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Cooperation of Syndecan-2 and Syndecan-4 among Cell Surface Heparan Sulfate Proteoglycans in the Actin Cytoskeletal Organization of Lewis Lung Carcinoma Cells

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Syndecan-2 cooperates with integrin α5β1 in cell adhesion to a fibronectin substratum and regulates actin cytoskeletal organization in an expression level-dependent manner; Lewis lung carcinoma-derived P29 cells with high expression form stress fibers, whereas the same tumor-derived low expressers, LM66-H11 cells, form cortex actin [Munesue, S., Kusano, Y., Oguri, K., Itano, N., Yoshitomi, Y., Nakanishi, H., Yamashina, I., and Okayama, M. (2002) *Biochem. J.* 363, 201–209]. In this study we examined the participation of other cell surface heparan sulfate proteoglycans in this signaling. The two clones expressed syndecan-1, -2 and -4, and glypican-1 at similar levels except for syndecan-2. Treatment of cells with phosphatidylinositol-specific phospholipase C or immobilized anti-syndecan-1 antibodies demonstrated that neither glypican-1 nor syndecan-1 was involved in this signaling, indicating that individual cell surface heparan sulfate proteoglycans have functional specificity. Stimulation with immobilized anti-syndecan-2 or -4 antibodies induced stress fiber formation in P29 cells but not in LM66-H11 cells, despite the similar levels of syndecan-4 expression, suggesting that stress fiber formation required a threshold expression level of syndecan-2 acting downstream of syndecan-4. This was confirmed by cells in which syndecan-2 expression was artificially suppressed by antisense mRNA oligonucleotide treatment or elevated by cDNA transfection. This is the first report demonstrating that syndecan-2 and -4 cooperate *in situ* in actin cytoskeletal organization.

Key words: actin cytoskeletal organization, cell surface heparan sulfate proteoglycans, integrin α5β1, syndecan-2, syndecan-4.

Control of cell adhesion to the surrounding extracellular matrix is important in both physiological and pathological processes, including embryonic morphogenesis, maintenance of tissue homeostasis, wound healing, and tumor cell invasion and metastasis. These processes involve a variety of extracellular ligands, their interaction with cell membrane receptors, and subsequent downstream signal cascades. One class of cell adhesion receptors is the integrins, whose roles in extracellular matrix-associated adhesion and signaling are now well established (*1*–*5*). Recently, the syndecans, transmembrane heparan sulfate proteoglycans, which often work in cooperation with integrins, have received much attention as another class of cell adhesion receptors (*6*, *7*). The syndecan family is composed of four members and binds to a variety of soluble and solid extracellular effectors (*8*, *9*). Their expression is loosely limited as to cell types, that is, syndecan-1, -2, and -3 are the major syndecans of epithelial, fibroblastic and neuronal cells, respectively, whereas syndecan-4 is ubiquitous (*10*–*12*). Furthermore, during embryogenesis and morphogenesis, their expression is regulated spatially and temporally, and most cells and tissues express multiple syndecans, suggesting that the respective members might have their own individual functions, whether similar or distinct. However, their functional differences or specificities are still poorly understood.

As a cell adhesion receptor, syndecan-4 has been most studied in the family. It has been demonstrated that syndecan-4 is concentrated in focal adhesions together with integrins in cultured fibroblasts (*13*), and that it activates protein kinase C by binding with phosphatidylinositol-4,5-bisphosphate (*14*, *15*). Therefore, its function was suggested to be ensuring of the focal adhesion structure through recruitment of a variety of cytoplasmic proteins through protein kinase C (*16*–*18*). Meanwhile, we have found that syndecan-2 also acts as a cell adhesion receptor. Using mouse Lewis lung carcinoma-derived clones with different metastatic potentials, we observed that more than 85% of the heparan sulfate proteoglycans that bound to immobilized fibronectin was syndecan-2 (*19*), and demonstrated that syndecan-2 cooperates with integrin α5β1 by interacting with fibronectin through the $[IdoA(2OS)\text{-}GlcNS(6OS)]_6$ structure in its heparan sulfate side chains, and that it regulates the actin cytoskeletal organization of the cells on a fibronectin substratum (*20*). Two clones, P29 with low metastatic potential and

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high expression of syndecan-2, and LM66-H11 with high metastatic potential and low expression of syndecan-2, showed different actin architectures on adhesion to the fibronectin substratum; the former exhibited stress fiber formation, whereas the latter formed cortex actin. Furthermore, suppression of syndecan-2 in P29 cells, due to antisense oligonucleotide treatment, resulted in the formation of cortex actin (*20*), while overexpression of syndecan-2 on LM66-H11 cells due to cDNA transfection resulted in the formation of stress fibers (*21*). However, the level of integrin α5β1 expression did not change in either case (*20*, *21*). These results indicate that syndecan-2 regulated the ligand-binding signaling through integrin α5β1 in an expression level-dependent manner. However, we also found that both clones expressed the other three syndecans at the transcriptional level although the expression of syndecan-3 was extremely low, and that the expression levels were very similar between the clones except for that of syndecan-2 (*21*). In the present study, we used these clones to investigate the involvement of cell surface heparan sulfate proteoglycans other than syndecan-2 in actin cytoskeletal organization.

EXPERIMENTAL PROCEDURES

Materials—Human plasma fibronectin was purchased from Iwaki Glass (Tokyo). A recombinant fibronectin polypeptide, C-274 (Pro^{1239}–Ser^{1515}), with an RGD-containing Cell-I domain, was generously provided by TaKaRa Biomedicals (Otsu) (*21*, *22*). Monoclonal antibodies, F58–10E4 and F69–3G10, recognizing intact heparan sulfate and an epitope generated in heparitinase-digested heparan sulfate proteoglycans, respectively, along with heparitinase-I [EC 4.2.2.8] and chondroitinase ABC [EC 4.2.2.4], were purchased from Seikagaku Corp. (Tokyo). Phosphatidylinositol-specific phospholipase C [EC 3.1.4.10] from *Bacillus thuringensis* was obtained from Funakoshi (Tokyo). Antisense phosphorothioate oligonucleotides complementary to mouse syndecan-2 mRNA, and the corresponding sense and scrambled antisense phosphorothioate oligonucleotides were used as described (*20*).

Cell Culture—Low metastatic P29 and highly metastatic LM66-H11 cells cloned from mouse Lewis lung carcinoma (3LL) were maintained in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (GIBCO, NY), streptomycin (100 μg/ml), and penicillin (100 units/ml), as described previously (*19*). H11-SN2 and H11-Vec cells, which are LM66-H11 cells transfected with mouse syndecan-2 cDNA and the vector, respectively, were produced as described previously (*21*), and were cultured in the above medium supplemented with geneticin (800 μg/ml). Cell layers were rinsed with phosphate-buffered saline (PBS), and cells were harvested after incubation with 2 mM EDTA in PBS at 37°C for 10 min, followed by gentle flushing with a pipette. The suspended cells were subcultured or used for experiments.

Flow Cytometrical Assay—Cells [3×10^5 cells suspended in 50 μl of 0.2% bovine serum albumin (BSA)/DMEM] were incubated with antibodies or the respective non-immune sera at 4°C for 1 h with gentle agitation. After washing three times with PBS, they were exposed to FITC-conjugated second antibodies for 30 min. The labeled cells were washed, and then the fluorescence intensity was measured by flow cytometry, using an Ortho Cytoron (Ortho Diagnostic Systems).

Preparation of Antibodies to Ectodomains of Syndecan-1, -3, and -4—Rabbit antibodies, SN1Ab, SN3Ab, and SN4Ab, specific to the ectodomains of the mouse syndecan-1, -3, and -4 core proteins, respectively, were raised against the respective recombinant polypeptides, as described previously for the preparation of anti-mouse syndecan-2 (SN2Ab) (*20*). Briefly, recombinants were prepared by expressing cDNAs encoding the respective ectodomains of the mouse syndecan-1, -3, and -4 core proteins in *Escherichia coli*, XL1-Blue (Stratagene), using glutathione S-transferase (GST) gene fusion vector pGEX-2T (Amersham Pharmacia Biotech). The cDNAs were amplified by RT-PCR from polyadenylated RNA of P29 cells with primer pairs, which corresponded to the two ends of the ectodomain of each syndecan, 5′-GAGGATCCCAAATTGTGGCTGTAAATGTTCCTC-3′ and 5′-GAGAATTCTTCCTTCCTGTCCAAAAGGC-3′ for syndecan-1,5′-GAGGATCCGCTCAACGCTGGCGCAATG A-3′ and 5′-GAGAATTCCTCCTTCCGCTCTAGTATGC-3′ for syndecan-3, and 5′-GAGGATCCGAGTCGATTCGAGAGACAGAG-3′ and 5′-GAGAATTCCTCAGTTCTCTCAAAGATGTTGC-3′ for syndecan-4. GST-fused proteins were purified by glutathione-Sepharose affinity chromatography and digested with thrombin to cleave the fusion site. Recombinant proteins were separated by rechromatography on the same column and purified further on a Q-Sepharose column. Their N-terminal amino acid sequences were confirmed with a Model 492 protein sequencer (Applied Biosystems). Rabbits (Std: NZW, Japan SLC K.K., Hamamatsu) were immunized with the purified polypeptides to obtain antisera as described previously (*20*). IgG fractions precipitated with 50% ammonium sulfate from the antisera were further purified by Protein A-Sepharose 4FF chromatography. To obtain antibodies specific for each antigen, the IgGs were passed sequentially through HiTrap columns linked with the other three members of the syndecan family, and unbound fractions were finally applied to columns linked with the antigen itself. The bound materials were eluted with 0.1 M glycine HCl buffer, pH 3.0, the volumes were adjusted to the original serum volume, and the materials were then used as SN1Ab, SN3Ab, and SN4Ab. The specificities and avidities of the respective antibodies were tested by ELISA. The wells of a 96-well plate were coated with 2.5 μg/50 μl of the antigen polypeptides at 4°C overnight, and then blocked with 1% BSA/PBS at 37°C for 1 h. Antibodies at various concentrations were added and then the plates were incubated at 37°C for 1 h. After the wells had been washed with 0.05% Tween 20/PBS (PBST) 3 times, horseradish peroxidase-conjugated second antibodies were added and then the plates were incubated at 37°C for 1 h. Color was developed by the addition of H_2O_2 and TMB-ELISA (Life Technologies). The reaction was stopped by adding 2.0 M H_2SO_4, and then the absorbance at 450 nm was measured.

Western Blotting of Cell Surface Heparan Sulfate Proteoglycans—Cell surface proteoglycans were extracted from P29 and LM66-H11 cell layers with 2% Triton X-100/25 mM KCl/50 mM Tris-HCl, pH 7.3, containing 10 mM EDTA/10 mM *N*-ethylmaleimide/1 mM phenylmeth-

ylsulfonyl fluoride/0.036 mM pepstatin A as proteinase inhibitors, for 12 h on ice, and then purified as described previously (*20*). Briefly, after the removal of insoluble materials by centrifugation at 65,000 ×*g* for 30 min at 4°C, the proteoglycan fraction was obtained by DEAE-Sephacel column chromatography and then applied to an Octyl-Sepharose 4B column. The bound hydrophobic proteoglycans were eluted with a linear concentration gradient of 0–0.5% Triton X-100 in 4 M guanidinium HCl/50 mM Tris-HCl, pH 7.3. Cell surface heparan sulfate proteoglycans were purified from this fraction by immunoaffinity chromatography on an F58–10E4-linked HiTrap column. Samples were digested with heparitinase-I plus chondroitinase ABC to remove glycosaminoglycan side chains as described previously (*19*), and then subjected to SDS-PAGE followed by transfer to Hybond-P membranes (Amersham Pharmacia Biotech). The membranes were blocked with 10% skim milk in PBST for 1 h and then reacted with F69–3G10, SN1Ab, SN2Ab, SN3Ab, or SN4Ab for 1 h. After washing with PBST, the membranes were reacted with horseradish peroxidase-conjugated second antibodies for 1 h and then stained with Immunostain (Konica). Quantification of the bands was performed using the public-domain NIH Image program in a 256-gray-scale mode.

Amino Acid Sequence Analysis—The core proteins of cell surface heparan sulfate proteoglycans and recombinant polypeptides were electrophoresed, blotted onto ProBlott membranes (Applied Biosystems), and then stained with Ponceau S (Sigma-Aldrich). The stained bands were cut out and treated with 0.004% trypsin (Worthington Biochemical) at 37°C overnight. The peptides generated were separated by HPLC on a YMC-Pack AM303 column (Shimadzu, Kyoto) as described previously (*23*). The amino acid sequences of the peptide fragments were determined with a Model 492 protein sequencer (Applied Biosystems).

Treatment of Cells with Phosphatidylinositol-Specific Phospholipase C—Cells suspended in DMEM were digested with six concentrations (0, 0.5, 1, 5, 10, and 100 mU/ml) of phosphatidylinositol-specific phospholipase C at 37°C for 15 min, and then separated from the supernatants by centrifugation. The supernatants were desalted by dialysis against water and then concentrated by lyophilization. Aliquots of the cells were then inoculated onto fibronectin-coated cover glasses, incubated for 1 h in the presence or absence of the enzyme at the different concentrations, and then fixed and stained to visualize the actin cytoskeleton as described below. The hydrophobic proteoglycans were extracted from the remaining cells as described above. Samples were digested with or without heparitinase-I plus chondroitinase ABC, and the core proteins thus generated were electrophoresed and immunoblotted with F69–3G10 as described above.

Actin Cytoskeleton Staining—Cover glasses were coated with: (i) fibronectin (50 μg/ml); (ii) combinations of C-274 (500 μg/ml) and antibodies, *i.e.* SN1Ab (dilution, 1:900), SN2Ab (1:150), SN3Ab (1:150), or SN4Ab (1:15); or (iii) the individual antibodies alone overnight at 4°C, and then blocked with 0.2% BSA/PBS at room temperature for 30 min. The concentrations of the antibodies coating the cover glasses were determined as the avidity of each antibody, as calculated from the results of ELISA (Fig. 2A). Cells (5×10^3 cells in 50 μl of 0.2% BSA/DMEM) were inoculated onto the cover glasses and then incubated for 1 h at 37°C under a humidified 5% CO_2 atmosphere. They were then fixed in 3.7% paraformaldehyde containing 0.1% Tween-20 for 5 min, and treated in 0.1 M NH_4Cl for 10 min at room temperature. Actin filaments were stained with rhodamine-conjugated phalloidin (Molecular Probes) for 30 min at room temperature, and specimens were observed under a fluorescence microscope. When necessary, the number of cells per unit area was determined.

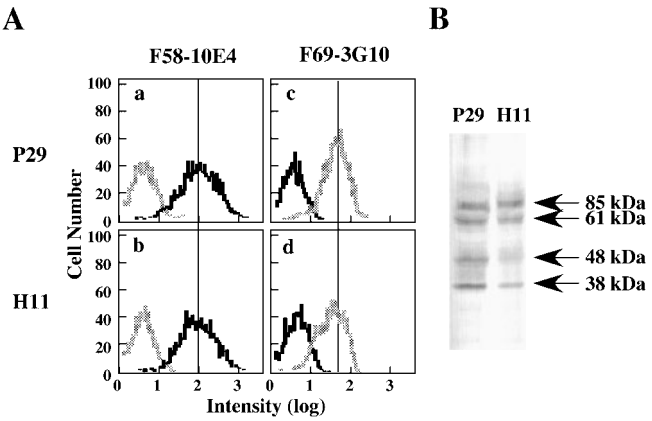
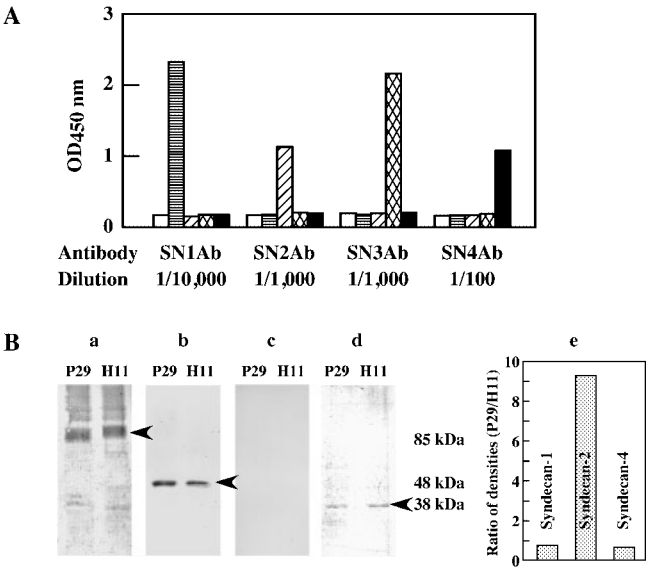


Fig. 1. **Expression of cell surface heparan sulfate proteoglycans on P29 and LM66-H11 cells.** (A) Flow cytometrical analysis. P29 (a, c) and LM66-H11 (H11) (b, d) cells were treated with (gray line) or without (black line) heparitinase-I (100 mU/ml) for 15 min at 37°C. The cells were reacted with F58-10E4 (a, b) or F69-3G10 (c, d) for 1 h at 4°C, followed by reaction with FITC-conjugated second antibodies for 30 min. The intensity of fluorescence was measured with a flow cytometer. (B) Western blot analysis. The hydrophobic heparan sulfate proteoglycans purified from P29 and LM66-H11 (H11) cells were digested with heparitinase-I (40 mU/ml) plus chondroitinase ABC (40 mU/ml) to obtain the core proteins, which were subjected to SDS-PAGE and then transferred to a membrane. The membrane was treated with F69-3G10 for 1 h, incubated with horseradish peroxidase-conjugated second antibodies for 1 h and then stained with Immunostain.

RESULTS

Identification of Cell Surface Heparan Sulfate Proteoglycan Species Expressed on P29 and LM66-H11 Cells—Our previous report (*21*) clearly demonstrated that the level of syndecan-2 expression is a primary factor in the induction of different types of actin cytoskeleton in Lewis lung carcinoma-derived cells adhered to a fibronectin substratum. However, the other members of the syndecan family were also transcribed in the two clones, although the levels in the two clones were very similar. Thus, we first analyzed the cell surface expression of all heparan sulfate proteoglycans. Flow cytometrical analysis demonstrated that the expression levels on the cell surface were not significantly different between the two clones (Fig. 1A). The epitope for F58–10E4, which recognized *N*-sulfated glucosamine in heparan sulfate (*24*), disappeared completely on heparitinase-I digestion (Fig. 1A, a and b). F69–3G10, on the other hand, only reacted after the enzyme digestion (Fig. 1A, c and d). This anti-



C $G^{43}FSLSDVP^{50}$
$L^{96}QA^{98}$
$T^{121}LQEAFPGAFGDLYTQN^{137}$
$L^{424}PEVMG^{429}$

Fig. 2. **Identification of cell surface heparan sulfate proteoglycans produced by P29 and LM66-H11 cells.** (A) Specificity and avidity of the synthesized antibodies against four mouse syndecans. The wells of a 96-well plate were coated with 2.5 μg of BSA (white bars), or the ectodomain of recombinant polypeptides of syndecan-1 (horizontally lined bars), -2 (slantly lined bars), -3 (crossed lined bars), or -4 (black bars). Various concentrations of SN1Ab, SN2Ab, SN3Ab and SN4Ab were reacted at 37°C for 1 h, followed by the addition of the horseradish peroxidase-conjugated second antibodies. Color was developed by TMB-ELISA. The data shown are representative in showing absorbance of around 1 to 2 at 450 nm. The fractions indicate the dilutions of the antibodies, the original serum volume being taken as 1. (B) Western blot analysis of syndecans. The proteoglycans purified from P29 and LM66-H11 (H11) cells were digested with heparitinase-I plus chondroitinase ABC to obtain the core proteins, which were subjected to SDS-PAGE, transferred to a membrane, and then immunostained with SN1Ab (a), SN2Ab (b), SN3Ab (c), or SN4Ab (d). The densities of the syndecan-1, -2, and -4 bands were quantified using the NIH Image program, and the ratios of the respective bands in P29 and LM66-H11 cells were calculated (e). (C) N-Terminal amino acid sequences of the four fragments derived from the 61 kDa protein (Fig. 1B) digested with trypsin. The numerals indicate the amino acid positions in mouse glypican-1.

body recognizes unsaturated disaccharides at the nonreducing ends of heparan sulfate chains, which are generated on heparitinase-I digestion. Therefore, its reactivity is thought to reflect the number of heparan sulfate chains. These results suggest that the difference in syndecan-2 expression between the two clones is small considering the expression of all cell surface heparan sulfate proteoglycans. We next analyzed the molecular species of cell surface heparan sulfate proteoglycans of the two clones. The core proteins generated from the hydrophobic proteoglycans on digestion with heparitinase-I plus chondroitinase ABC were separated by SDS-PAGE and then immunoblotted with F69-3G10 (Fig. 1B). Four bands corresponding to molecular masses of 85, 61, 48, and 38 kDa were detected for both clones. Among them, the 48 kDa protein exhibiting different expression levels in the two clones was identified as syndecan-2 (*25*). To identify the other materials, we produced polyclonal antibodies, SN1Ab, SN2Ab, SN3Ab, and SN4Ab, specific to recombinant polypeptides of the ectodomains of syndecan-1, -2, -3, and -4, respectively. The avidities of these four antibodies varied within a range of 10^2 in dilution (Fig. 2A). Western blot analyses with the respective antibodies revealed that the 85 and 38 kDa bands were syndecan-1 and -4, respectively (Fig. 2B, a and d), and confirmed that the 48 kDa band was syndecan-2 (Fig. 2B, b). Syndecan-3 was not detectable in either clone (Fig. 2B, c). Comparison of the individual band densities between P29 and LM66-H11 cells demonstrated that the expression levels are very similar between the two clones except for that of syndecan-2 (more than 9 times higher in P29 cells) (Fig. 2B, e), consistent with their mRNA expression levels (*21*).

To identify the 61 kDa band material, we attempted to determine its N-terminal amino acid sequence but were unable to do so, suggesting that the N-terminus of the intact molecule was blocked. Accordingly, we digested it with trypsin and obtained four fragments. As the N-terminal amino acid sequences of the fragments were homologous to partial sequences of mouse glypican-1, the 61 kDa band was identified as glypican-1 (Fig. 2C). With this procedure, using a sample prepared from 1.5×10^9 cells, the bands of syndecan-1, -2, and -4 were not detectable on separation by electrophoresis, suggesting that glypican-1 is the most abundant cell surface heparan sulfate proteoglycan. Therefore, we next examined the participation of glypican-1 in actin cytoskeletal organization.

Participation of Glypican-1 in Actin Cytoskeletal Organization—The glypican family is another type of cell surface heparan sulfate proteoglycan that anchors to the cell surface through glycosyl phosphatidylinositol. Glypicans can thus be released from the cell surface by digestion with phosphatidylinositol-specific phospholipase C (PI-PLC). To determine the possible participation of glypican-1 in actin cytoskeletal organization, cells were treated with PI-PLC to remove it. As expected, the 61 kDa band material disappeared from the cell surface on PI-PLC digestion (Fig. 3A, lanes 2 and 3), and three bands corresponding to molecular masses of 61, 59, and 56 kDa (Fig. 3A, lane 7) were observed in the supernatant. The latter two band materials were small in quantity, but were detected in the supernatant without PI-PLC-digestion (Fig. 3A, lane 6), suggesting that degradation or shedding due to proteolytic enzyme(s) occurred naturally. PI-PLC digestion affected neither stress fiber formation in P29 cells (Fig. 3B, a and c) nor cortex actin formation in LM66-H11 cells (Fig. 3B, b and d) on a fibronectin substratum. No effect was observed at any enzyme concentration (up to 100 mU/ml) (data not shown). These results indicate that glypican-1 does not play a role in the actin cytoskeletal organization in these cells on fibronectin, despite its predominance among heparan sulfate proteoglycans on the cell surface.

Ability of Each Syndecan to Cooperate with Integrin α5β1 to Induce Stress Fiber Formation—Using a mixed substratum comprising C-274 and SN2Ab, we previously demonstrated that stimulation of syndecan-2 through its core protein was equivalent to that through its heparan

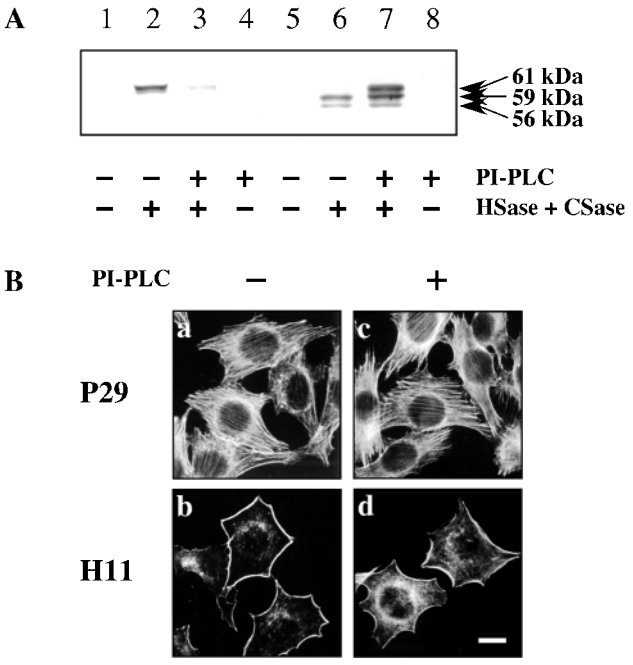


Fig. 3. **Participation of glypican-1 in actin cytoskeletal organization.** (A) Western blot analysis of glypican-1 in P29 cells before and after digestion with phosphatidylinositol-specific phospholipase C (PI-PLC). P29 cells were treated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) 10 mU/ml of PI-PLC for 15 min at 37°C. Cell (lanes 1–4) and supernatant (lanes 5–8) fractions were then treated with (lanes 2, 3, 6, and 7) or without (lanes 1, 4, 5, and 8) heparitinase-I (HSase) plus chondroitinase ABC (CSase). After being subjected to SDS-PAGE, samples were immunoblotted with F69-3G10. (B) Effect of PI-PLC digestion on the actin cytoskeletal organization of P29 and LM66-H11 cells on a fibronectin substratum. P29 (a, c) and LM66-H11 (H11) (b, d) cells treated with (c, d) or without (a, b) PI-PLC as described in (A) were inoculated on cover glasses coated with fibronectin, incubated at 37°C for 1 h, fixed and then stained with rhodamine-conjugated phalloidin. Bar, 20 μm.

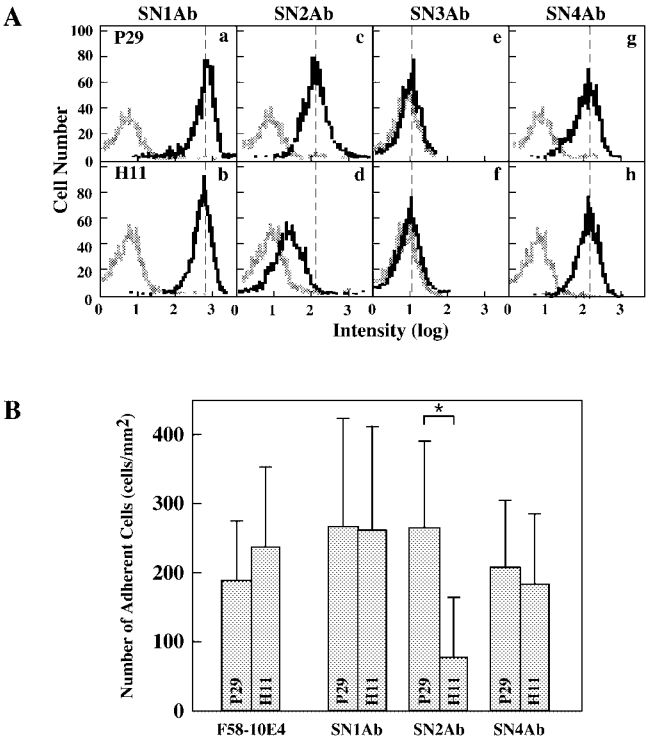


Fig. 4. **Comparison of cell surface expression of syndecans and cell adhesion to antibody-substrata between P29 and LM66-H11 cells.** (A) Flow cytometrical analyses of syndecans. P29 (a, c, e, g) and LM66-H11 (H11) (b, d, f, h) cells were treated with SN1Ab (a, b), SN2Ab (c, d), SN3Ab (e, f), or SN4Ab (g, h) for 1 h at 4°C, and then with FITC-conjugated second antibodies for 30 min. The intensity of fluorescence was measured with a flow cytometer. The peaks with gray lines are for control samples reacted with nonimmune serum as the first antibodies. (B) Attachment of cells to immobilized antibodies. P29 and LM66-H11 (H11) cells were inoculated onto cover glasses coated with F58–10E4, SN1Ab, SN2Ab or SN4Ab, and then incubated at 37°C for 1 h. Cells that become attached were fixed and stained with rhodamine-conjugated phalloidin. The number of cells adhering to each substratum was determined in six randomly selected areas in two different specimens. The asterisk indicates a significant difference ($p < 0.0001$).

sulfate side chains in actin cytoskeletal organization (*21*). Therefore, we carried out a similar assay to clarify the participation of the individual syndecans in this signaling. Firstly, we examined the cell surface expression of the individual syndecans (Fig. 4A). The expression levels of syndecan-1 (Fig. 4A, a and b) and syndecan-4 (Fig. 4A, g and h) were very similar, but syndecan-3 expression was scarcely observed in either clone (Fig. 4A, e and f). Only syndecan-2 exhibited significantly different expression levels between the two clones (about 10 times higher in P29 cells) (Fig. 4A, c and d). These results are consistent with the translation (Figs. 1B and 2B) and transcription levels (*21*). Next, to verify that the immobilized antibodies were able to function as solid ligands, we carried out a cell adhesion assay (Fig. 4B). P29 cells adhered to SN1Ab, SN2Ab, and SN4Ab at the same levels as to an F58-10E4 substratum, indicating that each substratum is able to function in the binding of cells through the respective antigens. Only the adhesion of LM66-H11 cells to SN2Ab was significantly lower than that of P29 cells ($p < 0.0001$), whereas the adhesion to other substrata was similar for the two clones, suggesting that cell adhesion to the immobilized antibodies reflected the cell surface expression levels of syndecans. We then analyzed the cytoskeletal organization of the cells on substrata comprising C-274 and each antibody.

On C-274, both cell types formed cortex actin, indicating that the signal mediated by integrin α5β1 alone resulted in cortex actin formation (Fig. 5, a and b). As expected from the very low expression levels of syndecan-3, SN3Ab did not affect this signaling pathway (Fig. 5, g and h), and the cells showed similar responses to those on C-274 alone. The fact that P29 cells, which hardly express syndecan-3, form stress fibers on a fibronectin substratum indicates that syndecan-3 is not essential for stress fiber formation. In spite of the higher expression levels of syndecan-1, both cell types on the substratum containing SN1Ab (Fig. 5, c and d) showed similar responses to those on C-274 alone, indicating that the stimulation of syndecan-1 was not sufficient to modify the integrin α5β1-signaling. SN2Ab was a sufficient stimulus for the induction of stress fiber formation in P29 cells (Fig. 5e) but not in LM66-H11 cells (Fig. 5f), as shown in our previous report (*21*), indicating that the

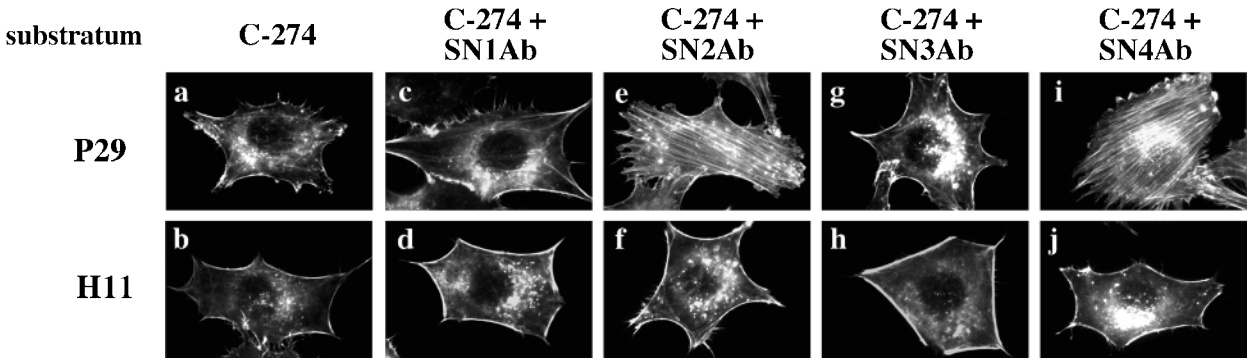


Fig. 5. **Ability of each syndecan to modify the signal through integrin α5β1 in actin cytoskeletal organization.** P29 (a, c, e, g, i) and LM66-H11 (H11) (b, d, f, h, j) cells were inoculated onto cover glasses coated with C-274 (a, b), or a mixture of C-274 and SN1Ab (c, d), SN2Ab (e, f), SN3Ab (g, h), or SN4Ab (i, j), and then incubated at 37°C for 1 h. Cells were fixed and stained with rhodamine-conjugated phalloidin. The photographs presented show typical actin cytoskeletal organization in the cells. Almost all the cells on each substratum exhibited the respective patterns in the figures.

ability of syndecan-2 to modify the signal through integrin α5β1 depends on its expression level. Although cell adhesion to SN2Ab reflected the expression level of syndecan-2 in the cells (Fig. 4B), the number of adherent cells on a mixed substratum of C-274 and SN2Ab was almost the same for the two clones (data not shown). This was expected because 91% of the P29 cells and 85% of the LM66-H11 cells adhered to the C-274 substratum (*21*).

Interestingly, the cytoskeletal organizations of the cells on a mixed substratum of C-274 and SN4Ab (Fig. 5, i and j) were very similar to those on C-274 and SN2Ab, in spite of the similar expression levels of syndecan-4 (Fig. 4A, g and h). The fact that P29 cells formed stress fibers on the mixed substratum of C-274 and SN4Ab indicated that stimulation of syndecan-4 by the antibodies was sufficient to modify the signal through integrin α5β1 (Fig.

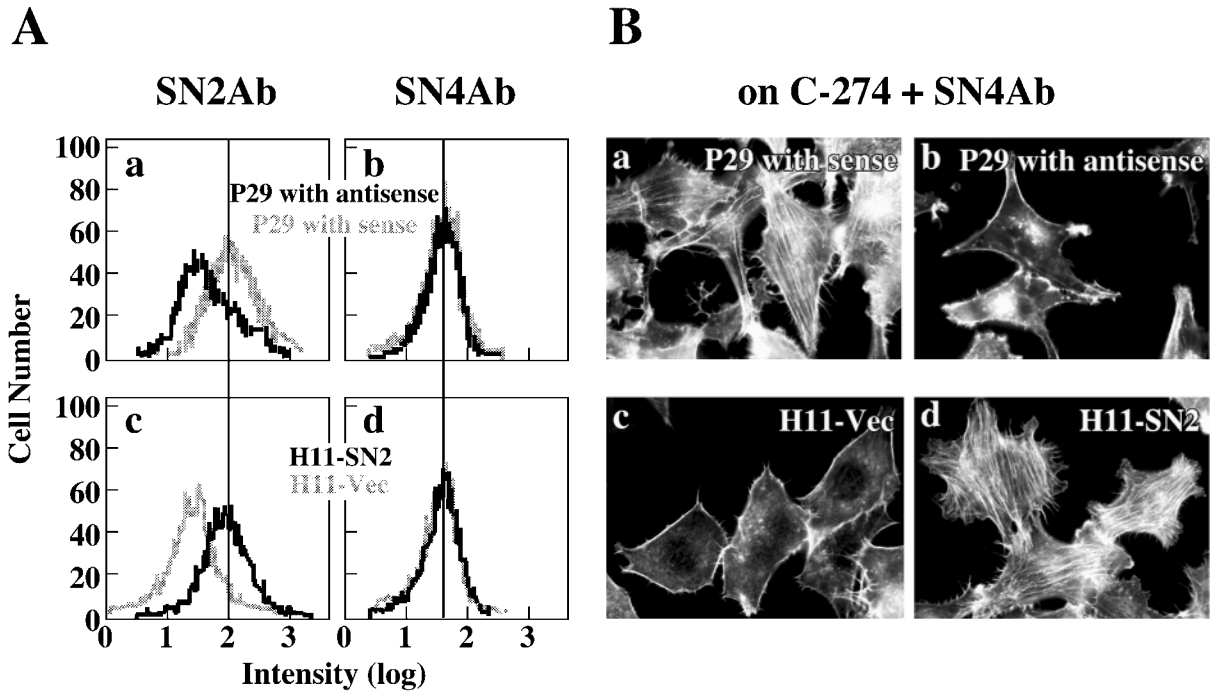


Fig. 6. **Requirement of syndecan-2 expression for stress fiber formation induced by syndecan-4-stimulation.** (A) Flow cytometrical analyses of syndecan-2 and -4 expression on cells in which syndecan-2 expression had been manipulated artificially. P29 cells (a, b) pretreated with antisense (black line) or sense (gray line) oligonucleotides of syndecan-2 mRNA for 4 days, and the cloned transfectant of LM66-H11 cells (c, d) with cDNA of syndecan-2 (H11-SN2, black line) or the vector only (H11-Vec, gray line), were treated with SN2Ab (a, c) or SN4Ab (b, d) for 1 h at 4°C, and then treated with FITC-conjugated second antibodies for 30 min. The intensity of fluorescence was measured with a flow cytometer. (B) Actin cytoskeleton in cells on a mixed substratum of C-274 and SN4Ab. P29 cells treated with sense (a) or antisense (b) oligonucleotides of syndecan-2 mRNA and H11-Vec (c) or H11-SN2 (d) were inoculated onto cover glasses coated with the mixed substratum, and then incubated at 37°C for 1 h. The cells were fixed and stained with rhodamine-conjugated phalloidin.

5i). However, LM66-H11 cells were not induced to form stress fibers (Fig. 5j), suggesting that the signal through syndecan-4 is correlated with syndecan-2 in an expression level-dependent manner, and that syndecan-2 might be situated downstream of syndecan-4. These results suggest the possibility of crosstalk between syndecan-2 and syndecan-4.

Implication of Syndecan-2 in the Syndecan-4 Signaling Pathway—To assess the possible interaction between the two species of syndecans, cells in which syndecan-2 expression was artificially regulated were plated on a substratum comprising C-274 and SN4Ab. Treatment of P29 cells with the antisense oligonucleotide of syndecan-2 mRNA for 4 days resulted in suppression of syndecan-2 expression (Fig. 6A, a), but did not significantly affect the expression of syndecan-4 (Fig. 6A, b) or syndecan-1 (data not shown). Conversely, transfection of syndecan-2 cDNA into LM66-H11 cells increased the expression of syndecan-2 (Fig. 6A, c), but not that of syndecan-4 (Fig. 6A, d) or syndecan-1 (data not shown). The cells with higher expression levels of syndecan-2, regardless of the clone or manipulation, were able to induce stress fiber formation on a mixed substratum of C-274 and SN4Ab (Fig. 6B, a and d). In contrast, the cells with low expression levels of syndecan-2 failed to form stress fibers but formed cortex actin instead (Fig. 6B, b and c). These results confirmed that the expression level of syndecan-2 regulates the signal through syndecan-4, strongly suggesting that there is crosstalk between the two syndecans, and that syndecan-2 might be situated downstream of syndecan-4.

DISCUSSION

Our previous reports (*20*, *21*) clearly demonstrated that the signal organizing the actin cytoskeleton in Lewis lung carcinoma-derived cells adhering to a fibronectin substratum is mediated through integrin α5β1 and syndecan-2 in a dual receptor system. Signal transduction is possible both independently and cooperatively for each receptor. Thus, a signal mediated only by integrin α5β1 or syndecan-2 resulted in either the formation of cortex actin or filopodia, whereas a signal mediated by both receptors resulted in stress fiber formation. Furthermore, it became evident that the signals had no effect when the expression level of syndecan-2 had not reached a threshold, regardless of whether syndecan-2 acts independently or cooperatively with integrin α5β1. In our earlier study, we also found that other members of the syndecan family are transcribed in these cells. This prompted us to explore the possibility of their participation in this signaling pathway.

In the present study, we demonstrated that the two clones expressed four types of cell surface heparan sulfate proteoglycans; syndecan-1, syndecan-2, syndecan-4, and glypican-1. The most predominant one, glypican-1, clearly showed no participation in the signaling pathway linked to actin cytoskeletal organization in Lewis lung carcinoma cells. Functional differences between syndecans and glypicans have been demonstrated by several studies involving transfection techniques. For example, overexpression of syndecan-1, -2, or -4 on ARH-77 cells resulted in non-invasive and type I collagen-binding cells, whereas glypican-1 overexpressers were invasive and did not bind to collagen as the parent cells did (*26*). Moreover, 293T cells transfected with syndecan-2 or -4, but not glypican-1, adhered to the heparin binding domain of the laminin α3 chain (*27*). These phenomena might be expected since signaling mediated by syndecans is thought to be transduced through their clustering, which is caused extracellularly by binding with ligands and intracellularly by binding with PDZ proteins (*28–32*), whereas glypicans are not transmembrane-type but glycosyl phosphatidylinositol-anchored proteoglycans. From these results, it is clear that glypicans perform a function(s) distinct from that/these of syndecans.

The second question is whether the individual members of the syndecan family co-existing on the same cells function differently. To clarify this, there are the following three requirements: (i) cells exhibiting expression of multiple syndecans; (ii) tools able to stimulate individual syndecans; and (iii) a bioassay system. The Lewis lung carcinoma system used here satisfies all three criteria: (i) Lewis lung carcinoma cells express syndecan-1, -2, and -4; (ii) we have prepared antibodies that can act as solid ligands for the respective syndecans; and (iii) we have already established an assay system for distinguishing different signaling. The results obtained here clearly show that each syndecan plays a different role. Syndecan-1 does not appear to be essential for the signal transduction that is dependent on substratum adhesion. The fact that syndecan-1 is located on the lateral surface of basal cells in cuboidal and columnar epithelia (*33*) would suggest that it plays a role in cell-to-cell adhesion. This idea is supported by the reports of the loss of syndecan-1 in poorly differentiated tumors (*34*, *35*).

The participation of syndecan-4 as a cell adhesion receptor in actin cytoskeletal organization is very interesting. The stimulation by immobilized C-274 and SN4Ab was sufficient to induce stress fiber formation in P29 cells but not in LM66-H11 cells, despite the similar levels of syndecan-4 expression. Furthermore, the two clones with artificially altered syndecan-2 expression levels formed converse actin cytoskeletal organizations on the same substratum. These results strongly suggest that the signal through syndecan-4 is regulated downstream by syndecan-2. Couchman and colleagues found that binding of phosphatidylinositol-4,5-bisphosphate (PIP_2) to the variable region of the cytoplasmic domain of syndecan-4, but not that of syndecan-2, regulates protein kinase Cα (PKCα) activity, and that the activated PKCα recruits cytoplasmic proteins to focal adhesions (*15*). Zimmermann *et al.* reported that the PDZ domains of syntenin, which are able to bind to the EFYA sequence common to all members of the syndecan family, concentrate PIP_2 and syndecans at the plasma membrane (*36*). It has also been reported that a synthetic peptide of the syndecan-2 cytoplasmic domain, but not that of syndecan-4, can be a substrate for PKCα, and that the level of phosphorylation is dependent on multimerization of the peptide (*37*, *38*). Furthermore, we previously demonstrated that a serine residue(s) of the syndecan-2 cytoplasmic domain purified from P29 cells is phosphorylated (*25*). Taken together, these results suggest that the signal through syndecan-4 is regulated downstream by syndecan-2 in a quantity-dependent manner, and that there may be a possible cascade in which PKCα activated by

syndecan-4 phosphorylates the cytoplasmic domain of syndecan-2. This supposition strongly supports the report (*39*) that syndecan-4-null fibroblasts form focal adhesions and stress fibers on a fibronectin substratum, even if syndecan-4 is evident in focal adhesions of normal fibroblasts, such as human and rat embryo fibroblasts (*13*). In this signal transduction, syndecan-2, rather than syndecan-4, seems to be a key molecule.

One possible origin of such a functional difference among syndecans is the specificity of the ligand binding site of heparan sulfate of each molecule. Among the cell surface heparan sulfate proteoglycans produced by P29 cells, syndecan-2 accounts for more than 85% of the syndecans that can bind to immobilized fibronectin (*19*). Furthermore, the cells bind to the Hep-II domain of fibronectin through the $[IdoA(2OS)\text{-}GlcNS(6OS)]_6$ structure in heparan sulfate chains (*20*). At least in Lewis lung carcinoma cells, this structure or cluster(s) of the structure in heparan sulfate side chains may be unique to syndecan-2. The functional specificity of syndecans can also be explained by the difference in cytoplasmic proteins bound to the cytoplasmic domain of each syndecan stimulated with extracellular ligands. Syntenin (*28*, *32*), CASK/LIN-2 (*29*, *30*), and synectin (*31*) can bind to the EFYA sequence common to all members of the syndecan family through their PDZ domains. However, as with synbindin (*40*) and ezrin (*41*, *42*) for syndecan-2 as well as syndesmos (*43*) for syndecan-4, several cytoplasmic proteins perhaps bind specifically to their cytoplasmic variable regions. Thus, the entire spectrum involving both combinations of various extracellular and intracellular binding proteins and the specific phosphorylation of their cytoplasmic domains appears to play a role in determining the functional specificity of each syndecan. In any case, this is the first report that clearly demonstrates that cell surface heparan sulfate proteoglycans expressed on the same cell function differently and clarifies the cooperation of syndecan-2 and syndecan-4 in actin cytoskeletal organization.

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