Phosphorylation of a membrane-intercalated proteoglycan, syndecan-2, expressed in a stroma-inducing clone from a mouse Lewis lung carcinoma

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We previously reported that a mouse Lewis lung carcinoma-derived stroma-inducing clone, P29, highly expresses a syndecan-like proteoglycan exhibiting specific binding to fibronectin, a major constituent of the interstitial matrix formed by the induced stromal cells, via its heparan sulphate chains [Itano, Oguri, Nakanishi and Okayama (1993) J. Biochem. (Tokyo) **114**, 862–873]. On metabolic labelling of the proteoglycan with [^{32}P]P_i, followed by identification of the radiolabelled material using glycanases, almost all the isotope was found to have been incorporated into a core portion of molecular mass 48 kDa, which was generated by digestion with heparan sulphate lyase I plus chondroitin ABC lyase. Immunoblotting of the core protein with a monoclonal antibody, F58-6G12, demonstrated that the proteoglycan was mouse syndecan-2. CsCl-density-gradient centrifugation after mild treatment of liposome-intercalated ^{32}P-labelled syndecan-2 with trypsin resulted in clear separation of the radioactivity into a bottom fraction containing all the glycosaminoglycans (accounting for 40 % of the total radioactivity) and a top fraction containing liposome-associated peptides (60 %). The former isotope was shown to be linked covalently to both heparan sulphate and chondroitin sulphate chains, probably at their bridge regions. The latter was mostly attributed to phosphoserine, the one and only phosphorylated amino acid released on acid hydrolysis of this proteoglycan, strongly suggesting that the phosphorylation occurs at a specific serine residue(s) in the cytoplasmic domain of the core protein.

INTRODUCTION

Epithelial–mesenchymal interactions play an important role in morphogenesis [1–4], and are accompanied by dynamic changes in the extracellular matrix [5,6]. The response of cells to these changes must involve integral surface molecules that recognize their ligands and initiate signal transduction intracellularly. Some of the best characterized molecules of this type comprise the syndecan family of transmembrane heparan sulphate proteoglycans, which are considered to mediate the linkage between the extracellular matrix and the actin cytoskeleton [7,8]. Because their ligand-binding site is heparan sulphate with very varied structures, they can select various interstitial extracellular matrix constituents [7,8] and heparin-binding growth factors as ligands [8–10]. The cytoplasmic domain of the core protein appears to associate with the detergent-insoluble actin cytoskeleton [11,12]. However, the nature of the putative signals generated on their binding to ligands and the mechanisms of signal transduction remain unknown.

Syndecans also mediate the tumour–stromal interaction that is important for epithelial carcinogenesis [13–16]. All human cancers show various degrees of a fibrotic stromal response of host tissues. However, the cellular and molecular events regulating the tumour-associated stromal response remain largely unclear. In previous studies we found that *in vivo* selection of different metastatic clones from a mouse Lewis lung carcinoma (3LL) resulted in the selection of clones exhibiting distinctly different extracellular matrix-dependent tumorigenesis [15–18]; the growth of the highly metastatic clones, LM60-D6 [17,18] and LM66-H11 [15], which elicited no significant stromal response and formed a well-organized basement membrane, depended on their own basement membrane *in vivo*, whereas a low metastatic clone, P29, with the capacity to elicit the host stromal response, exhibited tumorigenesis dependent on the interstitial matrix formed by the induced stromal cells [15,16]. Moreover, we found that a membrane-intercalated syndecan-like proteoglycan exhibiting specific binding to fibronectin, a major constituent of the induced interstitial matrix [16], via its heparan sulphate chains, was expressed about six times more abundantly in P29 cells than in LM66-H11 cells [15].

In order to obtain a clue as to the mechanism of the cellular response of P29 cells to the interstitial matrix through the fibronectin-binding proteoglycan, which was identified immunochemically as mouse syndecan-2 in this study, we examine here the possibility of phosphorylation at the cytoplasmic domain of the core protein, as in the cases of other classes of receptors.

MATERIALS AND METHODS

Cell culture

The low metastatic P29 cell line established from a mouse Lewis lung carcinoma (3LL) was routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum (Gibco, New York, NY, U.S.A.), 100 μg of streptomycin/ml and 100 units of penicillin/ml, as described previously [15]. The cells were harvested with 2 mM EDTA in PBS.

Purification and radiolabelling of a syndecan-like proteoglycan

Confluent cultures of P29 cells (3×10^5 cells/cm^2) were doubly labelled metabolically with D-[6-^{3}H]glucosamine (1110 MBq/

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mmol; DuPont/NEN, Boston, MA, U.S.A.) at 1.85 MBq/ml and [^{32}P]P_i (carrier-free; Japan Radioisotope Association, Tokyo, Japan) at 18.5 MBq/ml in phosphate-free minimum essential medium supplemented with 10 % (v/v) dialysed foetal bovine serum, penicillin and streptomycin, for 2 and 12 h at 37 °C, in a humidified atmosphere of 5 % CO_2 in air. The cell layer was washed three times with Dulbecco's modified Eagle's medium, and then the radiolabelled macromolecules were extracted with 4 M guanidinium chloride/2 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, containing 10 mM EDTA, 10 mM *N*-ethylmaleimide, 1 mM PMSF and 0.036 mM pepstatin A as proteinase inhibitors [15] and 1 mM sodium vanadate, 80 mM sodium phosphate, 0.2 mM trifluoroperadine and 50 mM NaF as phosphatase inhibitors [19]. The extracts were then dialysed against 7 M urea/0.5 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, containing the same inhibitors on ice. A syndecan-like proteoglycan was purified from the urea-soluble fraction with the aid of ^{3}H radioactivity, as described previously [15]. Briefly, the urea-soluble fraction was applied to a column (1 cm × 10 cm) of DEAE-Sephacel (Pharmacia Biotech., Tokyo, Japan). The column was washed with 7 M urea/0.5 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, and then the bound materials were eluted with a linear gradient of 0.1–1.0 M NaCl in the same buffer. The proteoglycan fraction eluted at 0.35–0.65 M NaCl was applied to a column (0.7 cm × 5 cm) of octyl-Sepharose CL-4B (Pharmacia Biotech.). The column was washed with 4 M guanidinium chloride/50 mM Tris/HCl, pH 7.3, and then the bound materials were eluted with a linear gradient of 0–0.5 % (v/v) Triton X-100 in the same buffer. The hydrophobic proteoglycan fraction eluted at 0.15–0.3 % (v/v) Triton X-100 was applied to a column (0.5 cm × 5 cm) of fibronectin (from human plasma; Iwaki Glass, Funabashi, Japan)-linked Sepharose 4B. The column was washed with 0.1 M NaCl/0.5 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, and then the bound materials were eluted with a linear gradient of 0.1–0.5 M NaCl in the same buffer. The fibronectin-binding proteoglycan fraction eluted at 0.15–0.3 M NaCl was brought to 4 M guanidinium chloride/0.5 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3. Solid CsCl (0.5 g/ml) was added to give an initial density of 1.45 g/ml, and then a density gradient was formed by centrifugation at 170000 ***g*** for 72 h at 15 °C. The syndecan-like proteoglycan was collected in the bottom fraction with a density of more than 1.47 g/ml. The purified proteoglycan was dialysed against 4 M guanidinium chloride/0.5 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, and then applied to a column (1 cm × 120 cm) of Sepharose CL-4B in the same buffer.

Detection of incorporation of [^{32}P]phosphate into the syndecan-like proteoglycan

To determine the incorporation of [^{32}P]phosphate into the syndecan-like proteoglycan, the purified proteoglycan was digested with proteinase-free heparan sulphate lyases I (EC 4.2.2.8) and II (gifts from Dr. K. Yoshida, Tokyo Institute of Seikagaku Corp., Japan), proteinase-free chondroitin ABC lyase (EC 4.2.2.4) (Seikagaku Corp.), or a combination of the three enzymes, and the materials generated were analysed by SDS/PAGE with a gradient (4–20 %) gel, as described previously [20]. After drying, the gels were exposed to high-sensitivity X-ray film (Fuji HA; Fuji Film, Tokyo, Japan) for an appropriate period at −80 °C,

To determine phosphorylated amino acids, the purified proteoglycan was hydrolysed with 6 M HCl under N_2 for 1 h at 110 °C [19]. The hydrolysate was subjected to two-dimensional electrophoresis on a thin-layer plate with phosphoserine, phosphothreonine, phosphotyrosine and P_i as internal references [19]. Phosphoamino acids and P_i were located by staining with ninhydrin and ammonium molybdate reagents respectively. The stained plate was then exposed to high-sensitivity X-ray film for an appropriate period at −80 °C.

To locate a phosphorylation site(s) along the molecule, the purified proteoglycan was dissolved in 4 M guanidinium chloride/75 mM octyl glucoside/50 mM Tris/HCl, pH 7.3, containing 5 mg/ml L-α-phosphatidylcholine (Nacalai Tesque, Kyoto, Japan) and NBD-phosphatidylchloine (Funakoshi, Tokyo, Japan). The sample was dialysed against the same solution without detergent to form liposomes and then divided into two portions. The samples prepared were dialysed against 50 mM Tris/HCl, pH 7.3, incubated with or without trypsin (50 μg/ml) for 15 min at 25 °C, followed by the addition of soyabean trypsin inhibitor, and then adjusted to 4 M guanidinium chloride/50 mM Tris/HCl, pH 7.3. Solid CsCl (0.22 g/ml) was then added to the samples to give an initial density of 1.25 g/ml, and a density gradient was formed by centrifugation at 170000 ***g*** for 72 h at 15 °C.

To examine the incorporation of [^{32}P]phosphate into glycosaminoglycan side chains, the glycosaminoglycan-bearing portion was treated with 0.3 M NaOH/1 M $NaBH_4$ at 25 °C for 12 h to release glycosaminoglycan chains, followed by extensive digestion with Pronase. After dialysis against water and freeze-drying, the sample was dissolved in water containing heparan sulphate and chondroitin sulphate A as internal references, and then subjected to two-dimensional electrophoresis on a cellulose acetate membrane with external references of heparan sulphate, chondroitin sulphate A and hyaluronic acid. After staining with Alcian Blue, the membrane was exposed to high-sensitivity X-ray film for an appropriate period at −80 °C.

Immunoblotting of core proteins

A core protein sample prepared by digestion of the purified proteoglycan with a combination of heparan sulphate lyases I and II, and chondroitin ABC lyase was subjected to SDS/PAGE and then transferred to an Immobilon Transfer Membrane (Millipore Corp., Bedford, MA, U.S.A.). The membrane was blocked with 10 % (w/v) skimmed milk in PBS containing 0.05 % (v/v) Tween 20 (PBST) for 1 h at room temperature, and then washed with PBST. The membrane was incubated for 1 h at room temperature with monoclonal antibody {mouse anti-(human syndecan core proteins), F58-6G12 (2.5 μg/ml) or F58-2E9 (5.0 μg/ml) [21]} in PBST containing 5 % (w/v) skimmed milk. After being washed with PBST, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgGs (DakoPatts, Copenhagen, Denmark) in PBST containing 5 % (w/v) skimmed milk. Immunoreactivity was visualized by colour development using Chemiluminescence Reagent (DuPont/NEN).

RESULTS AND DISCUSSION

Detection of the phosphorylation of a syndecan-like proteoglycan

Our previous study demonstrated that a Lewis lung carcinoma-derived stroma-inducing clone, P29, highly expresses a syndecan-like proteoglycan exhibiting specific binding to fibronectin, a major constituent of the interstitial matrix formed by the induced stromal cells, via its heparan sulphate side chains [15]. In the present study, to examine whether, like other classes of receptors, this proteoglycan has a phosphorylation site(s) in the cytoplasmic portion of the core protein, P29 cells were doubly labelled metabolically with [^{32}P]P_i and [^{3}H]glucosamine by incubation for

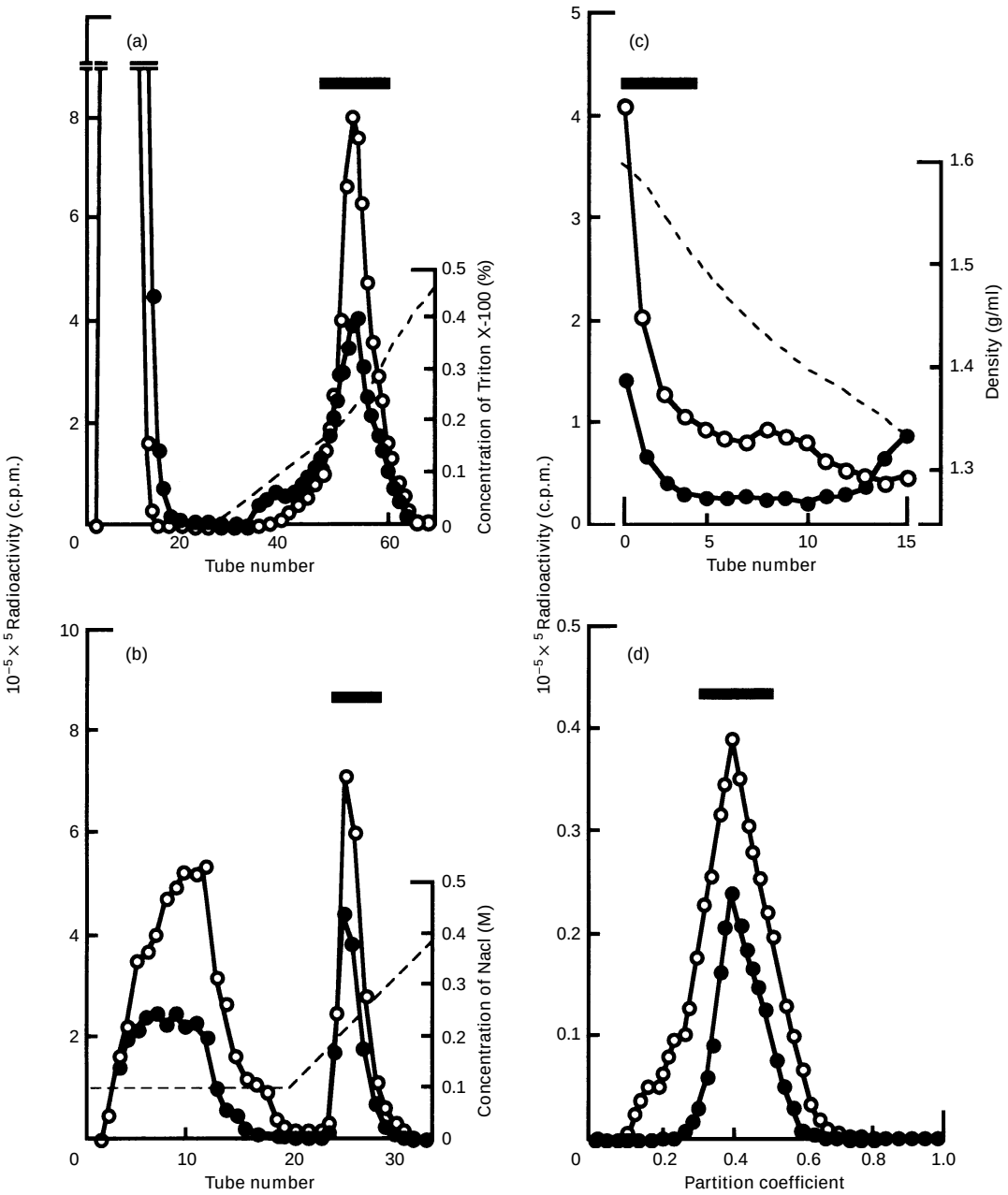

Figure 1 Isolation and purification of a $^{32}P/^{3}H$-labelled syndecan-like proteoglycan

Confluent cultures of P29 cells were double labelled metabolically with [^{32}P]P_i and D-[6-^{3}H]glucosamine, and radiolabelled macromolecules were extracted from the cell layer with 4 M guanidinium chloride/2% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, containing proteinase and phosphatase inhibitors as described in the Materials and methods section. The extract was then dialysed against 7 M urea/0.5% (v/v) Triton X-100/50 mM Tris/HCl, pH 7.3, containing the same inhibitors, on ice. A syndecan-like proteoglycan was purified from a urea-soluble fraction by chromatography on DEAE-Sephacel (results not shown), octyl-Sepharose CL-4B (**a**), fibronectin-linked Sepharose 4B (**b**), followed by CsCl-density-gradient centrifugation (**c**) and gel chromatography on Sepharose CL-4B (**d**). ^{32}P (●) and ^{3}H (○) radioactivity in an aliquot of each fraction was measured. Dashed lines in (**a**), (**b**) and (**c**) show linear gradients of 0–0.5% (v/v) Triton X-100 and 0.1–0.5 M NaCl and the densities of the fraction respectively. Recovery of ^{32}P- and ^{3}H-labelled materials in the proteoglycan fraction at each purification step was more than 80%. The fractions shown by the bars were pooled for further purification.

12 h. Under the culture conditions used, the cells exhibited a linear incorporation of [^{35}S]sulphate into macromolecules up to 24 h [15]. On purification of the syndecan-like proteoglycan with the aid of the ^{3}H-labelled one (Figure 1), a small but significant amount of ^{32}P radioactivity was co-purified with the ^{3}H-labelled syndecan-like proteoglycan (Table 1). To confirm that the ^{32}P radioactivity was incorporated into the molecule, we digested the purified $^{32}P/^{3}H$-labelled proteoglycan with glycosaminoglycan-degrading enzymes and then subjected the digest to SDS/PAGE, followed by autoradiography to detect only ^{32}P radioactivity (Figure 2A). The intact molecule migrated as a broad band corresponding to a molecular mass of more than 230 kDa (lane 1). Digestion with heparan sulphate lyase I plus II resulted in conversion of the ^{32}P-labelled macromolecule into materials

Table 1 Purification of a $^{32}P/^{3}H$-labelled syndecan-like proteoglycan

Results in parentheses are percentage of radioactivity applied. Total recovery of ^{3}H was 3.0% and total recovery of ^{32}P was 0.04%.

Step	Fraction	$10^{-3} \times {}^{3}H$ (c.p.m.)	$10^{-3} \times {}^{32}P$ (c.p.m.)	$^{32}P/^{3}H$
DEAE-Sephacel	Proteoglycan (34.3)	23393 (8.2)	753508	32.21
Octyl-Sepharose CL-4B	Hydrophobic proteoglycan	6258 (33.1)	3979 (0.7)	0.64
Fibronectin-Sepharose 4B	Fibronectin-binding proteoglycan	1913 (35.6)	1114 (30.8)	0.58
CsCl-density-gradient centrifugation	High-density proteoglycan ($\rho > 1.47$ g/ml)	984 (62.2)	517 (57.7)	0.53
Sepharose CL-4B	Syndecan-like proteoglycan	722 (87.2)	298 (78.4)	0.41

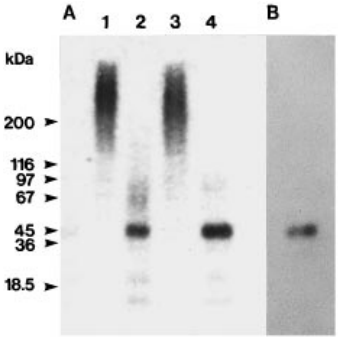

Figure 2 Detection of incorporation of [^{32}P]phosphate into the syndecan-like proteoglycan and immunochemical identification of its molecular species

The purified $^{32}P/^{3}H$-labelled syndecan-like proteoglycan was subjected to SDS/PAGE before and after digestion with various glycanases, and then autoradiographed for ^{32}P radioactivity only (**A**). Lanes 1–4 show the intact proteoglycan and the digests with heparan sulphate lyase I plus II, chondroitin ABC lyase and all three enzymes respectively. The same gel as lane 4 was run and stained with monoclonal antibody F58-6G12, which is specific to the core protein of syndecan-2 [21], as described in the Materials and methods section (**B**).

migrating to the positions of a heparan sulphate-free chondroitin sulphate proteoglycan with a molecular mass of 90–50 kDa and a 48 kDa core portion (lane 2). On digestion with chondroitin ABC lyase, the chondroitin sulphate-free heparan sulphate proteoglycan migrated only slightly faster than the intact molecule (lane 3). Treatment with the three enzymes together gave the core portion with bridge regions (lane 4). These patterns were essentially the same as those obtained for the [^{35}S]methionine/[^{3}H]glucosamine-labelled proteoglycan [15]. The molecular mass of the core protein was nearly the same as that of the core protein of human fibroglycan, recently named syndecan-2 [7,8]. Moreover, immunoblotting analyses demonstrated that the core protein cross-reacted with monoclonal antibody F58-6G12, which is specific to the core protein of syndecan-2 (Figure 2B), but not with monoclonal antibody F58-2E9, which is specific to those of both syndecan-1 and syndecan-3 (results not shown). These results indicate that the syndecan-like proteoglycan with fibronectin-binding ability is mouse syndecan-2. Thus the above results further indicate that syndecan-2 produced by the P29 clone consists of two forms, a hybrid form having heparan sulphate and chondroitin sulphate

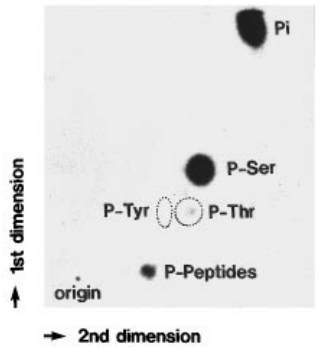

Figure 3 Identification of the phosphorylated amino acids of the core protein of syndecan-2

An acid hydrolysate of $^{32}P/^{3}H$-labelled syndecan-2 was subjected to two-dimensional electrophoresis on a thin-layer plate with phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr) and inorganic phosphate (Pi) as internal references. After staining the authentic materials as described in the Materials and methods section (dotted line), the plate was subjected to autoradiography.

chains on the same core protein, and another form having only heparan sulphate chains [15], and that the ^{32}P radioactivity was incorporated into both forms.

Identification of the phosphorylation site of the syndecan-2 proteoglycan

To examine the possibility of phosphorylation at the core protein, we hydrolysed $^{32}P/^{3}H$-labelled syndecan-2 with HCl and then subjected the hydrolysate to two-dimensional electrophoresis. Three ^{32}P-radioactive spots appeared (Figure 3), the two major spots co-migrating with phosphoserine and P_i, and the minor one with probably a phosphorylated small peptide [19], indicating that the core protein is only phosphorylated at a serine residue(s).

Next, to examine whether the phosphoserine residue(s) is located in the cytoplasmic domain of the core protein, $^{32}P/^{3}H$-labelled syndecan-2 was intercalated into liposomes, and then analysed by CsCl-density-gradient centrifugation before and after mild treatment with trypsin. Before trypsin treatment, the ^{32}P and ^{3}H radioactivities were mostly collected with liposomes in a top fraction with a density of less than 1.10 g/ml (Figure 4a). Since the molecular density of syndecan-2 was more than 1.47 g/ml (see Figure 1c and Table 1), it indicated that the proteoglycan was intercalated into the liposomes. After trypsin treatment (Figure 4b), 60% of the ^{32}P radioactivity was collected in the top fraction with the liposomes, but without any significant ^{3}H radioactivity, whereas the ^{3}H-labelled glycosaminoglycan-bearing portion associated with the remaining 40% of the ^{32}P radioactivity was collected in the bottom fraction, suggesting that trypsin cleaves at a site between a glycosaminoglycan-bearing ectodomain and a liposome-intercalated domain of the core protein. This is consistent with the fact that the core proteins of all syndecans have dibasic proteinase-susceptible sites in their ectodomains immediately adjacent to the transmembrane domains [7,8]. Consequently, the ^{32}P radioactivity associated with the liposome-intercalated domain may be attributed to the phosphoserine, and that of the glycosaminoglycan-bearing portions to another site(s).

Heparan sulphate and chondroitin sulphate of different origins were previously reported to be phosphorylated at position C-2 of the xylose residues of their bridge regions [22,23]. To examine this possibility, peptide-free glycosaminoglycan chains were

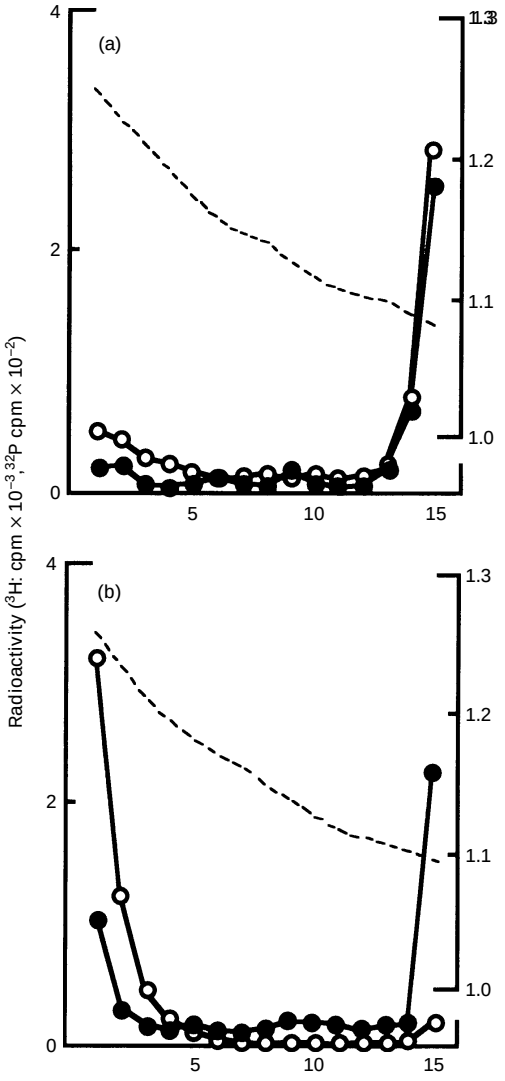

Figure 4 Location of a phosphoserine residue(s) in the cytoplasmic domain of the core protein of syndecan-2

^{32}P/^{3}H-labelled syndecan-2 intercalated into liposomes was incubated without (**a**) or with (**b**) trypsin. The initial density was adjusted to 1.25 g/ml by the addition of solid CsCl, and then a density gradient was formed by ultracentrifugation. ^{32}P (●) and ^{3}H (○) radioactivity in each fraction was measured. ––––, Densities of the fractions.

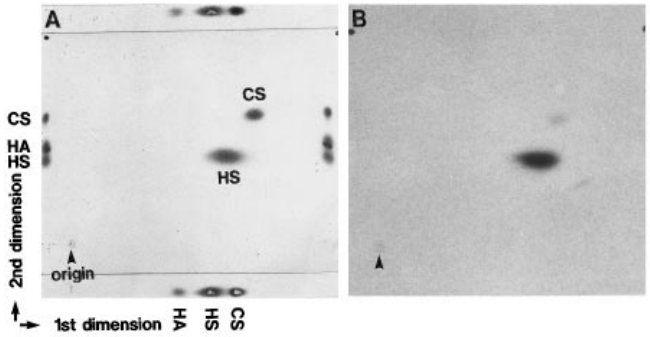

Figure 5 Phosphorylation of glycosaminoglycan chains of syndecan-2

Peptide-free glycosaminoglycan sample released by β-elimination reaction of the radiolabelled material of the bottom fraction obtained on CsCl-density-gradient centrifugation was subjected to two-dimensional electrophoresis on a cellulose acetate membrane with internal and external references including heparan sulphate (HS), chondroitin sulphate A (CS) and hyaluronic acid (HA). (**A**) and (**B**) show the staining of the authentic materials with Alcian Blue and the autoradiogram of ^{32}P radioactivity respectively.

obtained from the ^{32}P/^{3}H-labelled material in the bottom fraction of the trypsin digest by means of the β-elimination reaction, followed by extensive digestion with Pronase. More than 85% of both the ^{32}P- and ^{3}H radioactivity was recovered in the dialysate, suggesting the incorporation of both isotopes into glycosaminoglycan chains. On two-dimensional electrophoresis, which separated authentic glycosaminoglycans from each other (Figure 5A), the ^{32}P-labelled material was separated into two radioactive spots (Figure 5B), a major one co-migrating with heparan sulphate and a minor one migrating with chondroitin sulphate A, reflecting the relative contents of these two glycosaminoglycans in this proteoglycan [15]. Furthermore, digestion of the sample with heparan sulphate lyase I plus II or chondroitin ABC lyase resulted in the disappearance of the corresponding radioactive spot (results not shown), indicating that the ^{32}P radioactivity associated with the glycosaminoglycan-bearing portion is due to phosphate residues linked covalently to both heparan sulphate and chondroitin sulphate chains, and mainly attributable to the P_i released on acid hydrolysis of the proteoglycan (see Figure 3). From these results we concluded that the ^{32}P radioactivity of the liposome-intercalated domain was mostly due to the phosphoserine. It should be mentioned that the results obtained from a 2 h incubation system were qualitatively the same as those from the 12 h incubation system described above: that is, more than 90% of the ^{32}P radioactivity incorporated into syndecan-2 in the former system was demonstrated to be incorporated into the phosphoserine and glycosaminoglycan chains as described above. However, in the shorter incubation, there was a tendency for incorporation of the isotope more into the phosphoserine(s) than into the glycosaminoglycan chains.

In conclusion, we have clearly demonstrated that the core protein of syndecan-2 was phosphorylated at only a serine residue(s) in the cytoplasmic domain. In this regard, it is noteworthy that the reported amino acid sequences of the core proteins of the human and rat syndecan-2 molecules show that their cytoplasmic domains contain the amino acid sequence RKPS [24,25], which corresponds to the potential phosphorylation motif, RXXS, for cAMP- and cGMP-dependent protein kinases [26–28]. Thus the serine residue in the RKPS sequence may be the phosphorylation site in the core protein of mouse syndecan-2. It is also of note that the cytoplasmic domain of the syndecan-2 core protein has an amino acid sequence, KKDEGSY [24,25], which corresponds to the tyrosine kinase phosphorylation consensus sequence, KXXEXXY [25], but no phosphorylated tyrosine could be detected in the syndecan-2 proteoglycan examined here.

The reported amino acid sequences of the core proteins of syndecans show that the cytoplasmic domains of syndecans-1 and -3 contain potential phosphorylation sites such as SLEE [29] and TLEE [30] respectively which correspond to the casein kinase II phosphorylation consensus sequence, S(T)XXE [30]. In addition, the tyrosine kinase phosphorylation consensus sequence, KXXEXXY [25,30], is well conserved in the cytoplasmic domains of the core proteins of the reported four syndecan proteoglycans [7,8,31]. The presence of these potential phosphorylation sites in the cytoplasmic domains has tempted speculation that phosphorylation is a means of regulation

[7,8,25,30,31]. However, there has been no report on phosphorylation of the core proteins of syndecans. We now provide the first direct evidence that the mouse syndecan-2 proteoglycan exhibiting specific binding to fibronectin [15] is actually phosphorylated at its cytoplasmic domain.

Finally, it is appropriate to discuss briefly a biological role for syndecans in relations to their binding to fibronectin and phosphorylation of the core proteins. We have preliminary reported that the co-operation of integrin $\alpha 5\beta 1$ and syndecan-2 is crucial for the formation of stress fibres in P29 cells on cell adhesin to a fibronectin substrate [32]. Couchman and his colleagues have proposed on the basis of their extensive studies using mainly fibroblasts that for the formation of focal adhesions and stress fibres, which was triggered by attachment of the cells to fibronectin, two signals derived from interactions of the RGD cell-binding domain/integrins and also the C-terminal heparin-binding domain/membrane-intercalated heparan sulphate proteoglycans are essential [33]. Furthermore, on the basis of the recent finding that syndecan-4 is a ubiquitous component of focal adhesions in various cell types, they propose that it may be involved in focal adhesion formation, possibly through activation of protein kinase C [34]. Syndecan-2, the cytoplasmic domain of which is most closely homologous to that of syndecan-4, appears to be distributed uniformly along cell membranes in fibroblasts, but not to be concentrated in their focal adhesions [35]. Thus, if the mechanisms underlying focal adhesion formation in fibroblasts act also in P29 lung carcinoma cells to form the structures, syndecan-2 described here may be involved in the formation of stress fibres at sites other than focal adhesions, because it is a major fibronectin-binding proteoglycan among several membrane-intercalated heparan sulphate proteoglycans produced by P29 cells [15] and is apparently involved in stress-fibre formation [32]. The relationship between the phosphorylation of syndecan-2 and the formation of focal adhesions and stress fibres in P29 cells is currently under investigation.

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