is the principal endogenous membrane-associated proteoglycan in Schwann cells. Incorporation into the total pool of cellular proteoglycans was also measured. As assessed by  $^{35}\text{SO}_4$  labeling, syndecan-1 expression increased the total glycosaminoglycan content of the cells by  $\sim 70\%$  and accounted for less than one-half of the total glycosaminoglycan synthesized by the cells (Table I). The amount of syndecan-1 associated glycosaminoglycan was  $\sim 1.7$  times the amount associated with the lipid anchored proteoglycan. When this value was adjusted for the numbers of glycosaminoglycan at-

tachment sites—five for rat syndecan-1 (Cizmeci-Smith et al., 1992) vs three for the lipid anchored proteoglycan (David et al., 1990)—then the two proteoglycans were present at approximately equivalent concentrations (assuming equal chain lengths and sulfation).

### Discussion

We have investigated the effects of expression of the transmembrane proteoglycan syndecan-1 in cultured Schwann cells. The most obvious alteration that was observed in response to syndecan-1 expression was a conversion of the cells to a highly flattened cuboidal morphology. This was in contrast to the typical morphology of cultured Schwann cells, i.e., small spindle-shaped cells with long thin neurite-like processes. There was little apparent effect of syndecan-1 expression on the growth properties of the cells, except for a decrease in the confluent density (unpublished observations). We attributed this difference to the large increase in area occupied by individual cells, leading to contact inhibition of cell proliferation at lower cell densities. Syndecan-1 expression also resulted in a major reorganization of cytoskeletal structures within the cells, although under basal conditions there was no apparent colocalization of cell surface syndecan-1 with cytoskeletal structures.

The morphological conversion of the Schwann cells was most likely caused by increased adhesiveness of the cells to the substratum. Syndecan-1 has been proposed to function as a receptor for interstitial type extracellular matrix (Saunders and Bernfield, 1988). In our studies, increased spreading caused by syndecan-1 expression was observed on the non-biological substratum poly-L-lysine, as well as the matrix adhesive proteins fibronectin and laminin. Syndecan-1 has been reported to bind *in vitro* to fibronectin (Saunders and Bernfield, 1988) as well as other ECM molecules including collagen type I (Koda et al., 1985), thrombospondin (Sun et al., 1989), and tenascin (Salmivirta et al., 1991). Interestingly, we have found that the Schwann cell expressed syndecan-1 binds only very weakly to fibronectin or laminin *in vitro* (Chernousov and Carey, 1993). These findings suggest, therefore, that it may be difficult to draw firm conclusions about the biological functions of these molecules from *in vitro* binding data alone. The precise mechanism by which syndecan-1 expression alters Schwann cell spreading is not known. Additional receptors would also appear to be involved, since focal contacts are present, and syndecan-1 did not colocalize with these structures. The effects we observed in living cells may result from the cooperative interactions of the cell surface proteoglycan with other adhesive systems (e.g., integrins). It has been proposed that proteoglycans of the syndecan family may function as “coreceptors” that stabilize or strengthen binding interactions mediated by other “high affinity” receptors (Bernfield et al., 1992). Whether syndecans and integrins cooperate to mediate adhesion in this manner is not known. We have shown previously that Schwann cell spreading on laminin-coated surfaces is inhibited partially by anti-integrin antibodies, and essentially completely by a combination of anti-integrin and heparin (Carey et al., 1990). Alternatively, the adhesion could result from the combined action of many weak binding interactions mediated by proteoglycans sequestered on the cell surface.

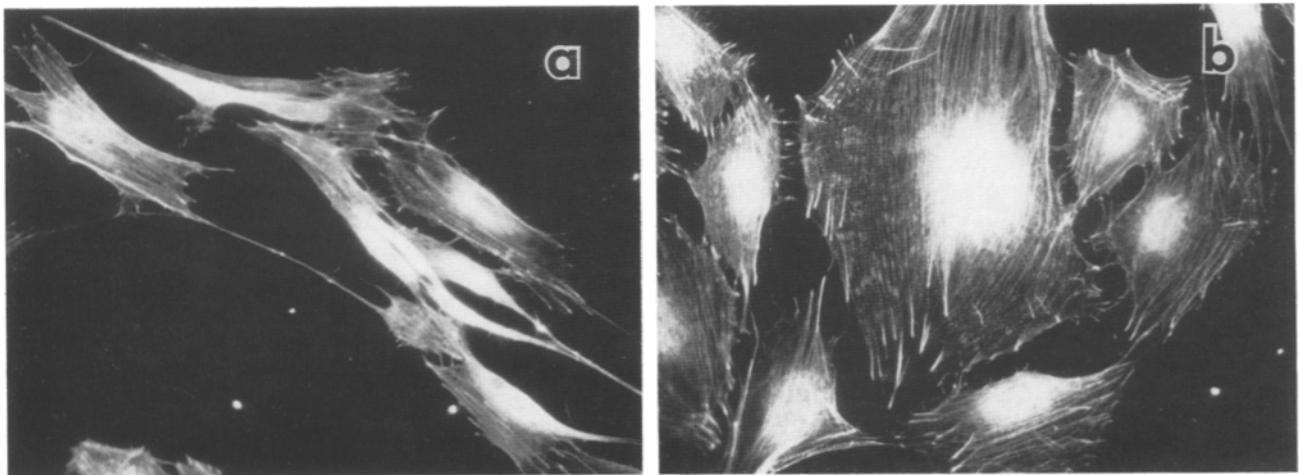


**Figure 7.** Effects of anti-syndecan-1 antibodies on the morphology of syndecan-1 expressing Schwann cells. A clonal line of syndecan-1 expressing Schwann cells (*B*, *C*, *E*, and *F*) or control Schwann cells (*A* and *D*) growing on cultures dishes coated with human plasma fibronectin (*A-C*) or mouse tumor laminin (*D-F*) were incubated in normal growth medium or growth medium containing 10  $\mu\text{g/ml}$  affinity purified anti-syndecan-1 antibodies (*C* and *F*) for 48 h. Bar, 100  $\mu\text{m}$ .

For example, patching of cell surface syndecan, which we observed during cell spreading, may increase the local concentration of the proteoglycan and drive the equilibrium toward association with extracellular ligands.

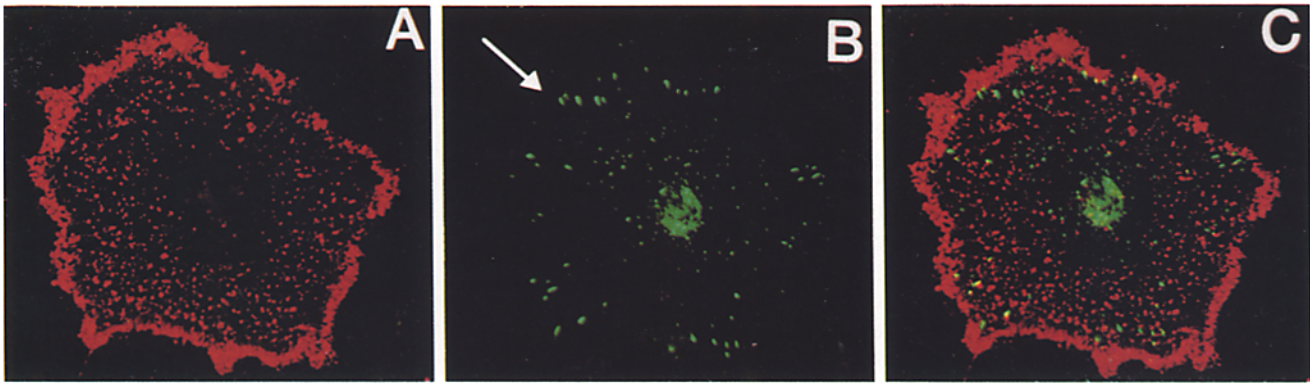
The observed effects of syndecan-1 expression on Schwann cells did not appear to result simply from a large increase in the content of cell surface glycosaminoglycans. We estimated from measurements of  $^{35}\text{SO}_4$  incorporation that the stably expressing Schwann cells had  $\sim 70\%$  more total glycosaminoglycan than control cells. The amount of syndecan-1 in the cells measured by this method was roughly equal

to the amount of the lipid anchored heparan sulfate proteoglycan. While it is possible that this level of increase could significantly alter cell adhesion to the extent observed in our cells, we think this is unlikely. This suggests that the nature of the core protein has an important effect on the biological activity of the glycosaminoglycans. This is a potentially important finding, since it has been difficult to demonstrate functional specificity of different membrane proteoglycans by analysis of their binding interactions *in vitro*. The structural diversity of the core proteins and their highly regulated patterns of expression make it unlikely that all membrane as-

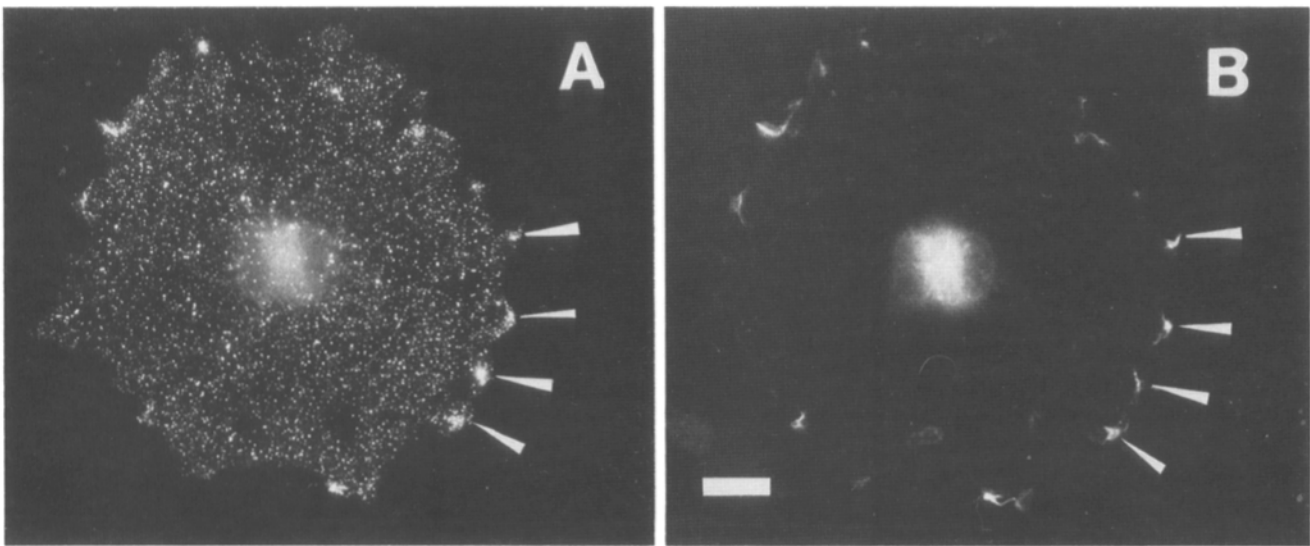


**Figure 8.** Cytoskeletal organization in control and syndecan-1 expressing Schwann cells. Subconfluent cultures of control Schwann cells (*a*) and a clonal line of syndecan-1 expressing Schwann cells (*b*) were stained with rhodamine-conjugated phalloidin. Bar, 25  $\mu\text{m}$ .





**Figure 9.** Syndecan-1 and vinculin distribution in syndecan-1 expressing Schwann cells. Syndecan-1 expressing Schwann cells were stained with rabbit anti-syndecan-1 antibodies and mouse anti-vinculin antibodies. These were visualized by rhodamine anti-rabbit IgG (red), and fluorescein anti-mouse IgG (green), respectively. *A* and *B* show the staining patterns obtained with each antibody. *C* shows a superimposed image of the staining patterns. Anti-vinculin stains predominantly focal contacts (arrow) adjacent to, but not overlapping, the outer rim of syndecan-1 staining.



**Figure 10.** Colocalization of cell surface syndecan-1 and actin filaments during cell spreading on fibronectin. Syndecan-1 expressing Schwann cells were plated onto fibronectin-coated dishes and allowed to attach and spread for several hours. The cells were then stained with anti-syndecan-1 antibodies (visualized with rhodamine anti-rabbit IgG, *a*) and fluorescein-phalloidin (*b*). Note the patches of syndecan-1 staining at the lateral cell edges that correspond to sites of actin filament polymerization (arrowheads). Bar, 25  $\mu$ m.

sociated proteoglycans carry out the same functions. This illustrates, therefore, the value of assays using proteoglycans expressed in living cells. It is possible that the core proteins alter the functional consequences of glycosaminoglycan bind-

**Table I.**  $^{35}\text{SO}_4$  Labeling of Schwann Cell Proteoglycans\*

Proteoglycan	$^{35}\text{SO}_4$ cpm (% total)
Syndecan-1	46,400 (44)
LAPG	27,023 (25)
Other	33,028 (31)
Total	106,451

\* Syndecan-1 expressing cells (clonal line 1B) were labeled with  $^{35}\text{SO}_4$ ; incorporation into proteoglycans was determined by immunoprecipitation and gel permeation HPLC. LAPG is the lipid anchored Schwann cell proteoglycan (Carey and Stahl, 1990). "Other" indicates proteoglycans not immunoprecipitated by anti-syndecan-1 or anti-LAPG antibodies.

ing in subtle ways that cannot be differentiated by the fairly crude *in vitro* binding assays. One obvious difference between syndecan-1 and the lipid anchored proteoglycan, for example, is the presence of a transmembrane and cytoplasmic domain in the former, affording it the opportunity to interact with intracellular proteins. The functional importance of these potential interactions are not yet understood. We should point out that Schwann cells also synthesize N-syndecan, but at amounts that are considerably lower than the syndecan-1 in the transfected cells. Thus, we cannot draw any firm conclusions about specific functions of syndecan family subtypes from these studies.

The effects of syndecan-1 expression on cytoskeletal reorganization could result indirectly from increases in substratum adhesivity. While interactions of cell surface proteoglycans (Carey and Todd, 1986), including syndecan-1 (Rapraeger et al., 1986), with the cytoskeleton have been

demonstrated in some cells, we did not observe any apparent colocalization of cell surface syndecan-1 with cytoskeletal structures under steady state conditions in spread cells. In contrast, we found that during active cell spreading there was an apparent transient association of cell surface syndecan-1 with actin filaments at the lateral cell edges. We have also found that colocalization of syndecan-1 with microfilaments can be induced in spread cells by aggregation of the cell surface proteoglycan with antibodies (Carey, D., R. Stahl, B. Tucker, K. Bendt and G. Cizmeci-Smith, manuscript submitted for publication). This raises the possibility that ligand mediated oligomerization of the proteoglycan during cell spreading could induce a transient cytoskeletal association that might influence cellular organization. This mechanism is attractive because it would allow for more dynamic regulation than would a stable long term association of the proteoglycan with cytoskeletal structures. Obviously, other adhesive receptor systems are also at work. An important area of future research will be to investigate the interactions between syndecans and other adhesive receptors.

The observed actin filament colocalization presumably involved coupling of the cytoplasmic domain of the proteoglycan core protein to microfilaments, but this has not been demonstrated directly. The cytoplasmic domains are the most highly conserved parts of the core proteins that comprise this gene family (Bernfield et al., 1992), which suggests that the cytoplasmic domains carry out an important biological function. This also suggests that other proteoglycans of this gene family would exhibit similar behavior, but this remains to be tested. By analogy with other cell surface transmembrane proteins that bind to cytoskeletal filaments, the binding of syndecan-1 to microfilaments would be indirect. For example, integrins interact with cytoskeletal structures, such as focal contacts, via binding of their cytoplasmic domain (Reszka et al., 1992) to the actin-binding protein  $\alpha$ -actinin (Otey et al., 1990). Binding of syndecan family proteoglycans to actin binding proteins in vitro has not been reported. Additional work in this area will be required to elucidate the specific molecular mechanisms that mediate the biological functions of transmembrane proteoglycans.

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## References

Bernfield, M., R. Kokenyesi, M. Kato, M. T. Hinkes, J. Spring, R. L. Gallo, and E. J. Lose. 1992. The biology of the syndecans. *Annu. Rev. Cell Biol.* 8:365-393.

Carey, D. J., and M. S. Todd. 1986. A cytoskeleton-associated plasma mem-

brane heparan sulfate proteoglycan in Schwann cells. *J. Biol. Chem.* 261:7518-7525.

Carey, D. J., and R. C. Stahl. 1990. Identification of a lipid-anchored heparan sulfate proteoglycan in Schwann cells. *J. Cell Biol.* 111:2053-2062.

Carey, D. J., D. M. Crumbling, R. C. Stahl, and D. M. Evans. 1990. Association of cell surface heparan sulfate proteoglycans of Schwann cells with extracellular matrix proteins. *J. Biol. Chem.* 265:20627-20633.

Carey, D. J., D. M. Evans, R. C. Stahl, V. K. Asundi, K. J. Conner, P. Garbes, and G. Cizmeci-Smith. 1992. Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan. *J. Cell Biol.* 117:191-201.

Chernousov, M. A., and D. J. Carey. 1993. N-syndecan from neonatal rat brain binds basic fibroblast growth factor. *J. Biol. Chem.* 268:16810-16814.

Cizmeci-Smith, G., V. Asundi, R. C. Stahl, L. J. Teichman, M. Chernousov, K. Cowan, and D. J. Carey. 1992. Regulated expression of syndecan in vascular smooth muscle cells and cloning of rat syndecan core protein cDNA. *J. Biol. Chem.* 267:15729-15736.

Cizmeci-Smith, G., R. C. Stahl, L. J. Showalter, and D. J. Carey. 1993. Differential expression of transmembrane proteoglycans in vascular smooth muscle cells. *J. Biol. Chem.* 268:18740-18747.

David, G., V. Lories, B. Decock, P. Marynen, J. J. Cassiman, and H. VandenBerghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111:3165-3176.

David, G., B. VanderSchueren, P. Marynen, J. J. Cassiman, and H. VandenBerghe. 1992. Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. *J. Cell Biol.* 118:961-969.

Elenius, K., A. Maatta, M. Salmivirta, and M. Jalkanen. 1992. Growth factors induced 3T3 cells to express bFGF-binding syndecan. *J. Biol. Chem.* 267:6435-6441.

Elenius, K., S. Vainio, M. Laato, M. Salmivirta, I. Thesleff, and M. Jalkanen. 1991. Induced expression of syndecan in healing wounds. *J. Cell Biol.* 114:585-595.

Gould, S. E., W. B. Upholt, and R. A. Koshier. 1992. Syndecan 3: a member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation. *Proc. Natl. Acad. Sci. USA.* 89:3271-3275.

Koda, J. E., A. Rapraeger, and M. Bernfield. 1985. Heparan sulfate proteoglycan from mouse mammary epithelial cells: cell surface proteoglycan as a receptor for interstitial collagen. *J. Biol. Chem.* 260:8157-8162.

Kojima, T., N. W. Shworak, and R. D. Rosenberg. 1992. Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. *J. Biol. Chem.* 267:4870-4877.

Lories, V., H. DeBoeck, G. David, J. J. Cassiman, and H. VandenBerghe. 1989. Heparan sulfate proteoglycans of human lung fibroblasts. *J. Biol. Chem.* 262:854-859.

Marynen, P., J. Zhang, J. J. Cassiman, H. VandenBerghe, and G. David. 1989. Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. *J. Biol. Chem.* 264:7017-7024.

Otey, C. A., F. M. Oavalko, and K. Burridge. 1990. An interaction between alpha-actin and the beta 1 integrin subunit in vitro. *J. Cell Biol.* 111:721-729.

Porter, S., M. B. Clark, L. Glaser, and R. P. Bunge. 1986. Schwann cells stimulated to proliferate in the absence of neurons retain full functional capability. *J. Neurosci.* 6:3070-3078.

Rapraeger, A., and M. Bernfield. 1985. Cell surface proteoglycan of mammary epithelial cells: protease releases a heparan sulfate-rich ectodomain from a putative membrane-activated domain. *J. Biol. Chem.* 260:4103-4109.

Rapraeger, A., M. Jalkanen, and M. Bernfield. 1986. Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. *J. Cell Biol.* 103:2683-2696.

Reszka, A., Y. Hayashi, and A. Horwitz. 1992. Identification of amino acid sequences in the integrin  $\beta$  cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321-1330.

Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375-413.

Salmivirta, M., K. Elenius, S. Vainio, U. Hofer, R. Chiquet-Ehrismann, I. Thesleff, and M. Jalkanen. 1991. Syndecan from embryonic tooth mesenchyme binds tenascin. *J. Biol. Chem.* 266:7733-7739.

Saunders, S., and M. Bernfield. 1988. Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. *J. Cell Biol.* 106:423-430.

Saunders, S., M. Jalkanen, S. O'Farrell, and M. Bernfield. 1989. Molecular cloning of syndecan, an integral membrane proteoglycan. *J. Cell Biol.* 108:1547-1556.

Sun, X., D. F. Mosher, and A. Rapraeger. 1989. Heparan sulfate-mediated binding of epithelial cell surface proteoglycan to thrombospondin. *J. Biol. Chem.* 264:2885-2889.