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Syndecan 4 Heparan Sulfate Proteoglycan Is a Selectively Enriched and Widespread Focal Adhesion Component

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Focal adhesion formation in fibroblasts results from complex transmembrane signaling processes initiated by extracellular matrix molecules. Although a role for integrins with attendant tyrosine kinases has been established, there is evidence that cell surface heparan sulfate proteoglycans (HSPGs) are also involved with an associated role of protein kinase C. The identity of the proteoglycan has remained elusive, but we now report that syndecan 4 (ryudocan/amphiglycan) is present in focal adhesions of a number of cell types. Affinity-purified antibodies raised against a unique portion of the cytoplasmic domain of syndecan 4 core protein recognized an HSPG of similar characteristics to those of syndecan 4. These antibodies stained focal adhesions only after cell permeabilization and recognized differing mammalian species. Syndecan 4 was associated with focal adhesions that contained either β_1 or β_3 integrin subunits and those that formed on substrates of fibronectin, laminin, vitronectin, or type I collagen. No focal adhesions were found that were vinculin-containing but lacked syndecan 4. In contrast, syndecan 2, whose cytoplasmic domain is closely homologous to syndecan 4, does not appear to be a focal adhesion component. Thus, syndecan 4 represents a new transmembrane focal adhesion component, probably involved in their assembly.

INTRODUCTION

Many anchorage-dependent cells attach, spread, and assemble focal adhesions on substrates coated with extracellular matrix molecules such as fibronectin, laminin, vitronectin, and type I collagen (Burridge *et al.*, 1988, 1992; Woods and Couchman, 1988; Hynes, 1990). Focal adhesions form where cytoskeletal microfilament bundles terminate at the membrane. They contain specialized cytoskeletal, membrane, and extracellular components and are stable points of adhesion to matrix molecules both in vitro and in vivo (Burridge *et al.*, 1988; Woods and Couchman, 1988; Hynes, 1990). Cell surface heparan sulfate proteoglycans (HSPGs) have been implicated in the formation of these structures. Polyclonal antibodies raised against liver microsomal HSPGs label focal adhesions and the membrane overlying stress fibers in addition to matrix fibrils (Woods *et al.*, 1984). A hydrophobic form of HSPG is retained in detergent-resistant cytoskeletal preparations (Woods *et al.*, 1985), and two types of mutant cells that lack, or have altered, HSPGs are unable to form focal adhesions in response to a fibronectin substrate (Couchman *et al.*, 1988; LeBaron *et al.*, 1988). In addition, focal adhesion formation can be promoted by the addition of heparin-binding fibronectin fragments to cells prespread on substrates of cell-binding Arg-Gly-Asp (RGD-containing) fragments (Woods *et al.*, 1986). The formation of focal adhesions appears to require activation of protein kinase C (PKC) (Woods and Couchman, 1992a,b), indicating that the cell surface HSPG involved may generate a transmembrane signal. The form of HSPG involved in this process has not previously been identified but should be expressed on the surfaces of those cells that form focal adhesions (e.g., fibroblasts and smooth muscle and endothelial cells) and may be concentrated in them.

Several different cell surface HSPGs can exist in any one cell type (reviewed in Lories *et al.*, 1989, 1992; Bernfield *et al.*, 1992; Couchman and Woods, 1993). Major transmembrane HSPG species in a variety of cells are the members of the syndecan family. These are,

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therefore, candidates for transducing the transmembrane signal to form focal adhesions. Fibroblasts can contain mRNA for syndecan 1 (Saunders *et al.*, 1989; Kojima *et al.*, 1992b), fibroglycan (syndecan 2) (Marynen *et al.*, 1989; Pierce *et al.*, 1992), and amphiglycan/ryudocan (syndecan 4) (David *et al.*, 1992; Kojima *et al.*, 1992b). Analysis of cell surface HSPGs expressed by fibroblasts indicate the presence of core proteins with Mr = 85k (syndecan 1), 48/90k (syndecan 2), 35k (syndecan 4), 125k (possibly syndecan 3) (Carey *et al.*, 1992; Gould *et al.*, 1992), and 64k. The latter is glypican, which is anchored in the membrane by a phosphoinositol linkage (David *et al.*, 1990). The major HSPGs synthesized appear to be glypican and syndecans 2 and 4 (Lories *et al.*, 1989, 1992; David *et al.*, 1992; Kojima *et al.*, 1992b). Published immunochemical studies on the distributions of these HSPGs have not shown their localization at focal adhesions, although syndecan 1 of epithelial cells is polarized basally and colocalizes with concentrations of microfilaments (Rapraeger *et al.*, 1986). In addition, we have extensively examined the distribution of these HSPGs using available antibodies against syndecans 1 and 2 and glypican (gifts from Dr. G. David, University of Leuven, Belgium) and have not found specific localization to focal adhesions.

Members of the syndecan HSPGs differ considerably in their extracellular domains but have high homology in their transmembrane and cytoplasmic domains (reviewed in Bernfield *et al.*, 1992; Couchman and Woods, 1993). Antibodies raised against different extracellular epitopes should specifically localize individual syndecans, but labeling of these epitopes at focal adhesions may be compromised by the small distance between the membrane and the substrate (~15 nm) (reviewed in Woods and Couchman, 1988). We, therefore, raised a polyclonal antiserum against a synthetic peptide specific for the cytoplasmic domain of syndecan 4. After affinity-purification, these antibodies specifically labeled focal adhesions of a range of cells on several different substrates. In contrast, an antiserum raised against rat liver HSPG (Pierce *et al.*, 1992), recognizing mainly fibroglycan (syndecan 2), strongly stained the general cell surface.

MATERIALS AND METHODS

Chemicals and Reagents

General chemicals were obtained from Sigma Chemical (St. Louis, MO), and tissue cultureware and media were from Fisher (Atlanta, GA). Affigel 102, Tween 20 (EIA grade), nitrocellulose, goat anti-rabbit, and anti-mouse IgG conjugated to alkaline phosphatase together with color developer were all obtained from Biorad Laboratories (Hercules, CA). Chemiluminescence reagents were obtained from Amersham (Arlington Heights, IL).

Production and Characterization of Antibodies Specific for Syndecan 4

Peptides of the sequence (C)LGKKPIYKK from the cytoplasmic domain of syndecan 4 were synthesized by the Cancer Center core facility at University of Alabama, Birmingham (U.A.B.). These were coupled by sulfo-LC-SPDP (Pierce, Rockford, IL) through the additional N-terminal cysteine to NH_2-groups of keyhole limpet hemocyanin, and 1 mg of conjugate emulsified with Freund's complete or incomplete adjuvant were used to immunize New Zealand rabbits at 3-wk intervals. Blood was taken from an ear vein at 2-wk intervals. Serum was prepared as previously (Couchman, 1987) and tested for labeling of cultured rat embryo fibroblasts. Active sera were affinity purified after ammonium sulfate precipitation by passage over the peptide coupled through sulfo-LC-SPDP to NH_2-groups of aminoalkyl agarose beads (Affigel 102). After elution with elution buffer (Pierce), antibodies were extensively dialyzed into phosphate-buffered saline (PBS) containing 0.02% sodium azide. Bovine serum albumin was added to 0.1% to stabilize the antibodies for storage at 4°C.

Antibody specificities were characterized by three methods: immunoblotting of whole cell lysates or total proteoglycans, immunoblotting of material immunoprecipitated by anti-syndecan 4 peptide antibodies, and chromatographic analysis of $^{35}SO_4$-labeled proteoglycans (Woods *et al.*, 1985) immunoprecipitated by the same antibody. Whole cell lysates were prepared by either 1) direct solubilization of monolayer cultures of rat embryo fibroblasts in 2% sodium dodecyl sulfate (SDS) in electrophoresis cocktail containing dithioerythritol or 2) with cell-matrix buffer (1% Tween 20, 4 M urea, 25 mM tris(hydroxymethyl)aminomethane [Tris]HCl pH 7.5 containing protease inhibitors 10 mM N-ethyl maleimide [NEM], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM benzamidine HCl, 0.1 M ϵ-amino-caproic acid, and 20 mM EDTA) followed by dialysis to heparinase buffer (0.1 M Na acetate pH 7.0, 0.1 mM Ca acetate, 0.1% Tween 20) for enzyme susceptibility studies. Total cell proteoglycans were extracted by scraping $^{35}SO_4$-labeled rat or human embryo fibroblast monolayers into cell-matrix buffer. After dilution with buffer lacking Tween 20 to reduce the detergent concentration to 0.1%, total proteoglycans were isolated under dissociative conditions as previously (Woods *et al.*, 1985) on DEAE-Sephacel (Pharmacia Biotech, Piscataway, NJ) except that 0.1% Tween 20 was used as detergent throughout. The column was washed sequentially with 0.2 M NaCl, 4 M urea in Tris-HCl pH 8.0, and 0.2 M NaCl, 4 M urea in 50 mM Na acetate pH 4.0 as previously (Woods *et al.*, 1985). Both of these contained protease inhibitors 20 mM EDTA, 10 mM NEM, and 0.2 mM PMSF. A final wash (10 column volumes) with 0.2 M NaCl without urea or protease inhibitors was performed before elution, because these interfere with ethanol precipitation of the proteoglycans. Bound proteoglycans were eluted with 4 M GuHCl, 0.1% Tween 20, 50 mM Na acetate pH 4.0 without protease inhibitors. Preparations from human fibroblasts to be used for immunoprecipitation with anti-syndecan 4 peptide antibodies followed by immunoblotting with monoclonal 8G3 anti-human syndecan 4 (David *et al.*, 1992) were subjected to two sequences of this dissociative isolation procedure.

Samples for Western blotting were precipitated with 4 vol ethanol at −20°C for up to 72 h, followed by solubilization in heparinase buffer. Equal volumes (15 μl) were either undigested or treated with 0.5 mIU heparinase III (EC 4.2.2.8, Seikagaku America, Rockville, MD), ±1 U heparinase II (Sigma Chemical), 5 mU chondroitinase ABC (protease free, EC 4.2.2.4, Seikagaku America), or all three enzymes together. Ovomucoid (50 μg/ml) from Sigma Chemical was added as protease inhibitor, and the samples were digested for 16 h at 37°C before addition of SDS-containing cocktail (Couchman, 1987) with dithioerythritol. Preparations were heated to 100°C for 2 min, and 0.4 mg iodoacetamide was added to each sample after cooling. Separation of proteoglycan or cell layer preparations was on 3–15% gradient SDS polyacrylamide gel electrophoresis (PAGE) minigels, followed by electrophoretic transfer at 50 V for 2 h to nitrocellulose. Membranes were blocked for 2 h at room temperature in 1% dried milk in PBS^- (PBS without calcium or magnesium) (Woods *et al.*, 1993) or Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Tris-HCl pH 7.5), both containing 0.02% azide. They were then incubated (16 h, 4°C) with primary antibodies in the same buffer containing 1% dried milk, 0.1% BSA, and 0.1% Tween 20 followed by washing and incubation (1 h, room temperature) with affinity-purified goat anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase diluted 1:2000.

Color development was according to manufacturer's protocol. Primary antibodies were affinity-purified anti-syndecan 4 (50 μg/ml), rabbit antiserum against rat liver HSPG (1:100 dilution) (Pierce *et al.*, 1992), rabbit antiserum against β_1 (a kind gift from Dr. S. Johansson, University of Uppsala, Sweden, 1:100 dilution), monoclonal anti-vinculin (1:400) (Sigma Chemical, Clone VIN-11-5), monoclonal anti-paxillin (5 μg/ml) (Zymed Laboratories, San Francisco, CA), and rabbit anti-rat plasma fibronectin (1:250 dilution). Minor differences in protocol were adopted to increase sensitivity when monoclonal anti-syndecan 4 (m8G3) (David *et al.*, 1992) was used. Membranes were blocked for 1 h in PBS^-, 1% milk, and 0.02% Na azide, incubated with 20 μg/ml 8G3 in PBS^-, 0.02% Na azide, and 0.1% Tween 20 for 1 h at 37°C and a further 2 h room temperature, washed extensively, incubated for 1 h at room temperature with goat-anti-mouse peroxidase (1:8000 in the same buffer), and washed and developed for chemiluminescence (ECL, Amersham) as per manufacturer's instructions.

For immunoprecipitation, $^{35}SO_4$-labeled total proteoglycans from rat or human fibroblasts were dialyzed to PBS^-, 0.1% Tween 20 and precleared by incubation with Protein A-Sepharose beads (Pharmacia Biotech) in the same buffer for 90 min before centrifugation and removal of supernatant. Affinity-purified antibodies (100 μg) against syndecan 4 were similarly incubated with Protein A-Sepharose beads, and the beads were washed 10 times by centrifugation and resuspension. Human proteoglycan preparations were also precleared as above with inactivated affinity-purified anti-syndecan 4 peptide antibodies. Precleared proteoglycan preparations were incubated for a further 90 min with 100 μg/100 μl Protein-A Sepharose slurry of affinity-purified anti-syndecan 4 peptide antibodies. The beads were washed and sedimented until no further radiolabel was removed (6–8 washes), then eluted in 100 μl 4 M GuHCl and 50 mM Na acetate pH 4.0 containing 1 mM mercaptoethanol, and dialyzed to appropriate buffer for enzyme treatments. In experiments to blot human material immunoprecipitated with polyclonal anti-syndecan 4 peptide antibodies with m8G3 anti-syndecan 4, 5 μg ovomucoid was added to the eluate before microdialysis over 12 h to heparinase buffer. The sample was divided into two equal aliquots, one of which was treated with 1 mIU heparinase III for 4 h at 37°C, before concentration and preparation for SDS-PAGE as before. In other experiments, proteoglycan preparations from rat cells were incubated (16 h, 4°C) with polyclonal affinity-purified antibodies to syndecan 4 that had been bound to Protein A-Sepharose and crosslinked by dimethyl pimelimidate, washed with binding buffer until no further radiolabel was removed, eluted with elution buffer, and dialyzed. Some samples were treated with chondroitinase ABC (0.1 U, 16 h, 37°C) as previously (Woods *et al.*, 1985) to degrade chondroitin and dermatan sulfate proteoglycans. Others were treated (16 h, 37°C) with heparinase II (1 U) and III (0.5 mIU) in heparinase buffer (0.1 M Na acetate, 0.1 mM Ca acetate pH 7.0, 0.1% Tween 20) to degrade heparan sulfate glycosaminoglycans. Nitrous acid and alkaline borohydride treatments were as previously described (Couchman *et al.*, 1985; Woods *et al.*, 1985).

Analysis of $^{35}SO_4$-labeled proteoglycans was by chromatography on 1 × 100-cm columns of Sepharose CL-4B run at 6 ml/h in 4 M GuHCl, 0.1% Tween 20, 50 mM Na acetate pH 4.0, or on 20-ml columns of Sephadex G-25 run in PBS^- containing 0.02 M Na azide and 0.1% Tween 20. V_o and V_t were determined by blue dextran and DNP-alanine respectively.

Cells and Labeling Procedures

The primary strains of rat and human embryonic fibroblasts have been previously described (Badley *et al.*, 1981; Woods *et al.*, 1986). Other cells used were rat aortic smooth muscle and porcine endothelial cells (both kind gifts from Dr. P. Bounelis, U.A.B.) and retinal pigmented epithelial cells (RPE) derived from fresh porcine retinas by trypsinization. All cells were grown in Alpha Minimum Essential Medium (MEM) containing fetal bovine serum (Intergen, Purchase, NY) at 5% (fibroblasts and smooth muscle cells) or 10% (endothelial and RPE cells). Cells were used at passages 2–6 from frozen stocks and shown to be free of mycoplasma contamination by staining with Hoechst 33257. In experiments monitoring adhesion to different substrates, cells were seeded in serum-free medium onto coated coverslips. Five micrograms of laminin from the Engelbreth-Holm-Swarm tumor (a gift from Dr. D.R. Abrahamson, U.A.B.), bovine vitronectin (prepared as in Yatohgo *et al.*, 1988), or Type I collagen (Vitrogen 100, Celtrix Laboratories, Palo Alto, CA) were dried onto glass coverslips, or they were incubated with 100 μg/ml of bovine fibronectin for 1 h as previously (Woods and Couchman, 1992a). Substrates were rehydrated when needed, washed with MEM (3 × 10 min), incubated with 0.1% heat-denatured bovine serum albumin (30 min), and washed (3 × 10 min) with MEM before addition of cells. Cells were allowed to attach for 3 h before fixation.

Cultures were fixed for 5 min with 3.5% paraformaldehyde in PBS^- containing 0.1% Tween 20, washed with PBS^- (10 min), quenched with 0.1 M NH_4Cl (10 min), and washed with PBS^- (10 min) before addition of primary antibodies. Prolonged fixation (15–20 min) did not allow labeling with antibodies against syndecan 4. Cultures were incubated at 37°C for 45 min with polyclonal antiserum (diluted 1:20) or affinity-purified antibodies (50 μg/ml) against syndecan 4, rat liver HSPG (1:20) (Pierce *et al.*, 1992), or monoclonal antibodies against vinculin (1:50) (Sigma Chemical), integrin β_1 (1:5) (Adeza Biomedical, Sunnyvale, CA), or integrin β_3 (1:100, a kind gift from Dr. P.J. Newman, The Blood Center of Southeastern Wisconsin, Milwaukee, WI) (Newman *et al.*, 1985). Samples were washed (3 × 15 min) with PBS^-, incubated with secondary antibodies (45 min, 37°C), and washed (3 × 15 min) with PBS^- before inspection. Secondary antibodies were 1:50 dilutions of $F(ab')_2$ fragments of goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC) (single labeling) or tetramethylrhodamine isothiocyanate (TRITC) (double labeling) and $F(ab')_2$ fragments of goat anti-mouse IgG conjugated to FITC (1:50 dilution, Cappel, Organon Teknika, West Chester, PA). In double immunofluorescent labeling, both primary antibodies were added together, as were secondary antibodies. No difference in labeling was noted when single labeled samples were compared to double labeled. Controls where both second antibodies were added after only one primary antibody showed only appropriate labeling, and no breakthrough of label from one fluorochrome wavelength to the other was seen. Cells were viewed on a Nikon Optiphot microscope (Garden City, NY) and photographed with Ilford HP5 film (Basildon, UK).

RESULTS

Activity of Antibodies Against the Syndecan 4 Cytoplasmic Domain

Antibodies raised against the synthetic peptide (C)LG-KKPIYKK recognized a single species of HSPG core protein in immunoblotting of cell layer proteoglycan preparations or whole cell lysates (Figure 1). The core protein had Mr = 44 k and could be visualized after treatment of rat proteoglycan preparations with heparinase III alone (Figure 1A, lane 2). Treatment of whole cell lysate with a combination of heparinase II and III (Figure 1B, lane 3) did not reveal any additional core proteins, nor any other cellular components reactive to the antibody, but led to the appearance of a nonspecific band (marked by a triangle in Figure 1B) present in the heparinase II and seen whenever this enzyme was present (lanes 1, 3, 4, 7) even in the absence of cellular material (lane 1). No core protein was seen after treatment with chondroitinase ABC (lane 3 in Figure 1A and 5 in B), and this treatment did not change the Mr seen after heparinase treatment (compare lanes 3 and 4 in Figure 1B). Although cDNA predicts a core protein size of 19.5 kDa, syndecan 4 core protein runs anomalously on SDS-PAGE (David *et al.*, 1992; Kojima *et al.*, 1992b)

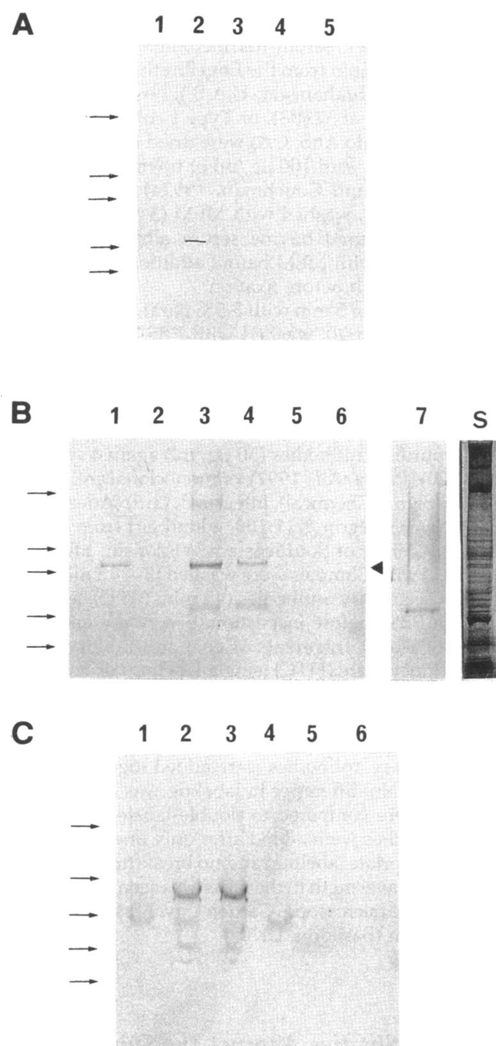

Figure 1. Immunoblotting characterization of anti-syndecan 4. Rat embryo fibroblast total proteoglycan (A and C) or whole cell lysate (B) preparations were probed with affinity-purified antibodies against (C)LGKKPIYKK (A and B) or antiserum against rat liver proteoglycans (C). (A) A single immunoreactive band is seen only after digestion of intact proteoglycan (lane 1) with heparinase III (lane 2) but not with chondroitinase ABC (lane 3). Heparinase III (lane 4) or chondroitinase ABC (lane 5) enzymes alone do not react with the antibody. (B) Additional treatment with heparinase II does not increase specific labeling. Heparinase II and III alone (lane 1) show nonspecific labeling (▲), which is present whenever heparinase II is used (lanes 3 and 4). No bands are visible in undigested lysates (lane 2) except at higher loading (lane 7). Treatment of lysate with heparinase II and III (lane 3), these enzymes plus chondroitinase ABC (lane 4), but not chondroitinase ABC alone (lane 5), reveals a single specific band. Lane 6 is chondroitinase ABC in the absence of lysate. S represents lysate stained with silver. (C) Antiserum against rat liver proteoglycans detects material in undigested proteoglycans (lane 1) with specific bands appearing after treatment with heparinase III (lane 2), heparinase III and chondroitinase ABC (lane 3), but not chondroitinase ABC alone (lane 4). Heparinase III and chondroitinase ABC enzymes alone are shown in lanes 5 and 6, respectively. The migration of molecular mass standards are shown by arrows and represent Mr = 215.5k, 105.1k, 69.8k, 43.3k, and 28.3k.

as do the other syndecans (Bernfield *et al.*, 1992). Intact syndecan 4 transfers poorly, even to Z-probe (David *et al.*, 1992) but sufficient transfer to nitrocellulose was achieved to detect a smear typical of proteoglycans on heavier loading (Figure 1B, lane 7). A polypeptide of the appropriate mass of syndecan 4 core protein was also recognized by anti-peptide antibody in undigested, reduced samples (lane 7, Figure 1B). This has also been previously reported (David *et al.*, 1992) and may represent unglycosylated core protein or a related polypeptide. This was not seen in proteoglycan preparations (Figure 1A, lane 1). In whole cell lysates, heparinase II and III treatment also yielded small amounts of higher mass polypeptides, possibly representing polymers of core protein (lanes 3 and 4, Figure 1B). Again, it has been previously noted that cell surface HSPGs readily oligomerize (David *et al.*, 1992; Kojima *et al.*, 1992a).

Antiserum against rat liver cell surface HSPG, which was used previously to isolate cDNA for the sequence of rat syndecan 2, detected three major core proteins after treatment of rat embryo fibroblast proteoglycans with heparinase III, of approximate Mr = 80, 49, and 44k (Figure 1C, lane 2). This pattern is nearly identical to that reported previously for this antiserum on rat liver preparations (Pierce *et al.*, 1992). After the previous interpretation of this data, it is probable that the 49 kDa protein represents syndecan 2 core protein (fibroglycan), and the 80-kDa protein may be syndecan 1. However, the HSPG of core protein 80 kDa is not a hybrid containing chondroitin sulfate chains (compare lane 3 that has additional treatment with chondroitinase ABC to lane 2), as syndecan 1 usually is. This proteoglycan is, furthermore, abundant in rat fibroblasts, whereas syndecan 1 is normally low in amount (Lories *et al.*, 1989, 1992) and, therefore, may represent a higher mass form of syndecan 2, as seen previously (Lories *et al.*, 1989). The identity of the 44-kDa protein core is unknown but may represent syndecan 4. The polypeptide of 68 kDa in each lane of proteoglycan material is possibly nonspecific.

Although the affinity-purified antibody against syndecan 4 peptide did recognize an appropriate HSPG and its core protein, we also showed (Figure 2) that it would not recognize other cellular components, even when heavily loaded (lane 7, Figure 1B) or at sufficient levels for other low level focal adhesion components to be detectable (Figure 2A). Cell lysates were probed with affinity-purified anti-syndecan 4 peptide (lane 1) or antiserum against rat liver proteoglycans (lane 2), and with a number of antibodies against known focal adhesion-associated components such as vinculin, paxillin, β_1 integrins, and fibronectin (lanes 3–6). These latter components could be readily detected by specific antibodies but were not recognized by the syndecan 4 peptide antibodies, which reacted only weakly with material at the interface between the stacking and resolving gels. This probably includes the undigested HSPGs.

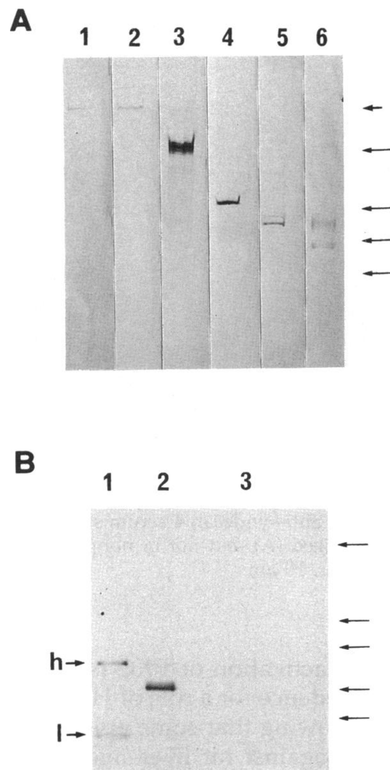

Figure 2. Affinity-purified antibodies against syndecan 4 peptide do not react with other proteins in cell lysates (A), but immunoprecipitate proteoglycans detectable by m8G3. (A) Material in rat cell lysate detectable by antibodies against syndecan 4 peptide (lane 1) or rat liver proteoglycan (lane 2) is seen only at the stacking/resolving gel interface (short arrow), whereas fibronectin (lane 3), vinculin (lane 4), $\beta1$ integrin subunit (lane 5), and paxillin (lane 6) can be readily detected. (B) Material immunoprecipitated by polyclonal anti-syndecan 4 peptide from human proteoglycan preparations and immunoblotted with monoclonal anti-human recombinant syndecan 4 shows only heavy and light chain from precipitating antibodies in undigested samples (lane 1), but a core protein after treatment with heparinase III. Enzyme alone is shown in lane 3. Molecular mass markers of Mr = 215.5k, 105.1k, 69.8k, 43.3k (and 28.3k in B) are denoted by arrows.

To confirm that the band of Mr = 44k detected by the anti-peptide antibody was indeed syndecan 4, we immunoprecipitated human embryo fibroblast proteoglycans from a preparation made under dissociative conditions (see MATERIALS AND METHODS) and subjected this to immunoblotting with monoclonal antibody m8G3. This monoclonal antibody was made against a fusion protein containing sequence from the ectodomain of human syndecan 4 and reacts with syndecan 4 on immunoblotting of human lung fibroblast cell surface proteoglycans (David *et al.*, 1992). In the absence of treatment with heparinase III (Figure 2B, lane 1), only two faint bands corresponding to heavy and light chains of the rabbit polyclonal anti-peptide antibodies used to immunoprecipitate the proteoglycan are visible. After heparinase III treatment, an additional band of Mr = 44k is evident (lane 2). This is not seen when enzyme alone is immunoblotted (lane 3).

As a further characterization of the polyclonal anti-syndecan 4 peptide antibodies, $^{35}SO_4$-labeled rat HSPGs were immunoprecipitated with affinity-purified antibodies. Analysis by Sepharose CL-4B chromatography of total proteoglycans before immunoprecipitation indicated the presence of several species (Figure 3A). After treatment with chondroitinase ABC, chondroitin sulfate disacharides eluted in the V_t, and the remaining radiolabel was polydisperse with a major peak at K_{av} = 0.4 and a shoulder at K_{av} = 0.29 (Figure 3A). After immunoprecipitation of total proteoglycans (Figure 3B) or affinity-purification of proteoglycans on a column of rabbit anti-syndecan 4 conjugated to Protein A-Sepharose, some aggregation of material was seen, resulting in material eluting near the V_o of the Sepharose CL-4B column. Aggregation of hydrophobic HSPGs resulting in elution near the V_o has been previously reported (Woods *et al.*, 1985; Kojima *et al.*, 1992a). The major peak, however, eluted with a K_{av} of 0.28, which is identical to the previously reported value for syndecan 4 (David *et al.*, 1992). The percentage of total $^{35}SO_4$-labeled material that was immunoprecipitated with the affinity-purified anti-syndecan 4 was ~3%. This was completely susceptible to treatment with heparinase II

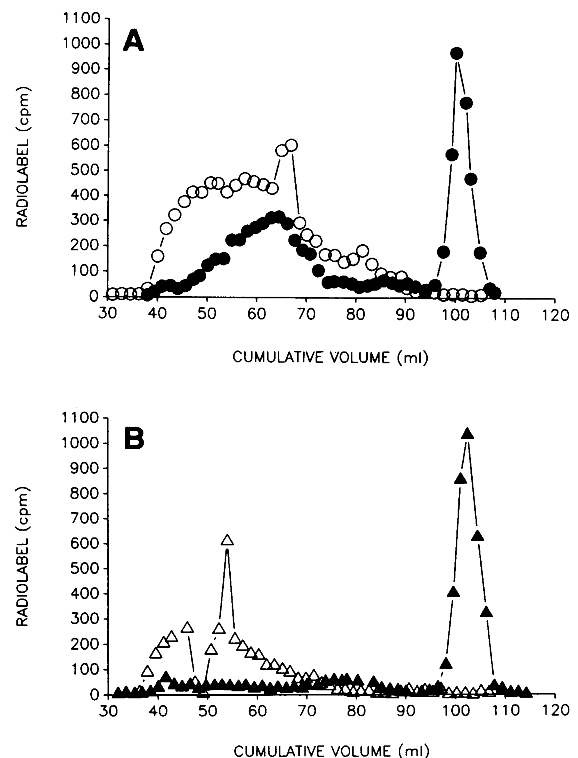

Figure 3. Chromatographic analysis of proteoglycans immunopurified by anti-syndecan 4. (A) Sepharose CL-4B profile of total $^{35}SO_4$-labeled proteoglycans before (○) or after (●) treatment with chondroitinase ABC. (B) Sepharose CL-4B profile of proteoglycans specifically immunoprecipitated with anti-syndecan 4 before (△) and after (▲) treatment with heparinase II and III. V_o = 38.0 ml, V_T = 101.5 ml.

and III, which resulted in radiolabel eluting near the V_t of the Sepharose CL-4B column (Figure 3B). When proteoglycans specifically purified on a column of rabbit anti-syndecan 4 conjugated to Protein A-Sepharose were analyzed by chromatography on Sephadex G-25, untreated material, and that treated with chondroitinase ABC, eluted in the V_o. No radiolabel was found in the V_t after chondroitinase treatment, whereas radiolabel was included in the column after treatment with nitrous acid. Heparan sulfate chains released from anti-syndecan 4 purified proteoglycans by alkaline borohydride treatment eluted with a K_{av} = 0.56 on a column of Sepharose CL-4B, similar to that reported for syndecan 4 (David *et al.*, 1992).

Localization of Syndecan 4 in Focal Adhesions

When cultures of rat embryo fibroblasts were labeled with antiserum against the syndecan 4 cytoplasmic peptide, focal adhesion-like structures were visible only after permeabilization of the cells (Figure 4, A and B). Labeling resembling focal adhesions was also seen in RPE cells (Figure 5A), human embryo fibroblasts (Figure 5C), rat smooth muscle cells (Figure 5E), and porcine endothelial cells (Figure 5F) using affinity-purified anti-syndecan 4. In contrast, antiserum against rat liver HSPGs (predominantly syndecan 2) (Pierce *et al.*, 1992) gave general membrane labeling of unpermeabilized cells. Labeling was enhanced by permeabilization, which also allowed occasional weak staining of focal adhesion-like plaques (Figure 5G).

Double labeling with vinculin antibodies was performed to confirm the distribution of syndecan 4 in focal adhesions. Codistribution of label was noted in all cell types, shown for RPE cells (Figure 5, A and B) and human embryo fibroblasts (Figure 5, C and D). The localization of syndecan 4 to focal adhesions was noted in normally grown fibroblasts (Figures 4–6, A and B), and those spread on fibronectin, laminin, vitronectin (Figure 6, C–E), or type I collagen, indicative of the presence of syndecan 4 in focal adhesions irrespective of the integrin species used. Indeed, double labeling of syndecan 4 (Figure 6, A, C, and E) with monoclonal anti-integrin β_1 (Figure 6B) in normally grown cells or with anti-integrin β_3 (Figure 6D) in cells on vitronectin substrates, confirmed some colocalization of syndecan 4 with both of these integrin subunits. Previous studies have indicated that either integrin β_1 or β_3 can be present in focal adhesions of cells when normally grown or seeded on vitronectin (Fath *et al.*, 1989). Interestingly, when normally grown cells were labeled for integrin β_1, some focal adhesions were negative for the integrin but positive for the presence of syndecan 4 (arrows in Figure 6, A and B). This was especially obvious in cells adherent to vitronectin substrates and labeled for integrin β_1 and syndecan 4 (Figure 6, E and F).

DISCUSSION

Many previous studies have indicated that cell surface HSPG(s) may be involved in focal adhesion formation, possibly through activation of PKC (see INTRODUCTION). Strong evidence for a role of HSPGs came from an early study showing that some epitopes recognized by an antiserum against rat liver microsomal HSPGs were concentrated in focal adhesions (Woods *et al.*, 1984). It was not possible at that time to identify which HSPG was present in adhesions, but the advent of sequence data for individual HSPG core proteins has now allowed us to generate specific probes for individual HSPGs. These show that syndecan 4 HSPG is selectively present and concentrated in focal adhesions.

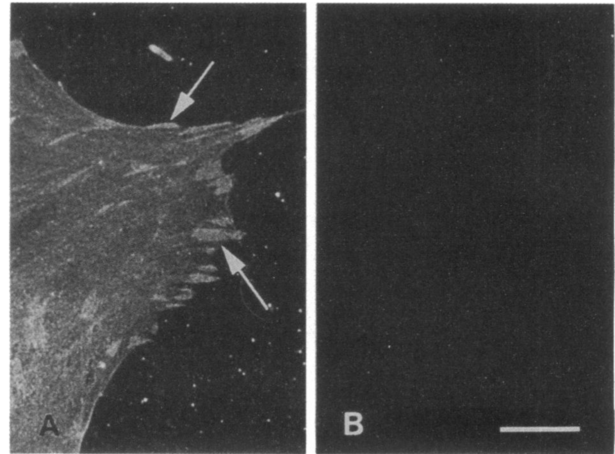

Figure 4. Syndecan 4 is present in focal adhesions. Immunofluorescent labeling with anti-syndecan 4 serum stains focal adhesions (arrows) in permeabilized (A), but not in nonpermeabilized (B) rat embryo fibroblasts. Bar, 10 μm.

Antibodies raised against a synthetic peptide with a sequence unique to the cytoplasmic tail of syndecan 4 specifically recognized a core protein of Mr = 44k after treatment with heparinase II and III, or after heparinase III alone in whole lysates and total proteoglycan preparations. This is similar to previous studies where syndecan 4 of endothelial and fibroblast cells (termed ryudocan [Kojima *et al.*, 1992a,b] and amphiglycan [David *et al.*, 1992] respectively) showed core protein size of approximately twice that predicted from cDNA data. The antibodies did not appear to recognize syndecan 2, which was visualized with an antiserum against rat liver HSPG (Pierce *et al.*, 1992). It also did not appear to recognize other cellular components, even those likely to be present in low amounts such as focal adhesion components, namely integrin β_1, vinculin, paxillin, or fibronectin, which could be immunoblotted in the same preparation. Affinity-purified rabbit antibodies against the cytoplasmic syndecan 4 peptide immunoprecipitated a single heparinase-sensitive HSPG, with a core protein of Mr = 44k that was detected by immunoblotting with a monoclonal antibody raised against recombinant syndecan 4 ectodomain. Furthermore, immunopurification of total proteoglycans with affinity-purified antibodies against the syndecan 4 peptide resulted in an HSPG

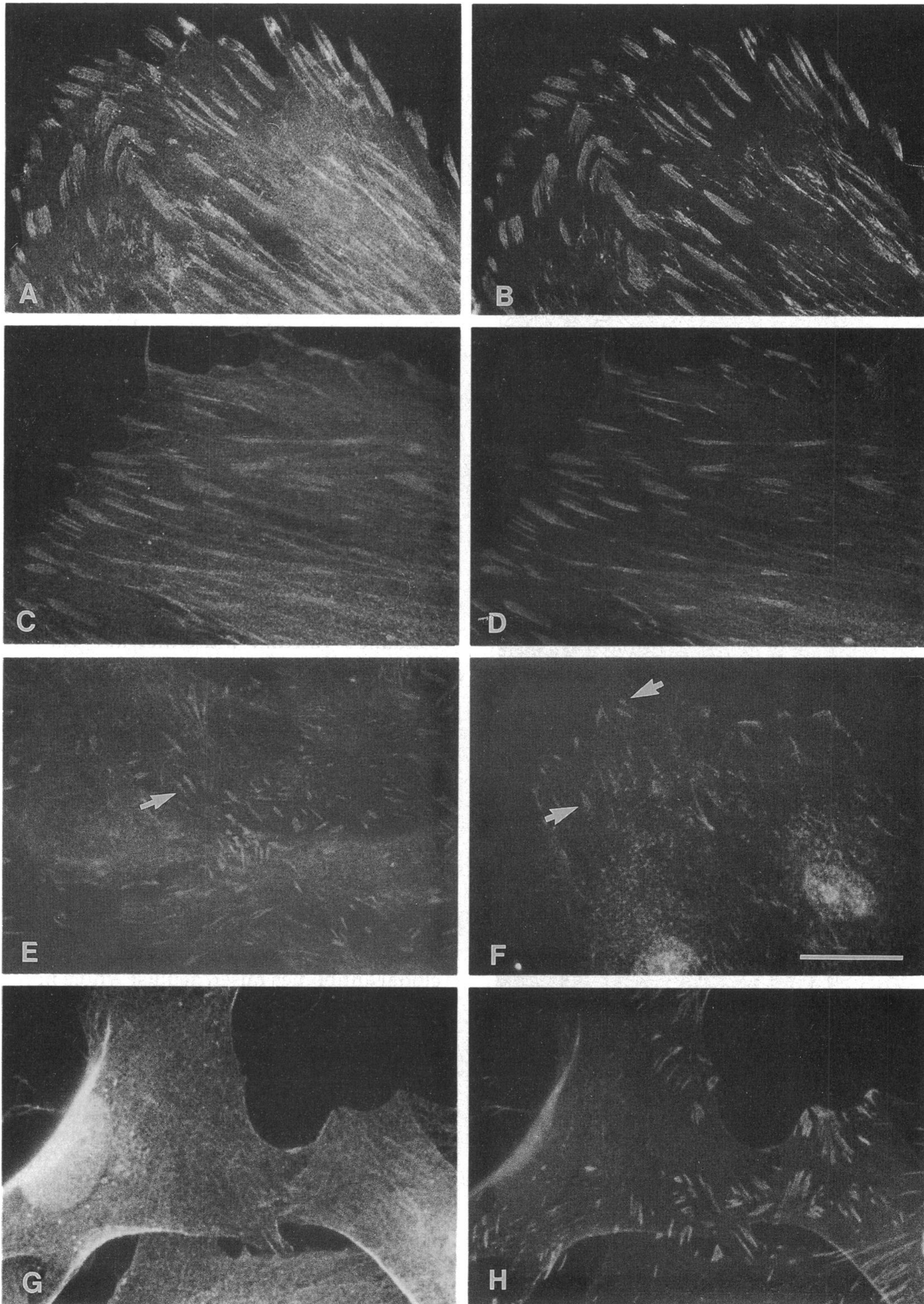

Figure 5. Syndecan 4, but not syndecan 2, codistributes with vinculin in focal adhesions. Affinity-purified anti-syndecan 4 stains focal adhesions in porcine RPE cells (A), human embryo fibroblasts (C), rat aortic smooth muscle cells (E), and porcine vascular endothelial cells (F). Double labeling with anti-vinculin confirms colocalization in RPE (B) and human embryo fibroblasts (D). Antibodies against liver HSPGs labels the membrane of rat embryo fibroblasts generally (G), but not focal adhesions that label with anti-vinculin (H). Bar, 10 μm.

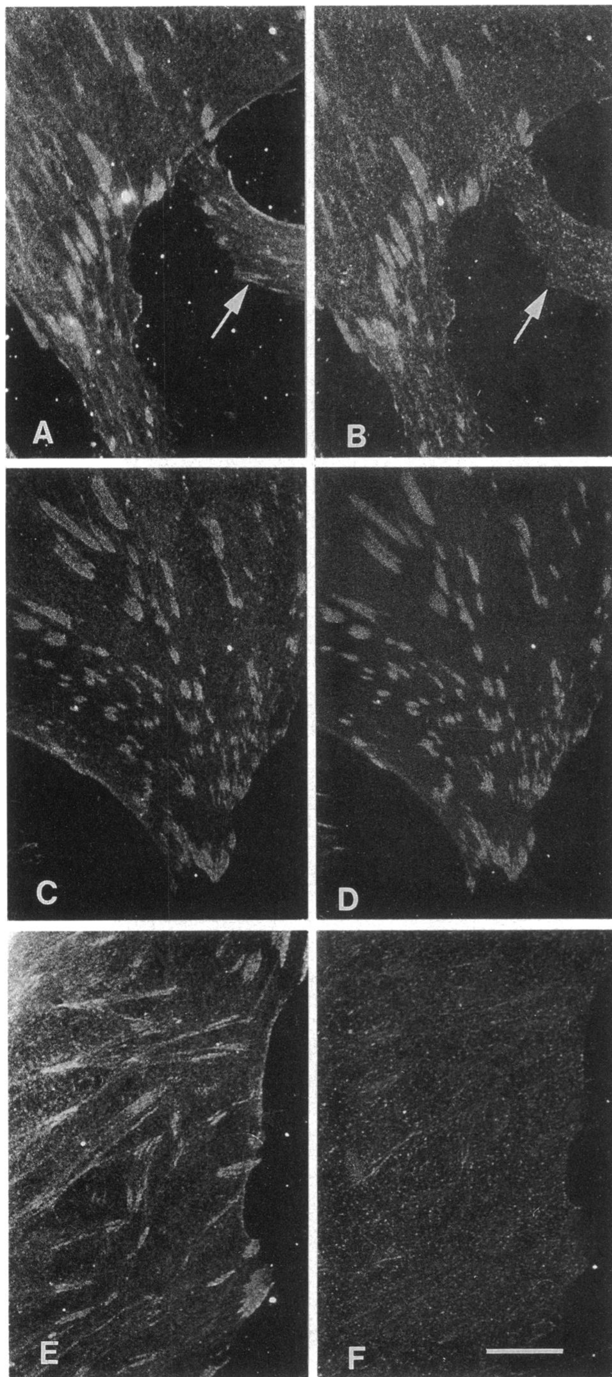

Figure 6. Syndecan 4 codistributes with integrin β_1 and β_3 in focal adhesions. Anti-syndecan 4 (A) labels focal adhesions in normally grown cells (A and B), and some of these adhesions, but not all (arrows), colabel with antibodies against integrin β_1 subunits (B). Anti-syndecan 4 (C and E) labels focal adhesions in cells spread on vitronectin (C–F). These colabel with antibodies against integrin β_3 (D) but not integrin β_1 (F) subunits. Bar, 10 μm.

that eluted on Sepharose CL-4B with the characteristics of amphiglycan (David *et al.*, 1992). It was completely susceptible to heparinase II and III, and the glycosaminoglycan chains had an approximate Mr = 50k. A marked tendency of immunoprecipitated proteoglycan to aggregate was noted. This may contribute to the anomalous behavior of the core protein on SDS-PAGE (David *et al.*, 1992; Kojima *et al.*, 1992b). Further analysis of anti-syndecan 4 affinity-purified material by Sephadex G25 chromatography indicated that it did not contain chondroitin sulfate but was susceptible to nitrous acid. This is consistent with the Western blotting data. Therefore, the antiserum raised against a synthetic peptide from syndecan 4 core protein cytoplasmic sequence, appears specific for, and has the capacity to recognize, deglycosylated and whole syndecan 4.

Rabbit polyclonal antibodies against syndecan 4, but not those against syndecan 2, labeled focal adhesions in a range of cell types. This is consistent with the high degree of conservation of cytoplasmic domains of syndecans across mammalian species (Bernfield *et al.*, 1992). Labeling only occurred if cells were permeabilized, confirming the intracellular nature of the epitope. Double labeling experiments confirmed that these adhesions contained vinculin and either integrin β_1 or β_3. Focal adhesions contained syndecan 4 in normally grown cells (i.e., in serum-containing medium), presumably adherent to vitronectin or fibronectin (Fath *et al.*, 1989), and in those plated on substrates of laminin, fibronectin, vitronectin, and type I collagen in serum-free medium.

In contrast, antibodies against rat liver HSPGs, mostly syndecan 2, labeled cell membranes in a general, rather uniform manner, with occasional focal adhesion labeling in permeabilized cells. The cytoplasmic domain of syndecan 4 shows some sequence homology to other members of the syndecan family. Indeed, syndecan 4 is most closely homologous to syndecan 2. Thus, polyclonal antibodies raised against whole fibroglycan (syndecan 2), as was the case with the antibodies against rat liver HSPG, may have some limited reactivity to cytoplasmic sequences common to more than one syndecan. The ability of the antiserum raised against rat liver cell surface HSPGs to recognize three different polypeptides after heparinase treatment was seen with rat embryo fibroblasts here, and rat liver previously (Pierce *et al.*, 1992). One of these (Mr = 44k) could be syndecan 4 by virtue of mass and regions of sequence homology to syndecan 2. All this would explain the occasional, but weak, detection of focal adhesions by this antiserum, in addition to generalized membrane staining. The striking feature of staining with polyclonal syndecan 4-specific antibodies was the specificity of focal adhesion labeling. However, David *et al.* (1992), using the monoclonal 8G3 antibody generated against a syndecan 4 fusion protein did not report focal adhesion labeling in human skin fibroblasts, a finding with which we concur. It may be that antibodies against extracellular

domains of the proteoglycan cannot easily penetrate the focal adhesions (Woods and Couchman, 1988).

The role of HSPG in adhesion formation remains to be elucidated. Studies (Woods *et al.*, 1986; 1992a,b; Burridge *et al.*, 1992) have indicated that two signals are needed for complete adhesion of primary fibroblasts to fibronectin. One, through interaction of cells with the RGD-containing cell-binding domain, appears to trigger tyrosine phosphorylation of specific focal adhesion components including $pp125^{FAK}$ and paxillin (Burridge *et al.*, 1992; Kornberg *et al.*, 1992; reviewed in Hynes, 1992) and is sufficient for attachment and spreading, but not focal adhesion formation (Burridge *et al.*, 1992). The second signal, which stimulates focal adhesion formation in cells prespread on substrates coated with cell-binding fragments of fibronectin, occurs on the addition of heparin-binding fibronectin fragments (Woods *et al.*, 1986, 1992a,b) or of a heparin-binding synthetic peptide with the sequence WQP-PRARI from this domain (Woods *et al.*, 1993). This second signal may be through activation of PKC because inhibitors of this kinase prevent focal adhesion formation, and direct activation of PKC with phorbol esters can circumvent the need for heparin-binding moieties (Woods and Couchman, 1992a,b). Cell surface HSPGs appear to transduce the second signal because treatment with heparinase II and III can prevent the induction of focal adhesion formation by whole heparin-binding 31-kDa fibronectin fragment or synthetic peptide (Woods *et al.*, 1993). Because of its ubiquitous localization in focal adhesions as shown here, syndecan 4 may well be the receptor that transmits this second signal.

The formation of multimolecular complexes to convert initial low affinity interactions into high avidity complexes is increasingly understood to be a common feature of cell surface receptor activity. This appears to be case in cell adhesion and in lymphocyte activation (see e.g., reviews by Damsky and Werb, 1992; Hynes, 1992; Tanaka *et al.*, 1993). In cell-matrix interactions, low affinity integrin interactions may lead to adhesion for migration, which correlates with a lack of focal adhesions (Couchman and Rees, 1979). In contrast, additional ligation of cell surface HSPG with heparin-binding moieties may convert cells to a state with very stable focal adhesions and stress fibers, characteristic of stationary cells entering the growth cycle (Couchman and Rees, 1979). In this respect, syndecan 4 in focal adhesions may be another example of HSPGs acting as "coreceptors" or "presenter" molecules. It is now increasingly clear that HSPGs can bind a component such as growth factor or cytokine and present it to its ligand with higher affinity (reviewed in Kjellén and Lindahl, 1991; Yayon *et al.*, 1991; Bernfield *et al.*, 1992; Tanaka *et al.*, 1993).

No evidence for direct interactions of proteoglycans with integrins has been reported, and it may well be that the role of syndecan 4 in focal adhesion formation is indirect through interaction of its core protein with the cytoskeleton. Indeed the cytoplasmic domain of syndecan 4, but not that of syndecans 1–3, has regions of homology with that of integrin β_{1A}, which also localizes to focal adhesions. These include NXXY common to both and NPIYKSA (β_{1A}) or KPIYKKA (syndecan 4). These regions have been implicated in the ability of integrin β_{1A} to be inserted into focal adhesions (Reszka *et al.*, 1992) and are missing in integrin β_{1B}, which is not inserted into these structures (Balzac *et al.*, 1993). The precise localization sequence in integrin β_{1A} is not, however, clear (Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990). PIYK was part of the sequence used to generate syndecan 4 antibodies, but these antibodies do not recognize integrin β_1 by immunoblotting, and some adhesions that label for syndecan 4 do not contain integrin β_1. The role of these sequences in the concentration of syndecan 4 in focal adhesions is currently under investigation. In addition, we are currently determining which cytoskeletal components bind to the core protein of syndecan 4, how this may change on binding heparin-binding fibronectin fragments to the extracellular domain of the molecule and how this correlates with a requirement for PKC activity.

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