Control of extracellular matrix assembly by syndecan-2 proteoglycan

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SUMMARY

Extracellular matrix (ECM) deposition and organization is maintained by transmembrane signaling and integrins play major roles. We now show that a second transmembrane component, syndecan-2 heparan sulfate proteoglycan, is pivotal in matrix assembly. Chinese Hamster Ovary (CHO) cells were stably transfected with full length (S2) or truncated syndecan-2 lacking the C-terminal 14 amino acids of the cytoplasmic domain (S2ΔS). No differences in the amount of matrix assembly were noted with S2 cells, but those expressing S2ΔS could not assemble laminin or fibronectin into a fibrillar matrix. The loss of matrix formation was not caused by a failure to synthesize or externalize ECM components as determined by metabolic labeling or due to differences in surface expression of α5 or β1 integrin. The matrix assembly defect was at the cell surface, since S2ΔS cells also lost the ability to rearrange laminin or fibronectin substrates into fibrils and to bind exogenous fibronectin. Transfection of activated α5β1 integrin into α5-deficient CHO B2 cells resulted in reestablishment of the previously lost fibronectin matrix. However, cotransfection of this cell line with S2ΔS could override the presence of activated integrins. These results suggest a regulatory role for syndecan-2 in matrix assembly, along with previously suggested roles for activated integrins.

Key words: Syndecan, Heparan sulfate proteoglycan, Laminin, Fibronectin, Integrin

INTRODUCTION

Regulation of ECM structure and composition is crucial to tissue integrity. However, the molecular processes that control deposition of ECM are still poorly understood. Many studies have concentrated on the mechanisms of insertion and organization of soluble fibronectin into a disulfide-bonded insoluble pericellular fibrillar network. This is a multistep process, which is under cellular control, leading to characteristic patterns of fibrillar arrays dependent on cell type (Bultmann et al., 1998; Christopher et al., 1997; Sechler et al., 1997 and references therein). When soluble fibronectin is added to cells, it first binds to cell surface sites in a deoxycholate-soluble and reversibly bound pool. Over time, this becomes a deoxycholate-insoluble fibrillar matrix multimerized via cell-mediated disulfide exchange (McKeown-Longo and Mosher, 1984; Chen and Mosher, 1996) or factor XIIIa-mediated cross-linking (Barry and Mosher, 1989). Endogenous fibronectin may be similarly polymerized, with initial deposition acting as a nucleus for fibrillogenesis, forming small aggregates that move centripetally over the cell surface, enlarging to form small fibrils and eventually larger fibers around the cells (Ljubimov and Vasiliev, 1982; Sechler et al., 1997).

The major integrin involved in the deposition of a fibrillar matrix in many cells is α5β1 (Akiyama et al., 1989; Fogerty et al., 1990; Wu et al., 1993), which binds to the RGD recognition site in fibronectin, leading to exposure of a fibronectin self-association site and growth of fibrils in vivo (Hocking et al., 1994; Sechler et al., 1997). Treatment of cells with antibodies against α5 inhibited matrix assembly (Akiyama et al., 1989; Fogerty et al., 1990), and overexpression or reduction of α5β1 in CHO cells resulted in increased fibronectin matrix assembly (Giancotti and Ruoslahti, 1990) or its loss (Schreiner et al., 1989), respectively. Matrix formation could be restored in CHO B2 cells that lack α5 integrin by transfection with α5 (Wu et al., 1993) or activated α5β3 (lacking the GFFKR region of α5β3; Wu et al., 1995b). Transfection of these cells with α5β1 also increased fibronectin deposition, possibly indirectly through increased deposition of entactin (Wu et al., 1995a).

Although the ‘cell-binding’ domain of fibronectin (particularly the III-10 module) is needed for fibrillogenesis, heparin-binding domains of the molecule are also required (Bultmann et al., 1998; Christopher et al., 1997; Hocking et al., 1994; McDonald et al., 1987; Schwarzbauer, 1991; Woods et al., 1988). Addition of the amino-terminal 70 kDa region of fibronectin, which contains both heparin- and collagen-binding activities, will block the binding and assembly of intact fibronectin to putative ‘matrix assembly sites’ (Dzamba et al., 1994). Dimeric recombinant fibronectin molecules containing this region are incorporated into matrix fibrils when added to cells (Sottile and Wiley, 1994). The 70 kDa binding activity appears to reside in type I repeats 1-5 (i.e. the heparin-binding


**MATERIALS AND METHODS**

**Supplies, cell culture, antibodies and matrix purification**

All general chemicals were from Sigma Chemical (St Louis, MO). Coverslips and cultureware were from Fisher (Alltanta, GA). CHO B2 cells, which are α5β1-deficient (Schreiner et al., 1989), and CHO B2 cells transfected with activated δα5γα2β1 which has the α subunit cytoplasmic membrane proximal sequence GFFKR deleted (O’Toole et al., 1994) were kind gifts from Dr C. Wu (UBA, Birmingham, AL) and Dr M. H. Ginsberg (The Scripps Research Institute, La Jolla, CA).

CHO-K1 (LeBaron et al., 1988) and CHO B2 cells were cultured in Ham’s F12 medium (Mediatech, Charlottesville, VA) containing 10% fetal calf serum (FCS, Intergen, Purchase, NY). Vectors pcDNA3 and pCDNA3.1Zeo were purchased from Invitrogen (San Diego, CA). Bovine plasma fibronectin and vitronectin were prepared as previously described (Miekka et al., 1982; Yatogho et al., 1988).

All antibodies were diluted in phosphate-buffered saline (PBS): (8.0 g NaCl, 0.2 g KCl, 0.2 g KH2PO4, 1.15 g Na2HPO4 per liter, pH 7.2). Rabbit polyclonal antibodies against laminin-1 from EHS-tumor and against bovine plasma fibronectin have been previously described (McCarthy et al., 1993). F-Actin was detected with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR) at a dilution of 1:1000. Paxillin antibodies (dilution 1:100) were purchased from Zymed (San Francisco, CA). The mouse monoclonal antibody against hamster α2 integrin, PB1 (Brown and Juliano, 1988), was a generous gift from Dr R. L. Juliano, University of North Carolina, Chapel Hill, NC. Monoclonal antibodies against integrin β1 subunits were isolated from culture supernatants of 7E2 hybridoma obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology at the University of Iowa, Iowa City, IA, under contract number N01-HD-2-3-3144 from NICHD. A polyclonal antibody against a glutathione-S-transferase (GST; Pharmacia Biotech, Pescataway, NJ) fusion protein containing the extracellular and transmembrane domains of rat syndecan-2 (designated S2ΔR; Fig. 1) was raised in a New Zealand White rabbit as previously described (Koff et al., 1991). The antiseraum was affinity purified for immunofluorescence experiments (Koff et al., 1992) and shown to be specific for syndecan-2 by ELISA, western blotting and immunostaining (data not shown). A chicken antibody against the same construct was made by AVES Laboratories, Inc. (Tigard, OR) and the purified IgY was used at 1:75 dilution. Primary antibodies were detected with F(ab')2 fragments of goat anti-mouse, anti-rabbit, or anti-chicken conjugated to fluorescein isothiocyanate (FITC) or Texas Red (Cappel, Durham, NC) diluted 1:50.

**DNA and plasmid construction**

Full-length cDNA constructs for wild-type and truncated syndecan-2 in pcDNA3 vectors (Invitrogen) were constructed in collaboration with Drs J. T. Gallagher and G. J. Cowling (Paterson Cancer Institute, Manchester, UK). Fig. 1 shows the cytoplasmic domain of syndecan-2 and its closest homologue, syndecan-4. The C1 and C2 homology regions, the V region unique to each syndecan and the sequences for the partial (S2AS, S4AM) and full (S2AR) truncations of the cytoplasmic domains are indicated. A mammalian vector was constructed by GC clamp polymerase chain reaction of rat liver syndecan-2 (clone 17; Pierce et al., 1992) in Bluescript vector (Stratagene, La Jolla, CA), digested with the appropriate restriction endonucleases, and ligated into pcDNA3 (Invitrogen) using the 5’EcoRI and 3’XbaI sites. The oligonucleotides used for PCR of the syndecan-2 cDNA were 5’-gggaaatctctctacattctacagg-3’ and 3’-gggtctagattaatacattgag-5’. Syndecan-2 cDNA encoding truncated cytoplasmic domains were constructed using the identical 5’ primers but the following modified 3’ primer containing a new stop codon (underlined) adjacent to the XbaI site of pcDNA3: 3’-gggtctagattaatacattgagcaccacagcag-5’ (S2AR: full truncation) and 3’-gggtctagattaatacattgagcaccacagcagcagcag-5’ for the truncation ending at serine197 (S2AS). A partially truncated syndecan-4 construct ending at isoleucine169 (designated S4AM) was generated as described (Longley et al., 1999). All mutated constructs were confirmed by sequencing. Syndecan-2 was also ligated into the pcDNA3 vector in the antisense orientation. A 5’ fragment of 150 bp was excised using the polylinker HindIII site and an internal HindIII site. Excision added a unique HindIII site to the 3’ end. This construct was religated into pcDNA3 vector cleaved with HindIII, and fragment orientation was confirmed using the BamHI restriction digests.

**activity), but its binding and incorporation into fibrils requires prior occupany of α5β1 by the ‘cell’-binding domain (Dzamba et al., 1994; Fogerty et al., 1990) confirming the primary role of this integrin.**

Cell surface proteoglycans can augment the interaction of cells with fibronectin by glycosaminoglycan binding to heparin-binding domains (reviewed by Carey, 1997; Woods and Couchman, 1998). Indeed, cells that lack glycosaminoglycan synthesis cannot adhere normally to fibronectin (LeBaron et al., 1988) nor establish a fibronectin matrix (Chung and Erickson, 1997). The four-member syndecan family of heparan sulfate proteoglycans are a second class of transmembrane receptors for ECM components (reviewed by Bernfield et al., 1992; Carey, 1997; Woods and Couchman, 1998). Syndecan-4 (David et al., 1992b; Kojima et al., 1992) may augment integrin-mediated cell adhesion to ECM and determine cytoskeletal organization (Longley et al., 1999; Woods and Couchman, 1998), which correlates with matrix formation (Ali and Hynes, 1977; Woods et al., 1988; Wu et al., 1996). Syndecan-4 may signal through protein kinase C (Baciuc and Goetinck, 1995; Oh et al., 1997a,b), and protein kinase C activity is also needed for matrix assembly (Somers and Mosher, 1993). However, any role for syndecans in matrix assembly has not been investigated.

Syndecan-4 is only a minor component in cells, including fibroblasts that produce ECM (Kim et al., 1994). The major syndecan in fibroblasts is syndecan-2 (David et al., 1992b, 1993), whose function has not been well investigated. Syndecan-2 core protein can be phosphorylated both in vitro by protein kinase C (Oh et al., 1997c; Prasthofer et al., 1995), and in vivo (Itano et al., 1996). The phosphorylation site(s) appear to be serine197 and serine198. All syndecans have highly conserved cytoplasmic domains, with constant regions proximal (C1) and distal (C2) to the membrane, separated by a region (V) unique to each family member (Bernfield et al., 1992; Woods and Couchman, 1998). In syndecan-4, the V region mediates protein kinase C binding and activation (Oh et al., 1997a,b), and may, therefore, be responsible for the role of this syndecan in integrin-dependent adhesion. In syndecan-2, the V region forms the phosphorylation site (Oh et al., 1997c). We, therefore, reasoned that this site in syndecan-2 may play a role in augmenting integrin-mediated matrix assembly. cDNA constructs encoding full length syndecan-2 core protein, partial cytoplasmic truncation (to serine197) or total cytoplasmic domain deletion, were expressed in CHO-K1 cells and effects on matrix assembly were monitored. We also used constructs encoding equivalent syndecan-4 core proteins and show that syndecan-4 specifically controls the assembly of both fibronectin and laminin matrices.
S2: RMRKKDEGSYDL GERKPKSAAYQ KAPTKEFYA C1 V C2
S2A5: RMRKKDEGSYDL GERKPS
S2AR: R
S4: RMRKKDEGSYDL GKKPYKMK APTNEFYA
S4A1: RMRKKDEGSYDL GKKPI

Fig. 1. Cytoplasmic domain amino acid sequences of wild-type and truncated syndecan-2. All syndecan cytoplasmic domains have high homology in membrane proximal and distal regions (C1 and C2) with a unique variable (V) region in the center. EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain; S2 syndecan-2; S4, syndecan-4.

Transfections and selection for high expressors

CHO-K1 cells were transfected either with empty pcDNA3 vector (designated pcDNA3) or pcDNA3 vector containing wild-type syndecan-2 (S2), the partially (S2AS) or fully (S2AR) truncated syndecan-2, antisense syndecan-2 (S2A), or truncated syndecan-4 (S4A1). 7.5 μg plasmid cDNA prepared by miniprep were mixed with 15 μg lipofectamine (Life Technologies, Gaithersburg, MD) in 2.5 ml of serum-free medium and incubated with CHO-K1 cells as per suppliers instructions. Several independent stable transfecants for each construct were isolated over three passages in the presence of G418 (Life Technologies) at 500 μg/ml active gentamycin and gave identical results. Expression levels decreased with passage, so all experiments except Fig. 9 were performed at passages 3-6 from initial transfection or fluorescence activated cell sorting (FACS) selection (see below). CHO B2 cells were transfected with the S2AS construct using the pcDNA3 vector and selected with G418. Since FmBH or FmB-transfected CHO B2 were already G418 selected, the S2AS construct was cloned into pcDNA 3.1 vector containing a Zeocin resistance cassette using EcoRI- and XbaI-restriction sites.

Transfected cell lines showed phenotypic drift over time, and CHO B2 transfection was inefficient, so high expressors were selected for some experiments (Figs 2, 10) by FACS. Confluent cell monolayers were treated for 1 minute with 3 ml of cell dissociation buffer (Life Technologies). All further steps were performed at -4°C. Cells were suspended in 1 ml of Ham’s F12 plus 10% FBS, centrifuged at 1000 g (10 minutes), washed twice with PBS+ plus 5% FBS and incubated with rabbit anti-syndecan-2 antiserum (diluted 1:50) for 45 minutes. Cells were washed twice with PBS+ plus 5% FBS, centrifuged (1000 g, 10 minutes), incubated with FITC-conjugated F(ab’)2 fragment of goat anti-rabbit IgG (Cappel, diluted 1:50) and washed three times as above. Cells incubated only with secondary antibody served as controls. FACS was performed by the Flow Cytometry Core Facility of the UAB Multipurpose Arthritis and Musculoskeletal Disease Center using a Becton Dickinson FACS caliber machine and Cell Quest computer program. Cells comprising the 5% highest expressors were collected in Ham’s F12 medium and 10% heat-inactivated FBS, grown to confluency, and stocks were frozen at passage 2-3 after selection.

Treatment of cells with conditioned medium

Conditioned medium was collected every other day from S2AS transfected CHO-K1 cells, centrifuged (1,000 g, 10 minutes) and stored at +4°C. This was used daily for two passages to replace medium in cultures of CHO-K1 stably transfected with the empty pcDNA3 vector, before staining for laminin and fibronectin as described below.

Immunohistochemical analysis and microscopy

To monitor endogenous matrix assembly cells were grown on glass coverslips (10 mm diameter; Fisher) for 1-2 days (sparse cultures) or 3-5 days (confluent cultures) in culture medium. In some cases, exogenous bovine fibronectin (260 μg/ml) was added 18 hours after seeding, before fixation and staining at 3 days. In other experiments, cells were incubated on coated coverslips in culture medium for 24 hours. For experiments on reorganization of substrates into a fibrillar matrix, cells were grown on laminin or fibronectin coated coverslips for 4-24 hours. Coating of coverslips with 5 μg of laminin (Sigma) or vitronectin, 10 μg of fibronectin or 100 μg of type I collagen (Vitrogen 100, Collagen Biomaterials, Palo Alto, CA) and blocking with 0.1% bovine serum albumin have been previously described (Woods and Couchman, 1994). Cultures were fixed (30 minutes) with 3% glutaraldehyde (Sigma) for phase contrast microscopy or 3.5% freshly prepared paraformaldehyde in PBS+ (20 minutes) for fluorescence. Permeabilization, when indicated, was by incubation (10 minutes) with 0.1% Tween-20 (Bio-Rad, Richmond, CA) in PBS+. Labeling with primary and secondary antibodies, or with Texas Red-phalloidin was as previously described (Woods and Couchman, 1994). For double labeling, cells were incubated with a mixture of chicken anti-rat syndecan-2 and rabbit anti-fibronectin, followed by FITC goat anti-chicken Ig and Texas Red goat anti-rabbit IgG. Cells were monitored using a Nikon Optiphot microscope and photographed with Ilford HPS film. Where amounts of extracellular labeling were compared, constant time of exposure, film development and print conditions were used.

Biosynthetic labeling of cells and pulse chase experiments

pcDNA3 or S2AS cells (6x10⁶ cells per well) at passage 3-5 were seeded in 6-well plates. After attachment and spreading for 2 hours, cells were starved overnight in Hanks’ balanced salt solution (Mediatech, Charlottesville, VA). The next morning cells were pulsed with 100 μCi [35S]methionine/cysteine (ICN Biomedical Flow Laboratories) for 1 hour, and ’chased’ with Ham’s F12 medium plus 10% FBS for 0, 30, 60, 90 minutes or 24 hours to allow synthesis, export and incorporation into any ECM which was formed. Very little ECM is visible by immunofluorescence at 90 minutes. The supernatants were collected and the cell layers lysed with RIPA-buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.2 mM PMSF, 1 mM benzamidine-HCl). Supernatants and cell lysates were immunoprecipitated (Woods and Couchman, 1994) with rabbit anti-laminin or anti-fibronectin. Polypeptides were resolved by 3-15% SDS-PAGE reducing gradient gel, and gels were impregnated with ENHANCE (NEN), dried, and gels were impregnated with ENHANCE (NEN), dried, and exposed to film for 5 days at –80°C before development. Fluorographs were quantified using the IP Lab Gel software by Macintosh for densitometry and gel analysis. Data values are displayed in pixels.

Measurement of cell surface syndecan-2 and integrins

To examine syndecan-2 cell surface expression, FACS analysis was performed as described above. For integrin β1 cell surface expression levels, cells were harvested with 0.05% trypsin, washed with culture medium, followed by FACS buffer (1× PBS-, 5% FCS, 0.1% sodium azide) and incubated with mouse monoclonal antibodies 7E2 or PB1 for 45 minutes. Cells were washed three times with FACS buffer, incubated with FITC-conjugated anti-mouse IgG for 45 minutes, washed three times with FACS buffer, and fixed in 1% paraformaldehyde in FACS buffer.
Quantitation of syndecan-2

To quantitate syndecan-2 levels in cells transfected with the antisense syndecan-2 construct, identical numbers of CHO-K1, S2 and S2A cells were grown for 3 days to confluence in 100 mm dishes. Cells were washed with PBS and scraped into 1 ml of 100 mM NaCl, 1 mM CaCl2, 50 mM HEPES, pH 7.0 (David et al., 1992a) containing 1 mM benzamidined-HCl, 1 mM phenylmethylsulfonfluryl fluoride, 10 mM N-ethylmaleimide and 10 μg/ml leupeptin, centrifuged at 800 g and resuspended in 50 μl of the above buffer. Samples for digestion were incubated with 2.5 μl heparitinase (EC.4.2.2.8) and 5 μl chondroitinase ABC (EC.4.2.2.4) (Seikagaku Americ) overnight at 37°C. After addition of 5x SDS sample buffer with 2-mercaptoethanol, samples were boiled for 3 minutes. Samples were applied to a 1.5 mm thick SDS-PAGE gel, electrotransferred at 75 V for 2.5 hours, and immunoblotted onto Bio-Rad nitrocellulose membrane as previously described (Woods and Couchman, 1994). The membranes were fixed for 30 minutes with PBS containing 0.05% glutaraldehyde and blocked in TBS plus 5% non-fat dried milk overnight at +4°C. Primary antibody was affinity-purified rabbit anti-pan-syndecan antibody (0.5 μg/ml) generated against the carboxy-terminal 10 amino acids of syndecan-1. This recognizes all syndecan core proteins (Ott and Rapraeger, 1998) and was a generous gift from Dr A. C. Rapraeger (University of Madison, Wisconsin). After washing in TBS plus 0.1% Tween-20, bound antibody was visualized with affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution 1:5000) (Zymed, San Francisco, CA) diluted 1:50 for 1 hour, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5000) (Bio-Rad), and chemiluminescence (ECL, Amersham) as per manufacturer’s instructions. Since endogenous syndecan-2 levels in CHO cells are very low, blots were exposed for 30 minutes. Densitometric analysis of syndecan-2 core protein was normalized to cytokeratin levels determined by reprobing the same blots. Blots were stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 30 minutes at 50°C, washed twice with PBS, and checked for lack of residual label by chemiluminescence. After reblocking, the blot was reprobed with monoclonal mouse anti-cytokeratin antibody (Zymed, San Francisco, CA) diluted 1:50 for 1 hour, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5000) (Bio-Rad) as secondary antibody, and chemiluminescence (ECL, Amersham). The experiment was repeated three times. Signals were assessed using the Image Analysis Software of model GS-670 densitometer (Bio-Rad). Results are shown as mean ± standard error.

Quantitation of cell surface laminin

Identical numbers of CHO-K1, S2 and S2A cells were grown to confluence (4 days) in two 100 mm dishes per cell line. Cell monolayers of one dish per cell line were surface-labeled with 0.5 D. Identical numbers of CHO-K1, S2 and S2A cells were grown for 3 days to confluence in 100 mm dishes. Cells were washed with PBS and scraped into 1 ml of 100 mM NaCl, 1 mM CaCl2, 50 mM HEPES, pH 7.0 (David et al., 1992a) containing 1 mM benzamidined-HCl, 1 mM phenylmethylsulfonfluryl fluoride, 10 mM N-ethylmaleimide and 10 μg/ml leupeptin, centrifuged at 800 g and resuspended in 50 μl of the above buffer. Samples for digestion were incubated with 2.5 μl heparitinase (EC.4.2.2.8) and 5 μl chondroitinase ABC (EC.4.2.2.4) (Seikagaku Americ) overnight at 37°C. After addition of 5x SDS sample buffer with 2-mercaptoethanol, samples were boiled for 3 minutes. Samples were applied to a 1.5 mm thick SDS-PAGE gel, electrotransferred at 75 V for 2.5 hours, and immunoblotted onto Bio-Rad nitrocellulose membrane as previously described (Woods and Couchman, 1994). The membranes were fixed for 30 minutes with PBS containing 0.05% glutaraldehyde and blocked in TBS plus 5% non-fat dried milk overnight at +4°C. Primary antibody was affinity-purified rabbit anti-pan-syndecan antibody (0.5 μg/ml) generated against the carboxy-terminal 10 amino acids of syndecan-1. This recognizes all syndecan core proteins (Ott and Rapraeger, 1998) and was a generous gift from Dr A. C. Rapraeger (University of Madison, Wisconsin). After washing in TBS plus 0.1% Tween-20, bound antibody was visualized with affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution 1:5000) (Bio-Rad), and chemiluminescence (ECL, Amersham) as per manufacturer’s instructions. Since endogenous syndecan-2 levels in CHO cells are very low, blots were exposed for 30 minutes. Densitometric analysis of syndecan-2 core protein was normalized to cytokeratin levels determined by reprobing the same blots. Blots were stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 30 minutes at 50°C, washed twice with PBS, and checked for lack of residual label by chemiluminescence. After reblocking, the blot was reprobed with monoclonal mouse anti-cytokeratin antibody (Zymed, San Francisco, CA) diluted 1:50 for 1 hour, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5000) (Bio-Rad) as secondary antibody, and chemiluminescence (ECL, Amersham). The experiment was repeated three times. Signals were assessed using the Image Analysis Software of model GS-670 densitometer (Bio-Rad). Results are shown as mean ± standard error.

RESULTS

Syndecan-2 expression in wild-type and transfected CHO-K1 cells

Immunofluorescent labeling showed that syndecan-2 is present at low levels in CHO-K1 cells, distributed mainly diffusely over the surfaces (Fig. 2A) as previously seen for rat embryonic fibroblasts (Woods and Couchman, 1994). It did not appear to correlate in double staining with the distribution of extracellular matrix in CHO-K1 (see Fig. 4A) or rat embryo fibroblasts (not shown). CHO-K1 transfected with wild-type (S2) or partially truncated (S2ΔS) syndecan-2 had variably increased cell surface labeling, which was pronounced in FACS-selected cells (Fig. 2B.C). This was present in a linear punctate pattern. In S2A cells, ruffles, blebs, and other surface protrusions, which are increased, were intensely labeled (Fig. 2C). Cells transfected with the full (S2ΔR) truncation-construct also showed some increased labeling, but, even after FACS selection, phenotypic drift occurred rapidly within 3-4 passages leaving mainly very low expressors (Fig. 2D) Cells transfected with syndecan-2 antisense (S2A) vector showed very little labeling for syndecan-2 at the cell surface (Fig. 2E).

There was no difference in immunofluorescent labeling or FACS analysis for syndecan-2 in CHO-K1 cells transfected with empty vector pcDNA3 (not shown). Increased cell surface expression of syndecan-2 was seen in the S2, S2A and S2ΔR cells by FACS analysis (Fig. 3). Although the range of expression was large within populations, the mean fluorescence of cells transfected with any syndecan-2 sense construct was always well above that of control pcDNA3-transfected cells or wild-type CHO-K1 (Fig. 3). Therefore, constructs were expressed at the cell surface, although the increase in S2ΔR cells was less than that in S2ΔS or S2 cells (Figs 2, 3). Since endogenous syndecan-2 levels were already very low in CHO-K1 cells, quantitative immunoblots of cell lysates of CHO-K1 and S2A cells were performed instead of FACS to demonstrate downregulation of syndecan-2 in the S2A cells (Fig. 3D). The extracellular domains of syndecans differ between species, but their cytoplasmic domains are highly homologous (Bernfield et al., 1992). Due to limited reactivity of the polyclonal anti-rat syndecan-2 (against extracellular and transmembrane domains) to endogenous hamster syndecan-2, immunoblotting was performed with a polyclonal antibody that recognized all syndecan cytoplasmic domains (Ott and Rapraeger, 1998). Samples were treated with heparitinase and chondroitinase ABC to ensure complete digestion of glycosaminoglycan chains. Fig. 3D shows that syndecan-1 was the major syndecan in CHO-K1 cells, with minor amounts of syndecan-2 and syndecan-4. Syndecan-2 core protein, represented by an approximately 48 kDa polypeptide as seen in previous studies (Ott and Rapraeger, 1998; Pierce et al., 1992), was reduced in S2A cells, but increased in S2 cells. Quantitative analysis normalizing to cytokeratin is shown in Fig. 3D.

Matrix assembly in CHO-K1 and transfected cells

CHO-K1 or pcDNA3 cells assemble an extracellular fibrillar matrix that contains both laminin and fibronectin. The distributions of laminin and fibronectin were similar in non-transfected cells, and altered coordinately with transfection, so results are shown here only for laminin. In sparse cultures of CHO-K1 or pcDNA3 (not shown) cells, a three-dimensional network of matrix fibers and smaller fibrils were visualized by staining non-permeabilized cells with anti-laminin (Fig. 4A). Additional label resembling the distribution of endoplasmic reticulum/Golgi was evident in permeabilized cells (Fig. 4B).
Syndecan-2 controls matrix assembly

Syndecan-2 controls matrix assembly

S2 cells had a more epithelial cell morphology, but the amount of either laminin (Fig. 4C) or fibronectin (not shown) assembled into extracellular matrix fibrils appeared normal. The distribution pattern was slightly altered, however, with a matrix that was restricted to cell-cell contact areas with reduced fibrils on top and underneath the cells. Their internal labeling pattern (Fig. 4D) was similar to that of CHO-K1 cells. In contrast, S2ΔS cells showed an almost complete lack of extracellular labeling for laminin (Fig. 4E) or fibronectin (not shown) except for areas of cell damage during processing, although staining of permeabilized S2ΔS cells (Fig. 4F) revealed intracellular label similar to that of CHO-K1 and S2 cells. Overexpressing the S2ΔR construct in non-selected cell populations showed only a slightly reduced fibrillar matrix (Fig. 4G). However, analysis of these cells showed few that stably expressed high levels of this construct. Most cells were low expressors (Figs 2, 3). The same reduction in laminin and fibronectin deposition by high expressors of S2ΔR (detected by double immunostaining of syndecan-2 and matrix glycoproteins; data not shown) was seen as for S2ΔS. It has been shown that syndecans with a totally truncated cytoplasmic domain may be abnormally processed, while partially truncated constructs may not be (Miettinen et al., 1994). Therefore, only the S2ΔS cells were monitored further. Cells expressing a syndecan-4 core protein that is truncated in its V region (S4ΔI) retained the ability to assemble a normal matrix (Fig. 4H). In very dense cultures of CHO-K1 (Fig. 5A) or S2 (not shown) cells, an elaborate ECM was assembled, but S2ΔS cells established only patchy, sparse, non-fibrillar deposits of laminin and fibronectin-containing matrix at the basal surface with a few fibrils between cells (Fig. 5B). S2A cells also had a greatly reduced, but not absent, fibrillar matrix (Fig. 5C). This intermediate result is consistent with an approximate one third reduction in detectable syndecan-2 (Fig. 3D). Thus, transfection with truncated syndecan-2 or antisense-syndecan-2 constructs reduces endogenous matrix assembly. Double staining of CHO-K1, S2 and S2ΔS cells showed no colocalization of fibronectin and syndecan-2 (not shown). Quantitation of biotinylated, cell surface laminin showed equal amounts in CHO-K1 and S2 cells, but S2ΔS cells had 50-65% less (Fig. 6).

Cells use specific integrin receptors when adhering to different extracellular matrix glycoproteins and vitronectin receptors are normally used by cells cultured in the presence of serum (Fath et al., 1989). To determine if ligation of various integrins could override the S2ΔS matrix defect, CHO-K1 and S2ΔS cells were seeded onto laminin-1, fibronectin, vitronectin and type I collagen substrates. Both cell lines spread very well on fibronectin and vitronectin, but poorly on laminin-1 and type I collagen. Staining for endogenously-derived laminin or fibronectin (not shown) showed that ECM deposition was similar in each case with the S2ΔS cells being unable to deposit a significant amount of fibrillar ECM irrespective of substrate.

It was also possible that the S2ΔS cells secreted a factor that indirectly caused proteolysis or internalization of matrix...
components. However, similar degrees of proteolytic cleavage of laminin were noted for pcDNA3 and S2ΔS cells (see below). In addition, incubation of control pcDNA3 cells with conditioned medium from S2ΔS cells did not affect subsequent matrix assembly even after continuous treatment for two passages.

Matrix synthesis and export in normal and transfected CHO-K1 cells

To determine if decreased matrix assembly by the S2ΔS cells was due to decreased laminin and fibronectin synthesis or export, pulse chase immunoprecipitation experiments were performed.

S2ΔS cell lysates contained less laminin (Fig. 7A) but more fibronectin (Fig. 7B) than vector-only transfected pcDNA3 cells after 30 minutes. However, both S2ΔS and pcDNA3 cells had similar cellular laminin and fibronectin content at 90 minutes (Fig. 7). Since a constant amount of antibody was used at each time point, the ratio of cold to radiolabeled material decreased with time, resulting in increased cpm being immunoprecipitated. These increases were similar in both cell types, further indicating similar processing and export. Proteolytically cleaved fragments of laminin and fibronectin were present in immunoprecipitates of both S2ΔS and pcDNA3 cell supernatants. This pattern of laminin degradation is typical for cleavage by plasmin (Liotta et al., 1981). When the laminin or fibronectin contents in supernatants and the corresponding cell lysates were quantified, there were no significant differences between pcDNA3 and S2ΔS cells (Fig. 7A,B). At these early time points, matrix fibrils are not visible by
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**immunofluorescence** (not shown), so the lysate represents mainly intracellular material plus any that is cell surface associated. The supernatant represents exported material. To further confirm a lack of extracellular matrix in S2ΔS cells, these and pcDNA3 cells were ‘chased’ for 24 hours to allow any ECM deposition, then briefly trypsinized to remove any extracellular label. Immunoprecipitation of duplicate lysates before and after trypsinization showed a loss of laminin and fibronectin from pcDNA3, but not S2ΔS cells (not shown).

**S2ΔS-cells have lost the ability to rearrange laminin or fibronectin substrates**

Since laminin and fibronectin synthesis and export appear similar between S2ΔS and CHO-K1 or pcDNA3 cells, but S2ΔS cells cannot form these into a fibrillar matrix, cell surface events may be disrupted in S2ΔS cells. To test this, CHO-K1, S2, S2ΔS and S2A cells were plated for 4 hours on coverslips coated with laminin or fibronectin and immunostained with anti-laminin or anti-fibronectin to detect the ability of these cells to rearrange substrates into a fibrillar matrix (Woods et al., 1988; Christopher et al., 1997). Spreading on either substrate did not differ with cell type. CHO-K1 (Fig. 8A) and S2 cells (Fig. 8B) reorganized either laminin (not shown) or fibronectin substrates into thick fibrils, especially at the cell edges and between adjacent cells (arrows). The S2 cells were more effective at substrate reorganization than wild-type CHO-K1 cells, whereas S2ΔS cells (Fig. 8C) almost completely lacked this ability. Substrates remained uniform, even after 24 hours, with dark areas representing the spread cells. S2A cells also had a reduced capacity to rearrange laminin or fibronectin into fibrils (Fig. 8D) with only a few fibrils (arrowed) being formed. All cell types could remove some of the substrate (evidenced as dark streaks), but only the CHO-K1, S2 and some of the S2A cells, but not the S2ΔS cells, redistributed this into fibers.

**Exogenous fibronectin partially restores matrix assembly in S2ΔS cells**

CHO-K1 cells can incorporate exogenously provided fibronectin into a matrix (Wu et al., 1995b). Addition of bovine plasma fibronectin to confluent cultures of wild-type CHO-K1 (Fig. 9A) and S2ΔS cells increased matrix assembly. However, the S2ΔS matrix was less substantial (Fig. 9B). When syndecan-2 levels were monitored in these S2ΔS cells, most showed no labeling, but some intermediate (arrowhead) and high expressors (arrow) were visible (Fig. 9C). The dual image further showed that those cells expressing intermediate or high levels of truncated syndecan-2 at the cell surface were virtually unable to accumulate a pericellular matrix. Only those cells staining weakly for syndecan-2 core protein could establish a matrix (Fig. 9D). This suggests that exogenous fibronectin in
high concentration may trigger ‘nucleation’ of matrix fibrils, and that this ‘nucleation’ is deficient in S2ΔS cells. Laminin matrix was not restored in S2ΔS cells following fibronectin addition (not shown), indicating the independent nature of laminin and fibronectin fibril formation.

The S2ΔS defect is not due to reduced cell surface integrins or lack of integrin activation

Assembly of laminin and fibronectin matrices in CHO-K1 cells is mediated by α6β1 and α5β1 integrins (Furtado et al., 1992; Wu et al., 1993). However, FACS analysis with anti-α5 and -β1 integrin (data not shown) showed no differences between CHO-K1 and S2ΔS cells in cell surface expression levels, even after FACS selection for high S2ΔS expression. An α6β1 integrin-deficient CHO cell line termed CHO B2 cannot assemble a regular fibronectin matrix (Schreiner et al., 1989; Wu et al., 1993). However, these cells did assemble a laminin matrix (Fig. 10A), albeit not as well organized as in CHO-K1 cells (Fig. 4A), possibly due to reduced cell spreading. This confirms that fibronectin and laminin matrix assemblies are regulated independently. Transfection of CHO B2 cells with the S2ΔS construct decreased laminin matrix formation (Fig. 10B) with extracellular laminin organized not as fibrils but only as amorphous aggregates. Thus, truncated syndecan-2 suppressed the fibronectin-independent assembly of a laminin matrix. Transfection of CHO B2 cells with activated αIIbβ3 integrin (O’Toole et al., 1994) did not change the laminin matrix (Fig. 10C), but restored fibronectin assembly (Fig. 10E), through the expression of a constitutively activated fibronectin-binding integrin (Wu et al., 1995c). However, when cells expressing activated integrin were cotransfected with the S2ΔS construct, laminin (Fig. 10D) and fibronectin (Fig. 10F) matrix assembly were both reduced, suggesting that S2ΔS expression could largely override even the effects of activated integrins.

Cytoskeletal changes in S2ΔS cells

The above experiments indicated that cell surface events other than the mere presence of matrix molecules and activated integrins are required for matrix assembly. Fibronectin fibrillogenesis occurs centripetally in cultured cells (Ljubimov and Vasiliev, 1982), and requires an intact microfilament cytoskeleton (Ali and Hynes, 1977; Wu et al., 1995b). Previous studies (Woods et al., 1988) had indicated a common requirement for both cell-binding and heparin-binding fibronectin domains for stress fiber and focal adhesion.

Fig. 5. Matrix assembly in transfected CHO-K1 cells in dense culture. CHO-K1 cells (A) assembled a network of matrix fibrils, whereas S2ΔS (B) and S2A (C) cells established only a patchy, sparse matrix with only a few fibrils (arrows). Cells were not permeabilized. Bar, 10 μm.

Fig. 6. Quantitation of cell surface laminin in CHO-K1, S2 and S2ΔS cell lysates. Immunoprecipitated, cell surface biotinylated laminin was detected by SDS-PAGE, electrophoretic transfer and streptavidin binding. In S2ΔS cells all laminin chains were decreased, whereas CHO-K1 and S2 cells had equivalent levels. Experiments were performed twice with similar results. Data are shown as a percentage relative to CHO-K1 ± s.d.
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We, therefore, tested whether the reduction in matrix assembly correlated with a reduction in cytoskeletal organization. Phase contrast microscopy (Fig. 11A-C) showed that S2ΔS cells (B) spread less well than CHO-K1 cells (A), and exhibited continuous ruffling. The morphology of S2ΔS cells resembled that of cells expressing a truncated syndecan-4 construct (S4ΔI, Longley et al., 1999; Fig. 11C). However, interference reflection microscopy (Woods et al., 1992) indicated that 60% of S2ΔS cells could still form focal adhesions. This is reduced in respect to CHO-K1 cells (70-75%) but higher than in S4ΔI cells, where only 26% of the population form these structures. Phalloidin labeling showed stress fibers present in CHO-K1 (Fig. 11D), with some reduction in S2ΔS cells (E), but this reduction was not as pronounced as in S4ΔI cells (F). Labeling with anti-paxillin confirmed the presence of focal adhesions in CHO-K1 (G) and S2ΔS (H) cells, but their lack in S4ΔI cells (I). Since the S4ΔI cells can still form a fibrillar matrix (Fig. 4H), but have a much less organized microfilament system than S2ΔS cells, control of matrix assembly seems not to be solely dictated by the cytoskeleton. Consistent with this, double labeling (not shown) indicated that microfilament bundles were not colinear with the linear punctate staining for syndecan-2 (Fig. 2).

DISCUSSION

The V region of the cytoplasmic domain of syndecan-2 core protein appears to be crucial for fibronectin and laminin matrix formation. Laminin is usually described as a basement membrane component, but it is found as fibrils in connective tissue (Timpl and Brown, 1996), and in cultured cells it can form a three-dimensional fibrillar matrix indistinguishable from that of fibronectin. Both fibronectin and laminin can bind entactin (Wu et al., 1988), so fibronectin and laminin matrices may be interconnected. However, in the case of CHO B2 and S2ΔS cells,
fibronectin and laminin matrix fibrils are regulated independently, but both depend on syndecan-2 proteoglycan. This suggests that syndecan-2 operates independently of the integrins involved.

Deletion of the C-terminal 14 amino acids of the cytoplasmic domain of syndecan-2 led to a marked diminution of matrix assembly. This was shown by immunofluorescence, where only short thin fibrils of laminin or fibronectin were detected, even in dense cultures. It was evident in several stably transfected lines of CHO-K1 cells, where most cells were low expressers of the truncated syndecan-2, and in cultures of the 5% highest expressors selected by FACS. Immunoprecipitation of biotinylated cell surface laminin confirmed an up to an almost two-thirds reduction in extracellular laminin. Inability of S2ΔS cells to form an endogenous matrix was not due to a defect in synthesis of either fibronectin or laminin, nor to decreased export of matrix components. Reduced matrix was also not due to proteolysis by secreted enzymes, since conditioned medium from S2ΔS cells had no effect on cells containing empty vector, and the pattern of laminin proteolysis was similar in pcDNA3 and S2ΔS culture supernatants. The decrease in matrix assembly was also not due to a lack of cell interaction with the matrix, since S2ΔS and S2A cells attached and spread similarly to CHO-K1 or pcDNA3 cells on matrix-coated substrates, and they could remove the substrate coat around them (Grinnell, 1986; Christopher et al., 1997). However, unlike CHO-K1 cells, S2ΔS cells were unable to form matrix fibrils with removed substrate fibronectin or laminin. S2A cells also showed a greatly reduced capacity to reorganize substrates, consistent with an approximate one third reduction in syndecan-2 core protein levels. When exogenous, soluble fibronectin was provided to S2ΔS cells, some matrix was then formed, but only by cells expressing low levels of S2ΔS construct. Those cells with high surface levels remained free of matrix. The data suggest that matrix ‘nucleation’ at the

![Fig. 8. S2ΔS cells cannot rearrange laminin or fibronectin substrates. Immunostaining with anti-fibronectin shows rearrangement by 4 hours of the fibronectin substrate by CHO-K1 cells (A). This was even more evident with S2 cells at 4 hours (arrows). In contrast, S2ΔS cells (C) did not reorganize the fibronectin substrate even after 24 hours, and S2A cells (D) showed reduced matrix reorganization after this time. Cells were not permeabilized. Fibrils are arrowed. Bar, 10 µm.](image)

![Fig. 9. Exogenous fibronectin can partially restore a fibrillar matrix. Addition of bovine plasma fibronectin increased the matrix of CHO-K1 cells (A) and to a lesser extent of S2ΔS cells (B). Cells in B were also labeled for syndecan-2 expression (C), and the double image is shown in D. The dual image (D) revealed fibronectin matrix only on the lower syndecan-2 expressors, while high expressors (arrowed in C) showed no matrix assembly. (C) Overexposed image to show limited labeling of low expressors. The high (arrow) and intermediate expressors (arrowhead) are indicated. Fibronectin was labeled with rabbit anti-fibronectin and Texas Red-conjugated goat anti-rabbit under identical conditions for A and B. Syndecan-2 was visualized with chicken anti-syndecan-2 ectodomain followed by FITC goat anti-chicken. Cells were passage 9 after FACS selection, when phenotypic drift has occurred, and were not permeabilized. Bar 10 µm.](image)
Integrins are required for both endogenous fibronectin matrix assembly (Fogerty et al., 1990; Wu et al., 1993) and for incorporation of exogenous fibronectin into deoxycholate-resistant matrices (Wu et al., 1995c). Much research has shown that fibronectin fibrillogenesis normally requires interaction of α5β1 with the RGD-containing domain of fibronectin, specifically the type III repeat 10 (reviewed by Bultmann et al., 1998; Schwarzbauer, 1991; Sechler et al., 1997; Wu et al., 1993). However, S2ΔS cells showed no alteration in surface levels of α5 and β1 integrin. Interactions of α5β1 with the RGD site do not seem to be sufficient for fibrillogenesis and secondary sites may be needed both at the cell surface and within fibronectin. The existence of a 'matrix assembly receptor' has been postulated for some time, but has been elusive. The studies reported here indicate that syndecan-2 may be this 'receptor', confirming previous indications for a role for heparan sulfate proteoglycans (Chung and Erickson, 1997).

Syndecans bind the heparin-binding domains of matrix molecules (reviewed by Bernfield et al., 1992; Carey, 1997), including fibronectin and laminin. Lack of interaction with the amino-terminal heparin-binding (Hep I) domain of fibronectin results in the formation of only short, thin fibrils (McDonald et al., 1987; Schwarzbauer, 1991) and the first five type I repeats (I1-5) and first type III repeat (III1) have all been implicated in matrix assembly (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; Sottile et al., 1991). Other studies indicate a role for type III repeats 12-14 of the Hep II high affinity heparin-binding site near the C terminus of the molecule (Bultmann et al., 1998) and our previous studies showed that both the integrin-binding domain and either the Hep I or Hep II domains were needed for reorganization of an exogenous fibronectin substrate into fibrils (Woods et al., 1988).

A 'synergy' site PHSRN in repeat III9 is needed for adhesion to fibronectin through α5β1 (Aota et al., 1994), and for efficient incorporation of recombinant fibronectins into fibers (Sechler et al., 1997). Fibrillogenesis initiates at the cell surface where receptor clustering provides a nucleus of fibronectin 'activated' by integrin binding into an unfolded form capable of self-association. This gives rise to short fibrils at the cell surface by 1 hour that increase in size and number. Additional fibronectin-fibronectin interactions generate deoxycholate-insoluble fibers over 16 hours forming a three-dimensional network of larger fibers extending away from the cell surface. Absence of the synergy site delays initiation of fibril formation, resulting in only very sparse short fibrils even at 16 hours, thus preventing fiber assembly. The need for the synergy site can be circumvented by activation of α5β1 with antibodies or Mn2+. We, therefore, monitored whether the presence of activated integrins would prevent the effect of transfection with truncated syndecan-2. However, S2ΔS appears to override even activated integrins, since CHO B2 cells expressing activated αIIbβ3 that normally allows fibronectin matrix assembly (Wu et al., 1995c) established only a sparse non-fibrillar matrix, if cotransfected with the truncated syndecan-2 construct.

The cytoskeleton may be involved in matrix fibrillogenesis. Treatment of cells with cytochalasin to disrupt the actin microfilament system prevents fibronectin matrix assembly even in cells expressing activated integrin (Wu et al., 1995c). The requirements for both integrin- and heparin-binding

**Fig. 10.** Overexpression of S2ΔS can override the effect of activated integrin. CHO B2 cells (A) assembled a laminin matrix, but CHO B2 transfected with S2ΔS (B) did not. Transfection of CHO B2 with constitutively activated αIIbβ3 integrin resulted in no change in laminin deposition (C) but a restoration of fibronectin matrix assembly (E). Cotransfection of activated αIIbβ3 and S2ΔS led to a reduction in deposition of both laminin (D) and fibronectin (F). Cells were not permeabilized, so only cell surface matrix (arrows) is seen. Bars, 10 μm.

cell surface may lie at the heart of the S2ΔS phenotype, and since this was not restricted to a single matrix component, syndecan-2 may be involved in signaling responses common to fibrillogenesis.
domains of fibronectin for matrix fibrillogenesis, or the reorganization of substrates into fibrils, parallels a similar need for both domains for focal adhesion and stress fiber formation in primary fibroblasts (Woods et al., 1988). Lysophosphatidic acid increases both stress fiber formation and fibronectin fibrillogenesis (Zhong et al., 1997), and protein kinase C activity is needed for both events (Somers and Mosher, 1993; Woods and Couchman, 1992). However, increased spreading induced by phorbol ester treatment did not result in increased matrix assembly (Sechler et al., 1997), indicating that the two events do not necessarily correlate. This is confirmed by our present studies where both de novo matrix assembly and the reorganization of surface bound matrix were disrupted in S2ΔS cells, but the cytoskeleton was not dramatically altered. Additionally, transfection with a partially truncated syndecan-4 construct (S4ΔI) had little or no effect on matrix assembly, even though these cells showed dramatically reduced focal adhesion and stress fiber formation.

The defect in matrix assembly did not occur if cells were transfected with a construct where the cytoplasmic domain was fully deleted (S2ΔR) except at very high expression levels, nor did transfection with full length syndecan-2 increase matrix assembly even at high expression levels. In contrast, changes were seen in S2ΔS populations before FACS selection, where modest increases in expression levels were noted. This argues that neither increased ectodomain expression levels, nor potential glycanation changes in ectodomains due to overexpression, are responsible for the definitive phenotype of the S2ΔS cells. The partial truncation mutant S2ΔS terminates at serine197 in the V region of syndecan-2 core protein. Synthetic peptides encompassing the syndecan-2 V region (LGERKPSSAAYQK) can be phosphorylated by protein kinase C at both serine197 and serine198, with the extent of phosphorylation varying with concentration (Oh et al., 1997c), and other studies have shown syndecan-2 phosphorylation in vitro (Prasthofer et al., 1995) and in vivo on serine (Itano et al., 1996). Phosphorylation of the V region may regulate matrix assembly. This would be compromised in both the S2ΔS and S2ΔR constructs, but only the S2ΔS cells exhibited an almost complete lack of matrix assembly. However, the S2ΔR construct may be more rapidly turned over at the cell surface as seen with syndecans totally lacking a cytoplasmic domain (Miettinen et al., 1994), thus allowing normal functioning of endogenous syndecan-2. All syndecans self-associate (Bernfield et al., 1992; Carey, 1997; Oh et al., 1997a,b; Woods and Couchman, 1998), and the association of the partially

![Fig. 11. Morphology of CHO-K1, S2ΔS cells and S4ΔI cells. Phase contrast microscopy shows well spread CHO-K1 cells (A), and some reduction in spreading of S2ΔS cells (B). This is more pronounced with S4ΔI (C) cells. Stress fibers are seen by labeling with phalloidin in CHO-K1 (D) and S2ΔS cells (E). Although stress fibers in S2ΔS cells are less substantial, the cytoskeleton is more organized than in S4ΔI cells (F). Paxillin staining shows focal adhesions in CHO-K1 (G) and S2ΔS cells (H), but their lack in S4ΔI cells (I). Bar, 10 μm.](image-url)
truncated core proteins with those of endogenous normal syndecan-2 may prevent the functioning of whole clusters of syndecans aggregated by ligand. The positive requirement for syndecan-2 in matrix formation is emphasized by the loss of matrix assembly in cells expressing antisense for syndecan-2. Since syndecan-4 V region binds protein kinase C and modulates its activity (Oh et al., 1997a,b), the possibility emerges that the V regions of syndecans function specifically by controlling different kinase systems.

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