The role of syndecan-2 in regulation of actin-cytoskeletal organization of Lewis lung carcinoma-derived metastatic clones

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INTRODUCTION

The extracellular matrix (ECM) is an extremely complex supramolecular structure, each of its components comprises several domains to allow them to interact with each other or with other ECM constituents and with cells. Cells generally interact with the ECM formed by themselves; epithelial cells interact with basement membranes and mesenchymal and stromal cells with interstitial-type matrix, but occasionally with allogenic ECM. Epithelial–mesenchymal or epithelial–stromal interactions play an important role in both physiological and pathological situations such as embryonic morphogenesis [1,2], wound healing [3] and tumorigenesis [4], and are accompanied by dynamic changes of ECM [5,6] to generate new cell–matrix interactions. In a metastatic process, tumour cells are always forced to interact with allogenic ECMs and these interactions are presumed to be important for the completion of metastasis.

The information encoded in an ECM is revealed by the interaction between individual ECM components and their respective receptors on the cell surface. It is well established that cell contact with a single ECM component initiates multiple signals affecting both cell behaviour and gene expression [7,8]. The interactions between fibronectin and integrin α5β1 have been studied extensively [9–11]. Their binding triggers the assembly of many cytoskeletal signalling proteins to form focal adhesions and re-organize actin cytoskeletons [12,13]. Recently, it has become clear that syndecans, a family of transmembrane heparan sulphate proteoglycans whose external glycosaminoglycan chains can bind various extracellular soluble and insoluble ligands and whose core protein cytoplasmic domain can transduce signals into intracellular events, can modify signals generated by integrin-mediated cell adhesion to fibronectin [14]. Syndecan-4 signals co-operatively with integrins for the assembly of focal adhesions in fibroblasts inoculated on a fibronectin substrate [15,16] in a Rho-dependent manner [17]. Although details of the signalling processes remain to be determined, several components, which appear to be involved in the signalling, have been identified. Couchman and co-workers [18–20] have demonstrated that the variable region in the cytoplasmic domain of syndecan-4 binds phosphatidylinositol 4,5-bisphosphate and protein kinase Cα (PKCα), and this interaction mediates its own multimerization, with resultant regulation of the intracellular distribution and activity of PKCα. Screening of a yeast two-hybrid library with a syndecan cytoplasmic domain identified several PDZ-containing proteins, syntenin [21], CASK/LIN-2 [22], synectin [23] and synbindin [24], which bind to the COOH-terminal EFYA sequence of syndecans, and syndemos, which binds to both the membrane proximal and the variable regions [25].

Recently, we demonstrated that Lewis lung carcinoma-derived low-metastatic P29 cells induced actin stress-fibre formation on adhesion to a fibronectin substrate [26]. The formation of actin

Key words: actin stress fibres, cell adhesion, fibronectin-recombinant polypeptides, heparan sulphate proteoglycans, integrin α5β1.

Abbreviations used: ECM, extracellular matrix; PKCα, protein kinase Cα; DMEM, Dulbecco’s modified Eagle’s medium; FCS, foetal calf serum; RT, reverse transcription.

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stress fibres was completely inhibited by the addition of GRGDS peptide, heparin, anti-heparan sulphate monoclonal antibody or heparan sulphate oligomer [IdoA(2-OSO₂)-GlcNSO₄(6-OSO₂)]₇, or treatment of the cells with heparitinase-I, suggesting that the signal to form actin stress fibres was transduced by a dual-receptor system with integrin α5β1 and heparan sulphate proteoglycans. Moreover, the fact that the selective suppression of the expression of syndecan-2 with an antisense oligonucleotide for the core protein of this proteoglycan resulted in the failure to form actin stress fibres, indicated that the syndecan-2 is an essential component for the induction of this cytoskeletal organization. These results are consistent with our previous findings that more than 85% of the cell-surface heparan sulphate proteoglycans purified from P29 cells by fibronectin affinity is syndecan-2 [27] and that among these proteoglycans only syndecan-2 has a serine residue(s) in the cytoplasmic portion of the core protein, which is phosphorylated [28].

The highly metastatic LM66-H11 clone derived from the same carcinoma from which the P29 clone was isolated, was established after 66 episodes of an in vitro selection on the basis of spontaneous metastatic potential, exhibiting an ECM-dependent tumorigenesis distinctly different from the P29 clone [27]. Thus P29 cells with a capacity to elicit a host-stromal response, exhibit tumorigenesis dependently on the well-organized basement membrane formed by tumour cells themselves [29–31]. Therefore in the LM66-H11 tumour, the tumour cells encounter fibronectin only when they are confronted with the basement membrane of tumour blood vessels, whereas P29 cells are constantly interacting with fibronectin in the immediate surroundings. In the present study, we demonstrated that cellular responses of the two clones to adhere on a fibronectin substratum in vitro reflected the difference in their ECM dependence in tumorigenesis and the difference of the responses was attributable to the expression level of syndecan-2 on the cells to modify the integrin α5β1-mediated signal. To determine the ligands which initiate signals and their respective receptors, we used as substrata the fibronectin-recombinant polypeptides, RGD-containing cell-binding domain (designated as C-274), C-terminal heparin-binding domain (H-271) and a fusion polypeptide of both (CH-271). Moreover, we demonstrated that overexpression of syndecan-2 in LM66-H11 cells resulted in the formation of stress fibres on fibronectin as did the P29 cells. These clones of epithelial origin do not produce fibronectin [32], thus providing a useful system for this type of analysis without any complications due to synthesis of endogenous fibronectin.

MATERIALS AND METHODS

Materials

Human-plasma fibronectin was purchased from Iwaki Glass (Tokyo, Japan). Three species of fibronectin-recombinant polypeptides (Figure 1), i.e. C-274 (Pro₁₁²⁹,Ser₁₁³¹) with the RGD-containing Cell-I domain, H-271 (Ala₁⁰⁰-Thr₁³⁶⁹) with the C-terminal heparin-binding Hep-II domain and CH-271, a fusion polypeptide of C-274 and H-271 with a methionine residue were generously supplied by TaKaRa Biomedicals (Otsu, Japan). Amino-acid sequences used here are numbered according to the system of Kornblitt et al. [33]. Goat anti-human integrin α5β1 antibody (AB1950) was purchased from Chemicon International (Temecula, CA, U.S.A.). Anti-heparan sulphate monoclonal antibody, F58-10E4, was a gift from Dr K. Yoshida of Seikagaku Corp., Tokyo, Japan. The rabbit anti-mouse syndecan-2 antibody (SN2Ab), specific to the ectodomain of syndecan-2, was prepared as described previously [26]. [Me²⁺H]thymidine (3.1 TBq/mmol), [%³⁵S]hosphate (carrier-free) and [%³²P]dCTP (111 TBq/mmol) were purchased from DuPont–NEN Research Product (Boston, MA, U.S.A.).

Cell culture

The low-metastatic P29 and highly metastatic LM66-H11 cells cloned from a murine Lewis lung carcinoma 3LL [27] were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) (Gibco, NY, U.S.A.), streptomycin (100 µg/ml) and penicillin (100 units/ml) at 37 °C in a humidified 5% CO₂ atmosphere. The syndecan-2 transfectants used in the present study were cultured in DMEM supplemented with 10% FCS G418 (800 µg/ml), streptomycin (100 µg/ml) and penicillin (100 units/ml) at 37 °C in a humidified 5% CO₂ atmosphere. The cells were harvested after incubation with 2mM EDTA in PBS (EDTA/PBS) for 10 min at 37 °C, followed by gentle flushing with a pipette and subcultured twice a week.

Metastasis assay and immunostaining of primary tumour tissues

The metastatic potentials of the clones were measured by injecting single-cell suspensions of 1.0 × 10⁵ or 2.0 × 10⁵ cells in 200 µl of DMEM into the tail vein intravenously or subcutaneously to the right abdominal flank of syngenic C57BL/6 6-week-old male mice, respectively. After 4 weeks, the animals were killed and the number of visible parietal nodules in the lung, fixed in Bouin’s solution, were counted.

The subcutaneous primary tumours were excised. Fresh frozen sections of 7 µm thickness were blocked with 1% normal goat serum (NGS, G-4877, Molecular Probes) and rabbit anti-human syndecan-2 antibody, F58-10E4, was a gift from Dr K. Yoshida of Seikagaku Corp., Tokyo, Japan. The rabbit anti-mouse syndecan-2 antibody (SN2Ab), specific to the ectodomain of syndecan-2, was prepared as described previously [26]. [Me²⁺H]thymidine (3.1 TBq/mmol), [%³⁵S]hosphate (carrier-free) and [%³²P]dCTP (111 TBq/mmol) were purchased from DuPont–NEN Research Product (Boston, MA, U.S.A.).

Actin-cytoskeleton staining

Cells (5 × 10⁵ cells in 50 µl of 0.2% BSA/DMEM) were inoculated on coverslips that had been coated with fibronectin (50 µg/ml), fibronectin-recombinant polypeptides (100 µg/ml) or combinations of C-274 (500 µg/ml) and antibodies 58-10E4 (15 µg/ml) or SN2Ab (0.7 µg/ml) overnight at 4 °C and blocked with 0.2% BSA/PBS for 30 min at room temperature. The cells were incubated for 1 h and then fixed in 3.7% paraformaldehyde containing 0.1% (v/v) Tween 20 for 5 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 fluorescent microscope.

Cell-adhesion assay

Cells growing in a logarithmic phase were incubated with [Me²⁺H]thymidine (74 kBq/ml) for 12 h. After washing thrice with PBS, the cells were harvested with EDTA/PBS. The radio-labelled cells (1 × 10⁴ cells) were suspended in 100 µl of 0.2%
Figure 1  Schematic models of fibronectin and the recombinant polypeptides used as substrata

Homology domain structures in fibronectin are represented by I, II, III and IIIICS for type-I, -II, and -III homology units, and type-III homology connecting segment, respectively. Functional heparin-, fibrinogen-, collagen- and cell-binding domains are abbreviated as Hep, Fib, Col and Cell, respectively. The open arrowhead shows the position of the SRGDS sequence. The recombinant polypeptide, C-274, was designed with 8th to 10th type-III homology and H-271 with 12th to 14th. CH-271 is a fusion form of C-274 and H-271 with a methionine residue (M). ED-A and ED-B indicate that extra domains arise from alternative splicing.

BSA/DMEM and inoculated in 96-well plates coated with fibronectin (50 μg/ml) or its recombinant polypeptides (100 μg/ml). After incubation for 1 h at 37°C, the plates were agitated on a microtube mixer at 1000 rev./min for 2 min to suspend unattached and loosely attached cells. The attached and unattached cells were solubilized separately in 1% SDS in 0.5 M NaOH. The radioactivity of an aliquot of each sample was measured in a liquid-scintillation counter (Beckman LS-9000).

Flow-cytometric assay

Cells (3 x 10⁶ cells suspended in 50 μl of 0.2% BSA/DMEM) were incubated with antibodies or respective non-immune sera for 1 h at 4°C with gentle agitation. After washing thrice with PBS, they were exposed to a FITC-conjugated second antibody for 30 min. The labelled cells were washed and the intensity of fluorescence was measured in a flow cytometer, Ortho Cytoron (Ortho Diagnostic Systems K.K.).

RT (reverse transcription)-PCR and Northern-blot analyses

Poly(A)+ RNAs were isolated from 1 x 10⁶ cells of each clone, using a QuickPrep micro mRNA Purification Kit (Amersham Pharmacia Biotech) and analysed by RT-PCR using a TaKaRa RNA LA PCR Kit (TaKaRa Biomedicals) in a thermal cycler, GeneAmp PCR System 9600-R (PerkinElmer). The amounts of poly(A)+ RNA templates (50 ng) and cycle numbers (28 cycles) for amplification were chosen in quantitative ranges where the reactions proceeded linearly. Temperatures and time periods employed for melting, annealing and extension were 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 1.5 min, respectively. Oligodeoxyribonucleotide primers employed were as follows: 5’-GAGAATTCACGCACGAGCTGAC-3’ (nucleotides 170–191 plus EcoRI adapter) and 5’-GAGTCGATTCGAGAGACTGAGG-3’ (complement of nucleotides 498–520 plus PstI adapter) for mouse syndecan-3 (U52826); 5’-GAGAATTTCGC-GAGTCGAGTCGAGACTGAGG-3’ (nucleotides 69–23 plus EcoRI adapter) and 5’-GACTGAGTGACCCAGCANCGACGAGC-3’ (complement of nucleotides 409–967), syndecan-2 (847–1175), syndecan-3 (370–931), or syndecan-4 (92–534), which had been labelled with [32P]dCTP by the random labelling method. After hybridization for 1 h at 42°C, the membranes were exposed to X-ray films and the films were scanned by CanoScan 600 (Canon, Tokyo). The amplification of the bands was performed with the public-domain NIH Image program in 256-grey-scale mode.

Transfection of syndecan-2 cDNA into LM66-H11 cells

The mouse syndecan-2 cDNA containing the entire coding region was amplified by RT-PCR with poly(A)+ RNA form P29 template as use 5’-GAGGATCCCTCTTTATATCCGGGTAGAG-3’ and 5’-GAGAATTCAGGTTCAAGGATGCTCTCCTATAGT-3’ (complements of nucleotides 487–1175) plus EcoRI sites respectively as indicated with underlines. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (5 μg/ml) and visualized under UV light. The RT-PCR products were sequence-verified.

Northern-blot analysis was performed as described previously [35]. In brief, 2 μg each of poly(A)+ RNAs from both clones were electrophoresed on a 1.5% agarose gel containing 1.1 M formaldehyde, transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech), and hybridized to a cDNA fragment of syndecan-1 (nucleotides 409–967), syndecan-2 (847–1175), syndecan-3 (370–931), or syndecan-4 (92–534), which had been labelled with [32P]dCTP by the random labelling method. After hybridization for 14 h at 42°C, the membranes were exposed to X-ray films and the films were scanned by CanoScan 600 (Canon, Tokyo). The quantification of the bands was performed with the public-domain NIH Image program in 256-grey-scale mode.
Affinity chromatography of purified syndecan-2

Sulphate-labelled syndecan-2 was isolated from both the clones as described below. P29 and LM66-H11 cells were labelled with [35S]sulphate (7.4 MBq/ml) in 10% FCS/Ham’s F-12 for 24 h at 37°C in a humidified 5% CO₂ atmosphere. The radio-labelled macromolecules were extracted from the cell layer with 7 M urea/0.5% (v/v) Triton X-100/0.15 M NaCl/50 mM Tris/HCl, pH 7.3, containing protease inhibitors, 10 mM EDTA/10 mM N-ethylmaleimide/1 mM PMSF/0.036 mM pepstatin A, for 12 h on ice. After removal of insoluble materials by centrifugation at 65000 g for 30 min at 4°C, the proteoglycan fraction was obtained on DEAE-Sepharose column chromatography and then applied to an Octyl-Sepharose 4B column. The bound hydrophobic proteoglycans were eluted with a linear-gradient concentration of 0–0.5 M NaCl in 0.5 column. Elution was with a linear gradient concentration of 0–0.5 Mglycine with 1 M NaCl obtained were applied to a fibronectin-linked Sepharose 4B column. Protein A-Sepharose was added and mixed. One hour later, the samples was precipitated from this hydrophobic proteoglycan fraction with SN2Ab, as described previously [36]. Briefly, the sample was mixed with SN2Ab in 0.15 M NaCl/0.1% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3. Syndecan-2 was precipitated from this hydrophobic proteoglycan fraction with SN2Ab, as described previously [36]. Briefly, the sample was mixed with SN2Ab in 0.15 M NaCl/0.1% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, for 1 h at room temperature, and 50% Protein A-Sepharose was added and mixed. One hour later, the beads were precipitated by a centrifugation and washed with 1 M NaCl/0.1% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3. Syndecan-2 was eluted by 0.1% (v/v) Triton X-100/0.1 M glycine/HCl, pH 2.0, followed by neutralization with 1 M Tris/HCl, pH 9.0. The [35S]-labelled syndecan-2 samples thus obtained were applied to a fibronectin-linked Sepharose 4B column. Elution was with a linear gradient concentration of 0.1–0.5 M NaCl in 0.5% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3.

RESULTS

Metastatic potential and tumorigenesis of P29 and LM66-H11 clones

The metastatic potentials of the two clones used in the present study are shown in Table 1. Irrespective of the sites of injection, a significant difference in the number of lung foci was observed between the clones. These clones also exhibited strikingly different patterns in the localization of fibroactin in primary tumours (Figure 2). The low-metastatic P29 cells having a strong ability to induce host-stromal response [31] were immediately surrounded by a fibronectin-rich interstitial-type matrix formed by the induced host-stromal cells (Figure 2A). On the contrary, in the tumour formed by the highly metastatic LM66-H11 cells lacking the capacity to induce the host-stromal response, fibronectin was hardly observed in the extracellular compartment of tumour parenchyma except in the endothelial basement membrane of tumour blood vessels (Figure 2B). The results indicate that each clone grew in quite different microenvironments in vivo, especially in the interaction with fibronectin, which was an allogenic ECM component for them.

Table 1 Lung metastatic potentials of P29 and LM66-H11 clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Route of injection</th>
<th>No. of cells injected (× 10⁵)</th>
<th>No. of lung foci</th>
<th>Incidence</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>P29</td>
<td>i.v.</td>
<td>1.0</td>
<td>3/7</td>
<td>0.4</td>
<td>0–0–1</td>
<td></td>
</tr>
<tr>
<td>LM66-H11</td>
<td>i.v.</td>
<td>1.0</td>
<td>7/7</td>
<td>516.2</td>
<td>467–744</td>
<td></td>
</tr>
<tr>
<td>P29</td>
<td>s.c.</td>
<td>2.0</td>
<td>0/7</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM66-H11</td>
<td>s.c.</td>
<td>2.0</td>
<td>7/7</td>
<td>35.2</td>
<td>23–56</td>
<td></td>
</tr>
</tbody>
</table>

† i.v., intravenously.
‡ s.c., subcutaneously.
Syndecan-2-regulating actin-cytoskeletal organization

Figure 2 Localization of fibronectin in primary tumours

Primary tumours formed by subcutaneous injection of P29 (A) and LM66-H11 (B) cells were treated with antibody against fibronectin. The reactive components were visualized by an FITC-conjugated second antibody (green) and the nuclei were counterstained with propidium iodide (red). Arrowheads show basement membranes of tumour blood vessels. Bar, 50 \( \mu \)m.

Table 2 Adhesion of cells to substrata of fibronectin and fibronectin-recombinant polypeptides

<table>
<thead>
<tr>
<th>Substratum</th>
<th>P29</th>
<th>LM66-H11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>83.8 ± 1.88</td>
<td>81.3 ± 4.38</td>
</tr>
<tr>
<td>CH-271</td>
<td>81.3 ± 4.38</td>
<td>72.5 ± 3.75</td>
</tr>
<tr>
<td>C-274</td>
<td>76.3 ± 3.50</td>
<td>68.8 ± 1.88</td>
</tr>
<tr>
<td>H-271</td>
<td>76.3 ± 3.63</td>
<td>55.0 ± 5.00</td>
</tr>
</tbody>
</table>

Expression and fibronectin-binding affinity of syndecan-2 produced by P29 and LM66-H11 cells

The cell-surface expression of the two receptors was compared using flow cytometry (Figure 4). As expected, the expression levels of integrin \( \alpha 5 \beta 1 \) were almost the same in the cloned cell lines (Figures 4A and 4B), whereas that of syndecan-2 on P29 cells was approx. eight times higher in fluorescence intensity than that of LM66-H11 cells (Figures 4C and 4D). To compare the binding ability of the syndecan-2 proteoglycan which was isolated from the two cloned cell lines with fibronectin, \(^{35}S\)sulphate-labelled syndecan-2 precipitated with SN2Ab from the hydrophobic proteoglycan fractions was subjected to fibronectin-affinity chromatography (Figures 4E and 4F). The syndecan-2 proteoglycan from each of the clones was eluted with 0.22 M NaCl, indicating that the binding abilities of the two samples to fibronectin were the same. These results suggest that the formation of stress fibres in P29 cells, which was induced in co-operation of syndecan-2 and integrin \( \alpha 5 \beta 1 \), requires a certain threshold of syndecan-2 below which the formation of cortex-actin structure ensues, as observed in LM66-H11 cells in this study and in the antisense-treated P29 cells in the previous study [26].

Participation of syndecan-2 in actin-cytoskeletal organization

Most cells express more than one species of syndecans [37] and our previous study also suggested that P29 cells synthesized cell-surface heparan sulphate proteoglycans other than syndecan-2 [27]. Hence, the expression of mRNAs of all members of the syndecan family was examined in the two clones (Figure 5). The RT-PCR analysis showed that the two clones expressed all members of the syndecan family although the levels of syndecan-3 were extremely low (Figure 5A). Among them, only syndecan-2 exhibited different expression levels between the two clones, i.e. the expression in P29 cells was significantly higher.
Figure 4 Expression of integrin α5β1 and syndecan-2 on P29 and LM66-H11 cells

P29 (A, C) and LM66-H11 (B, D) cells suspended in 0.2% BSA/DMEM were treated with anti-integrin α5β1 antibody, AB1950 (A, B) or anti-syndecan-2 core protein antibody, SN2Ab (C, D) for 1 h at 4 °C and then FITC-conjugated second antibodies for 30 min. Intensity of fluorescence was measured with a flow cytometer. Peaks shown with dotted lines are for control samples reacted with non-immune sera as the first antibodies. [35S]Sulphate-labelled syndecan-2 purified from P29 (E) and LM66-H11 (E) cells was applied to a fibronectin-linked Sepharose 4B column and eluted with a linear-gradient concentration of NaCl to compare the binding affinity of the two samples with fibronectin.

Figure 5 Expression of mRNAs of a syndecan family in P29 and LM66-H11 cells

Expressions of mRNAs of syndecan-1, -2, -3, -4 and β-actin in P29 and LM66-H11 cells were analysed by RT-PCR (A) and by Northern blot (B). Their levels were normalized by the expression of β-actin.

than that in LM66-H11 cells. By Northern-blot analysis (Figure 5B), mRNA of syndecan-3 was not detected but that of other syndecans, syndecan-1 (2.4 kb), syndecan-2 (1.2, 2.1 and 3.6 kb) and syndecan-4 (2.3 kb) were detected in both clones. Three syndecan-2 mRNA species of different sizes have been reported in human foetal lung fibroblasts [38] and shown to result from different use of alternative polyadenylation signals. The densities of the bands were quantified using the NIH Image program and normalized with the amount of β-actin. The ratios of the individual densities of the P29 and LM66-H11 cell-derived syndecan-1, -2, and -4 bands were 1.00, 4.47 (ratio of total densities of three bands) and 1.47 respectively demonstrating a significant difference in the expression of syndecan-2 mRNAs.

To confirm the participation of syndecan-2 in actin-cytoskeletal organization, responses of the cells to mixed substrata comprising C-274 and antibodies against two portions of the syndecan-2 proteoglycan, heparan sulphate (FS8-10E4) and the ectodomain of core protein (SN2Ab), were assessed (Figure 6). When P29 cells were inoculated on a mixed C-274 and FS8-10E4 substratum, they spread extensively in association with the formation of stress fibres (Figure 6A), whereas those pretreated with heparitinase-I did not form stress fibres under the same conditions (results not shown), indicating a necessity for clustering of heparan sulphate proteoglycans, mediated by binding of their glycosaminoglycans to the ligand. Moreover, a mixed substratum of C-274 and SN2Ab exerted similar effects on the organization of the actin cytoskeleton in P29 cells (Figure 6B), whereas heparitinase-I digestion of the cells did not affect the formation of stress fibres (results not shown). These results lead us to speculate that clustering of syndecan-2 on the cell surface...
is sufficient to work co-operatively with integrin α5β1 for signal transduction to induce stress fibre formation, and both glycosaminoglycan- and core protein-mediated clusterings are equivalent in terms of this signal transduction. On the contrary, LM66-H11 cells failed to form stress fibres, but exhibited the formation of cortex actin on these two substrata (Figures 6C and 6D), suggesting that the clustering of syndecan-2 expressed on their surface was not sufficient to form stress fibres under the conditions used, or that the cells lack some components to complete the stress-fibre formation. This possibility has been investigated below.

**Induction of stress-fibre formation by overexpression of syndecan-2 in LM66-H11**

To confirm that syndecan-2 is specifically involved in the induction of stress-fibre formation in the present system, we inserted a full-length cDNA of the mouse syndecan-2 into LM66-H11 cells with its low expression and obtained a stable clone, H11-SN2, expressing it at a level similar to that of P29 cells (Figure 7B). On the contrary, the clone transfected only with the vector, H11-Vec, expressed syndecan-2 at a level similar to that of the parental LM66-H11 cells (Figure 7A). Its overexpression did not affect the expression of other syndecans and integrin α5β1 (results not shown). When H11-Vec cells were allowed to adhere to the fibronectin substratum, they formed cortex actin (Figure 7C) as did the parental cells (Figure 3B). On the other hand, H11-SN2 cells spread extensively in association with the formation of stress fibres (Figure 7D). This actin-cytoskeletal organization was indistinguishable from that of P29 cells on the same substratum (Figure 3A). These results indicate that LM66-H11 cells have all the features required for the induction of stress-fibre formation, except the expression of syndecan-2 beneath the threshold.

**DISCUSSION**

In the present study, using the fibronectin-recombinant polypeptides as substrata, we have clearly demonstrated that signal transduction by (1) integrin α5β1 alone results in the induction of cortex-actin structure in association with cell spreading; (2) syndecan-2 alone results in the formation of filopodia; and (3) both receptors cause the formation of stress fibres (Figure 8). Increasing evidence suggests that many heparin-binding growth factors are activated by binding to cell-surface heparan sulphate chains, acting as physiological co-receptors before binding to their intrinsic receptors [37,39–41]. Thus it is noteworthy that unlike co-receptors for soluble growth factors, syndecan-2 itself has the potential to induce the formation of fibroplasia by binding to an immobilized substratum, indicating that ECM-ligand-mediated clustering of syndecan-2 can trigger the cytoplasmic cascade for signalling to rearrange the actin cytoskeleton.

Another new finding is that the signals binding to fibronectin by integrin α5β1 alone induced the formation of cortex actin in the cells used in the present study. In addition, syndecan-2 can act co-operatively with integrin α5β1 to generate a new signal for the formation of stress fibres, which differs from the respective signals by syndecan-2 or integrin α5β1 alone. Moreover, the fact that the stress fibre formation was induced in P29 cells on adhesion to a mixed substratum composed of C-274 and not only anti-core protein of syndecan-2 antibody but also anti-heparan sulphate antibody, further corroborates our previous finding that syndecan-2 participates specifically in the induction of stress-fibre formation [26]. However, it remains to be elucidated how syndecan-2 and integrin α5β1 cross-talk with each other to generate a new signal in terms of the molecular mechanism.

The responses of LM66-H11 cells with low syndecan-2 expression to adhesion substrata indicated that a certain threshold in the expression of syndecan-2 might be required for co-operation with integrin α5β1. The expression level of syndecan-2 on P29 cells is sufficient to work co-operatively with integrin α5β1 to induce the formation of stress fibres on fibronectin. Thus an elevated expression of syndecan-2 in LM66-H11 cells by transfection of its cDNA caused the formation of stress fibres in co-operation with integrin α5β1. This also suggests that LM66-H11 cells have all the features required for the induction of stress fibres, except for a low expression of syndecan-2.

The facts that the mixed substratum composed of C-274 and SN2Ab is sufficient to induce the formation of stress fibres in P29 cells and treatment of P29 cells with the antisense oligonucleotide for syndecan-2 prevents the formation of stress fibres [26], indicate that a certain level of syndecan-2 is not only essential but also sufficient to receive the ECM signals co-operatively with integrin α5β1 to induce stress-fibre formation. Although the clones used in this study expressed all members of the syndecan family, the molecular species exhibiting specific binding to fibronectin or H-271 was syndecan-2 [26–28]. On the other hand, it has been well established that syndecan-4 signals co-operatively with integrin α5β1 to result in the assembly of focal adhesions and actin stress fibres in fibroblasts inoculated on to a fibronectin substratum [16,17,42]. The cytoplasmic domain of syndecan-4 binds phosphatidylinositol 4,5-bisphosphate and PKCα at its variable region, and this interaction mediates its own multimerization, with resultant regulation of the intracellular distribution and activity of PKCα [18,19]. We have recently demonstrated [43] that the cross-talk of syndecan-2 and -4 is
required for the induction of those cytoskeletons in cooperation with integrin α5β1. This cross-talk may be explained by a substrate–enzyme interaction, i.e. a phosphorylation reaction at the serine residue(s) of the syndecan-2 cytoplasmic domain, which is catalysed by PKCζ bound to the syndecan-4 cytoplasmic domain [20]. However, it should be noted that fibroblasts derived from syndecan-4-null mice have been recently demonstrated to form focal contacts and stress fibres on adhesion to fibronectin as observed in normal fibroblasts [44], indicating that syndecan-4 is not essential and can be compensated for by some other molecule(s). Thus all these results suggest that both syndecans appear to function co-operatively with integrin α5β1 for the assembly of focal adhesions and actin stress fibres on cell adhesion to the fibronectin substratum.

Finally, it is appropriate to discuss the correlation between the metastatic potential and the expression level of syndecan-2. An in vivo selection of different metastatic clones from Lewis lung carcinoma cells on the basis of spontaneous metastatic potential results in the selection of clones with different abilities to induce the host-stromal responses, and also different expressions of syndecan-2. Thus each of the cloned cell lines grows in quite different microenvironments in vivo and cells adapt to such a milieu by all the receptors that they express on the cell surface. In consequence, the two cloned cells behave differently. Indeed, the actin cytoskeleton formed in the highly metastatic LM66-H11 cells on adhesion to a fibronectin substratum is indistinguishable from that in the low-metastatic P29 cells whose expression of syndecan-2 is selectively suppressed by in vitro treatment with antisense oligonucleotides for its core protein [26]. Moreover, we have recently demonstrated that over-expression of syndecan-2 by transfection of its cDNA into LM66-H11 cells causes the formation of actin stress fibres in the cells on adhesion to fibronectin, which is indistinguishable from the actin cytoskeleton induced in P29 cells on fibronectin and, surprisingly, results in striking suppression of metastasis [43]. The results reported here consistently suggest that syndecan-2 plays a pivotal role in the determination of the degree of metastasis.

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Figure 8 Fibronectin-induced signalings mediated by integrin α5β1 and/or syndecan-2

The results obtained in this study show that cells adhering to the Cell-I domain of fibronectin by integrin α5β1 spread and form cortex actin. In contrast, the cells adhering to the Hep-II domain by heparan sulphate side-chains of syndecan-2 spread to a lesser extent and develop filopodia. When they adhere to fibronectin by both receptors, stress-fibre formation is induced. It is also revealed that there is a threshold level for syndecan-2 signalling to form filopodia or regulating integrin α5β1-mediated signalling to form stress fibres. However, this depends on the ability of a ligand to bind syndecan-2. Therefore, on a fibronectin substratum, P29 cells with high syndecan-2 expression form stress fibres and LM66-H11 cells with low expression form cortex actin.

Cortex Actin | Stress Fibers | Filopodia

Cytoplasmic compartment
Cell Membrane
Extracellular compartment

Integrin α5β1

Fibronectin
Cell-I domain
Hep-II domain
Syndecan 2

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