Syndecan-1 alterations during the tumorigenic progression of human colonic Caco-2 cells induced by human Ha-ras or polyoma middle T oncogenes

P Levy¹, A Munier¹, S Baron-Delage¹, Y Di Gioia², C Gespach², J Capeau¹ and G Cherqui¹

¹Laboratoire de Biologie Cellulaire, INSERM-U.402, Faculté de Médecine Saint-Antoine, 27 rue Chaligny, 75571 Paris Cédex 12, France; ²INSERM-U.55, Hôpital Saint-Antoine, 184 rue du Faubourg Saint-Antoine, 75571 Paris Cédex 12, France.

Summary The products of ras and src proto-oncogenes are frequently activated in a constitutive state in human colorectal cancer. In this study we attempted to establish whether the tumorigenic progression induced by oncogenic activation of p21°° and pp60°° in human colonic Caco-2 cells is associated with specific alterations of syndecan-1, a membrane-anchored proteoglycan playing a role in cell–matrix interaction and neoplastic growth control. To this end, we used Caco-2 cells made highly tumorigenic by transfection with an activated (Val 12) human Ha-ras gene or with the polyoma middle T (Py-MT) oncogene, a constitutive activator of pp60°° tyrosine kinase activity. Compared with control vector-transfected Caco-2 cells, both oncogene-transfected cell lines (1) contained smaller amounts of membrane-anchored PGs; (2) exhibited decreased syndecan-1 expression at the protein level only but not at the mRNA level and for syndecan-1, with decreased specific activity; (4) produced a syndecan-1 ectodomain with a lower molecular mass and reduced GAG chain size and sulphation; and (5) expressed heparanase degradative activity. These results show that the dramatic activation of the tumorigenic potential induced by oncogenic p21°° or Py-MT/pp60°° in Caco-2 cells is associated with marked alterations of syndecan-1 expression at the translational and post-translational levels.

Keywords: syndecan-1; Ha-ras; polyoma middle T; Caco-2 cells; tumorigenic progression

Human colorectal cancer, the second most common cause of death in developed countries, is now thought to result from a series of genetic alterations. The most frequent alterations consist of point mutations in members of the ras gene family (Bos et al., 1987) which encode closely related M, 21 000 guanine nucleotide-binding proteins (p21°°) or in activation of the pp60 phosphoprotein encoded by the proto-oncogene c-src (Bolen et al., 1987; Cartwright et al., 1990). The high frequency of these alterations strongly suggests that p21°° and pp60°° oncogenes, both of which are involved in growth factor signal transduction pathways, play a central role in human colon carcinogenesis.

Cell transformation is frequently associated with specific defects in the basement membrane, which play a critical role in tumour growth, invasion and metastasis (Liotta et al., 1986; Yamada, 1983). These defects result mainly from the presence of specific enzymes involved in degradation of extracellular matrix constituents (Matsrissan, 1992), and also from significant modifications in cell surface proteoglycans (PGs) (Iozzo, 1985). Specific qualitative and quantitative changes in PGs have been reported in various transformed cells. We previously demonstrated that the expression of PGs alters considerably in intestinal epithelial cells after oncogene immortalisation, representative of an early stage of neoplastic transformation (Ley et al., 1990). Elevated concentrations of hyaluronic acid have been demonstrated in breast carcinomas and human gliomas (Glimelius et al., 1978), and increased amounts of chondroitin sulphate (CS) PGs have been shown in cancer cells from liver (Kojima et al., 1975) and in colon cancer (Iozzo and Wight, 1982). Transformed cells are known to exhibit a reduced amount of undersulphated heparan sulphate (HS) (Winterbourne and Mora, 1978) and some authors have suggested that there is a relationship between decreased HS content and increased activity of heparanase, an enzyme which specifically degrades HS chains (Ricoveri and Capelletti, 1986; Nakajima et al., 1984). Such alterations in the amount and degree of sulphation of HS may help to reduce cell matrix adhesion and thus favour tumour cell shedding from primary tumours (Iozzo, 1988).

Among the HS PGs, the syndecans, which constitute most of the transmembrane PGs, appear to have a profound influence on fundamental features of cell behaviour such as adhesion, matrix anchorage and growth control (David, 1993). The syndecan family consists of at least four members that differ considerably in their extracellular domains but exhibit high degrees of homology in their transmembrane and cytoplasmic domains (Bernfield et al., 1992; Rapraeger, 1993). Syndecan-1, which is found mainly on the surfaces of epithelial cells in mature tissues, is the one that has been most thoroughly characterised so far (Saunders et al., 1989). It plays a key role in the maintenance of the epithelial cell phenotype by anchoring cytoskeletal actin to the extracellular matrix (ECM) (Rapraeger et al., 1986) and behaves like a membrane receptor, by binding to a variety of ECM components (Saunders and Bernfield, 1988; Sanderson et al., 1992a). Despite the importance of the interaction between transformed cells and the surrounding matrix as a regulatory factor controlling the growth of these cells, there are only a few studies dealing with the effects of malignant transformation on syndecan-1. Loss of syndecan-1 expression occurs in experimental tumours induced by UV-irradiation of murine skin (Inki et al., 1991), by glucocorticoid exposure of mouse mammary epithelial cells (Leppä et al., 1991; Kirjavainen et al., 1993) and in the malignant transformation of human keratinocytes (Inki et al., 1994).

However, there is to our knowledge, no information about the changes of syndecan-1 associated with ras and src oncogene-mediated neoplastic colorectal transformation. To address this issue, we used human colon carcinoma cells Caco-2 which had been transfected with a plasmid vector recombined either with an activated (Val 12) human c-Ha-ras gene (Caco-2-T cells), or with the cDNA encoding the Py-MT antigen, a constitutive activator of the tyrosine kinase of pp60°° (Caco-2-MT cells). Since parental Caco-2 cells are known to display a very low tumorigenicity (Rousett et al., 1980; Trainer et al., 1988), these ras- and Py-MT-transfected Caco-2 cells, recently shown to be highly tumorigenic in nude

Correspondence: P Levy

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mice (Chastre et al., 1993; Delage et al., 1993), provide very suitable models in which the two defects most frequently observed in human colon cancer are reproduced. In this study, we investigated whether the tumorigenic progression induced by oncogenic p21<sub>ras</sub> or Py-<sub>MT</sub>/pp60<sub>src</sub> in Caco-2 cells was associated with (1) a change in syndecan-1 expression; (2) alterations in syndecan-1 ectodomain glycosylation and sulphation; and (3) the expression of specific endoglycosidase degradative activity.

Materials and methods
Materials
Carrier-free Na<sub>2</sub>35SO<sub>4</sub> (270 mCi mmol<sup>-1</sup>) was purchased from New England Nuclear, Boston, MA, USA. Chondroitin ABC lyase (EC 4.2.2.4) was obtained from Seikagaku Fine Chemicals, Tokyo, Japan. PD-10 columns, Sepharose CL-4B and Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Upsala, Sweden. DEAE-Sephal was from Whatman Biochemicals, Maidstone, Kent, UK. 3-[3-cholamidopropyl]dimethyl-ammonio]-<sup>-1</sup>-propane-sulphonate (CHAPS), phenylmethanesulphonyl fluoride (PMSF), N-ethylmaleimide (NEM) and benzamidine were obtained from Calbiochem, San Diego, USA. Soybean trypsin inhibitor was from Sigma Chemical Company, St Louis, Missouri. Zeta-probe membranes were from Bio-Rad Laboratories, Richmond, USA and Immobilon-N membranes from Millipore, Bedford, MA, USA. All other reagents, obtained from Boehringer Mannheim, Indianapolis, IN, USA, were of the highest analytical grade.

Cell lines
Human colon carcinoma cells Caco-2 were transfected by electroporation, as previously described (Chastre et al., 1993). Homer 6, a plasmid vector containing a MoMSVLTR-driven G418 resistance gene, which was recombined either with the cDNA encoding the Py-<sub>MT</sub> antigen (pHO6MT1) or with a mutated (Val 12) human Ha-ras gene (pHO6T1) was used for cell transfection. Caco-2 cells transfected with the plasmids Homer 6, pHO6MT1 or pHO6T1, were designated as Caco-2-H, Caco-2-MT and Caco-2-T cells respectively (Chastre et al., 1993; Delage et al., 1993). The oncogene-transfected Caco-2-MT and Caco-2-T cells are regularly verified for overexpression of PKC-<sub>z</sub> mRNA transcripts (Delage et al., 1993), which is taken as an index of maintenance of functional oncogenes in these cell lines.

Cells were routinely grown at 37°C on 100 mm diameter dishes in a humidified incubator equilibrated with 5% carbon dioxide using Dulbecco's modified Eagle medium (DMEM) (4.5 g l<sup>-1</sup> glucose) supplemented with 15% fetal calf serum, 2 mM glutamine, 100 units ml<sup>-1</sup> penicillin and 100 mg ml<sup>-1</sup> streptomycin. For all the assays reported below, the cells were harvested at confluency.

Isolation of transmembrane PGs
Control Caco-2-H and oncogene-transfected Caco-2-MT and Caco-2-T cells were incubated with Na<sub>2</sub>35SO<sub>4</sub> (50 μCi ml<sup>-1</sup>) for 24 h. After labelling, cell monolayers were washed twice in ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS). An extraction protocol using a detergent cell lysis buffer was designed to obtain cellular fractions enriched in plasma membrane-associated PGs (Yanagishita and Hascall, 1984). Briefly, cells were extracted and sonicated in 8 M urea, 150 mM sodium chloride, 1% Triton X-100, 50 mM sodium acetate (pH 4.5), 5 mM EDTA, 5 mM benzamidine, 5 mM NEM and 1 mM PMSF (Yeaman and Rapraeger, 1993). The detergent extracts were then centrifuged at 600 x g to remove insoluble material. 35S-PGs in the supernatant were isolated by ion-exchange chromatography on DEAE-Sepha
cel. Columns were equilibrated with extraction buffer and eluted with a linear gradient of sodium chloride (0.05–1.0 M) in the same buffer. Fractions were assayed for 35S-radioactivity by scintillation counting in a LKB 1209 counter (ACS, Amersham, UK). The peak of radioactivity eluting in the salt gradient was dialysed extensively against 10 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100 and supplemented with PMSF at a final concentration of 0.1 mM (Yeaman and Rapraeger, 1993). Before hydrophobic chromatography, aliquots of the purified PGs were diluted 20-fold to reduce the detergent concentration.

Hydrophobic affinity chromatography
The hydrophobic properties of the detergent-extracted 35S-PGs purified by ion-exchange chromatography were determined by gel chromatography on Octyl-Sepharose CL-4B. Columns (2 ml) were equilibrated with 4 M guanidinium hydrochloride (Gdn HCl)–50 mM sodium acetate (pH 7.0). After washing with the same buffer, columns were eluted with a 50 ml gradient of Triton X-100 (0–0.8%) in the Gdn HCl buffer. Fractions (1 ml) were analysed for the Triton X-100 concentration by absorbance at 280 nm and for 35S radioactivity by liquid scintillation counting.

Biochemical characterisation of syndecan-1
Expression of syndecan-1 in control and oncogene-transfected Caco-2 cells was analysed by Northern blot and immunoblot analyses. For Northern blot analysis, RNA samples containing 30 μg of total RNA were electrophoresed through 1% agarose–6% formaldehyde gels, blotted onto Hybond-N nylon membranes (Amersham) and hybridised with a mega-prime-labelled cDNA probe specific for syndecan-1 (Saunders et al., 1989). After hybridisation, blots were washed at 65°C to high stringency, (0.1 x SSC, 0.1% SDS), and exposed to Kodak X-Omat AR film with intensifying screens at –80°C. RNA was quantified and normalised by differential densitometric scanning of syndecan-1 bands with the 28S rRNA bands (Lévy et al., 1994).

For immunoblot analysis, PG-enriched fractions were prepared from detergent extracts of cells and partially purified as indicated previously (Inki et al., 1994). Samples were fractionated by SDS–PAGE (2–20% gradient) in Tris borate buffer (Jalkanen et al., 1985), and electrophoretically transferred to Immunoblot membranes. Membranes were then probed with MAb 281–2, an antibody which recognises the core protein of syndecan-1 (generous gift from Dr M Bernfield, Harvard Medical School, Boston, MA, USA). Detection of immunoreactive protein was performed with the RPN Amersham enhanced chemiluminescence Western blotting detection system.

Isolation of the syndecan-1 ectodomain
The syndecan-1 ectodomain was isolated as described (Jalkanen et al., 1987). Briefly, after a 24 h incubation with 35S-sulphate, cells were scraped into cold PBS in the presence of proteinase inhibitors and centrifuged for 10 min at 300 g, and the pellet was exposed to trypsin (20 mg ml<sup>-1</sup>) for 10 min on an ice bath. The reaction was stopped by adding 100 mg ml<sup>-1</sup> of soybean trypsin inhibitor. After a further centrifugation, the trypsin-cleavable ectodomain was obtained in the supernatant (Rapraeger and Bernfield, 1985).

Quantification of syndecan-1
The syndecan-1 ectodomain was quantified by blotting on Zeta-Probe membranes. Briefly, aliquots of trypsin-released 35S-syndecan-1 ectodomain were brought to a final concentration of 8 M urea and equilibrated with loading buffer containing 10 mM Tris-HCl, 8 M urea, 0.1% Triton X-100 (pH 8.0) and were spotted onto Zeta-Probe membranes with a dot-blot microfiltration apparatus (Bio-Rad Laboratories), as described (Buee et al., 1991). After passive filtration, the membranes were extensively washed under vacuum with the
loading buffer, then with 50 mM Tris HCl, 150 mM sodium chloride (pH 8.0) and finally with water. The dots were cut out and bound radiolabelled syndecan-1 ectodomain was quantified by scintillation counting.

Glycosaminoglycan (GAG) chains of syndecan-1 were characterised by their differential susceptibility to nitrous acid deaminative cleavage and chondroitin ABC lyase degradation, as previously reported (Lévy et al., 1989). After nitrous acid degradation of the GAGs, CS chains remain, as judged by their sensitivity to chondroitinase digestion; alternatively, after chondroitin ABC lyase digestion of the GAGs, HS chains remain, identified by their susceptibility to nitrous acid degradation. After treatment, radioisotope incorporation into HS and CS was quantified by spotting samples onto Zeta-Probe membranes as described above and by counting the corresponding membrane spots.

**Gel filtration and ion-exchange chromatography**

Gel filtration of syndecan-1 ectodomain on Sepharose CL-4B (0.9 x 60 cm) was performed in 4 M Gdn HCl containing 50 mM sodium acetate, at a flow rate of 9 ml h⁻¹. Free GAG chains were desorbed from syndecan-1 ectodomain by β-elimination with alkaline borohydride, as previously described (Lévy et al., 1986). The HS and CS GAG chain sizes were determined after chromatography on Sepharose CL-6B (0.9 x 60 cm) by comparing the Kₚ values determined experimentally with a standard curve of log M, versus Kₚ for GAG chains of various known molecular weights (Wasteson, 1971). Void and total volumes of the sizing columns were marked by blue dextran and phenol red respectively.

Ion-exchange chromatography of the GAG chains of the syndecan-1 ectodomain was performed on a DEAE-Sephalac column (1 x 3 cm). After washing the column with 50 mM Tris-HCl, 50 mM sodium chloride (pH 6.8), elution was performed with a linear 50 mM gradient of 50-800 mM sodium chloride in the same buffer under a constant flow rate of 5 ml h⁻¹. Fractions (2 ml) were collected. The gradient was determined by conductivity measurements of the fractions (radiometer) and radioactivity was measured.

**Heparanase activity**

Preparation of 35S-labelled HS from Caco-2-H cells was described above. Caco-2-H cells were labelled with 35S-sulphate for 24 h. 35S-GAG were prepared by alkaline borohydride degradation as described above. GAGs were digested with chondroitin ABC lyase and the degradation products were removed by passing through a Sepharose CL-6B column equilibrated with 4 M Gdn HCİ containing 50 mM sodium acetate. The radioactive peak in the void volume was collected, dialysed and concentrated. This material treated with nitrous acid was completely degraded and was thus identified as pure HS.

**Cell extracts**

Once confluent, cell monolayers of Caco-2-H, Caco-2-MT and Caco-2-T cells were washed 3 times with PBS and detached from the dish by gentle shaking at 37°C in the presence of calcium- and magnesium-free PBS containing 2 mM EDTA. Cells were collected by centrifugation at 900 x g for 10 min at 4°C, washed 3 times with PBS and freeze-dried. The lyophilised cells were then dispersed in 0.1% Triton X-100 and sonicated for 30 s in an ice bath (Ricoveri and Cappellari, 1986). Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin in 0.1% Triton X-100 as the standard. Approximately 30 x 10⁵ d.p.m. of 35S-HS isolated from Caco-2-H cells were added to cell extracts (3 mg proteins) from Caco-2-H, Caco-2-MT or Caco-2-T cells. All digestions were performed at 37°C in 0.1 M sodium phosphate buffer (pH 6.0) containing 15 mM D-saccharic acid 1,4-lactone, an inhibitor of β-glucuronidase (Ricoveri and Cappellari, 1986). Digestions were carried out in duplicate using a boiling extract as a control. At the end of incubation, the mixture was centrifuged at 10000 x g for 5 min and the supernatant was applied to a Sepharose CL-6B column, as described above.

**Intact cells**

Equal amounts (30 x 10⁵ d.p.m.) of 35S-HS from control Caco-2-H cells were added to confluent cell monolayers of Caco-2-H, Caco-2-MT or Caco-2-T cells. The medium was buffered by adding 1 M Hepes (pH 7.4) to a final concentration of 10 mM. The cells were incubated for the indicated times with gentle-shaking. At the end of incubation, the medium was harvested, centrifuged at 10 000 x g for 5 min and fractionated, as described for cell extracts.

**Statistical analysis**

Results are means ± s.d. for the indicated numbers of experiments with different cell preparations.

**Results**

**Membrane-anchored PGs**

The PGs from control and oncogene-transfected Caco-2 cells were examined for the presence of lipophilic moieties by hydrophobic chromatography on Octyl-Sepharose CL-4B, a procedure currently used to identify PG species that are integral components of the plasma membrane. Chromatography of detergent-extracted PGs generated two sulphate-containing peaks (Figure 1). The first peak, which eluted before the beginning of the gradient, constituted the unbound fractions. The second peak, requiring a detergent-containing buffer to be displaced from the column, contained 35S-labelled PGs which bound to Octyl-Sepharose through hydrophobic interactions. This bound material comprised the lipophilic PGs. In control Caco-2-H cells (Figure 1a), 57.6% of the total 35S-labelled PGs bound to the column, and the hydrophilic PG fraction eluted at 0.14% Triton X-100. In contrast, in the oncogene-transfected Caco-2-MT cells (Figure 1b) and Caco-2-T cells (Figure 1c), only 40.5% and 37.2% of the total 35S-labelled PGs bound to the column, and the hydrophilic PG fractions eluted at Triton X-100 concentrations of 0.11% and 0.09% respectively. These results indicate that expression of Ha-ras or Py-MT oncogenes in Caco-2 cells induced a decrease in the amount of membrane-anchored PGs. As indicated in Table I, this decrease was selectively accounted for by a decrease in syndecan-1 since the amount of the other lipophilic PGs was not modified in oncogene-transfected Caco-2-MT and Caco-2-T cells.

**Syndecan-1 expression**

Among the membrane-anchored PGs, syndecan-1 is known to play a central role in the maintenance of the epithelial phenotype. We therefore examined the expression of this PG in Caco-2 cells to determine if it was altered by Ha-ras or Py-MT oncogenes. Figure 2 shows the results of Northern blot analysis using a cDNA probe specific for syndecan-1 (Saunders et al., 1989). In control Caco-2 cells, two mRNA transcripts of 2.6 and 3.4 kb were detected (Figure 2a), in accordance with the results reported for other cell types (Saunders et al., 1989; Kirjavainen et al., 1993; Inki et al., 1994). In oncogene-transfected Caco-2-MT and Caco-2-T cells, syndecan-1 mRNA transcripts were present in a relative abundance similar to that observed in Caco-2-H cells, as determined by densitometric scanning of the Northern blots and normalisation of the results with respect to the 28S signal (Figure 2a). These results indicate that neither Ha-ras nor Py-MT oncogenes modified the amount of syndecan-1 mRNA.

Figure 2b shows the results of immunoblot analysis using an anti-syndecan-1 antibody (Jalkanen et al., 1985). In all three cell lines, this antibody recognised a core protein of approximately 70 kDa, which is in accordance with the published molecular mass of syndecan-1 core protein.
Determined (Jalkanen level. The oncogenic p21ras and GAG were monitored by tritium-100, Triton X-100 (c) were labelled with Na$_3^{35}$SO$_4$. $^{35}$S incorporation into total membrane-anchored PGs was determined as indicated in Figure 1. $^{35}$S incorporation into syndecan-1 was determined after mild trypsin treatment of the cells. Other, lipophilic PGs expressed at the cell surface as trypsin-resistance PGs.

Table 1 $^{35}$S incorporation into lipophilic PGs

| Cell line | Total membrane-anchored PG (c.p.m.) | Syndecan-1 (c.p.m.) | Other (c.p.m.) |
|-----------|------------------------------------|---------------------|---------------|
| Caco-2-H  | 23 205 ± 2 575                     | 17 400 ± 1 635      | 5 800 ± 555   |
| Caco-2-MT | 15 650 ± 2 065                     | 9 565 ± 945         | 6 083 ± 630   |
| Caco-2-T  | 14 370 ± 2 125                     | 8 710 ± 920         | 5 660 ± 690   |

Control and oncogene-transfected Caco-2 cells were labelled with Na$_3^{35}$SO$_4$. $^{35}$S incorporation into total membrane-anchored PGs was determined as indicated in Figure 1. $^{35}$S incorporation into syndecan-1 was determined after mild trypsin treatment of the cells. Other, lipophilic PGs expressed at the cell surface as trypsin-resistance PGs.

Figure 1 Hydrophobic affinity chromatography of detergent-extracted PGs. DEAE-purified $^{35}$S-labelled PGs from control Caco-2-H (a) and oncogene-transfected Caco-2-MT (b) or Caco-2-T (c) cells were chromatographed on Octyl-Sepharose CL-4B. Columns were equilibrated with 4 M Gdn HCl–50 mM sodium acetate (pH 7.0) and eluted with a linear gradient of 0–0.8% (v/v) Triton X-100 in the same buffer. Fractions (1 ml) were analysed for Triton X-100 concentration by absorbance at 280 nm and monitored for radioactivity.

Figure 2 Northern blot and Western blot analyses of syndecan-1. In a, 30 μg of total RNA isolated from control Caco-2-H and oncogene-transfected Caco-2-MT or Caco-2-T cells were size-fractionated in a 1% agarose/formaldehyde gel and blotted onto Hybond-N membrane. The membrane was hybridised with a cDNA probe specific for syndecan-1 (Saunders et al., 1989), and then with a 28 S rRNA probe. In b, samples of PG-enriched fractions were fractioned by SDS–PAGE (2 to 20% gradient) and transferred onto an Immobilon-N membrane. The membrane was then probed with MAb 281-2, and immunoreactive protein was visualised using enhanced chemiluminescence. The band representing the core protein of syndecan-1 is indicated by an arrow. Corresponding molecular weight markers in kDa are shown on the left.

Specific activity of syndecan-1 ectodomain and its HS and CS GAG side chains

The $^{35}$SO$_4$-labelled ectodomain of syndecan-1 from control and oncogene-transfected cells was isolated as described in Materials and methods, and incorporated radioactivity was measured by dot blot assay. As reported in Table II, the specific activity of the syndecan-1 ectodomain (expressed as d.p.m. mg$^{-1}$ of protein) was markedly lower in oncogene-transfected Caco-2-MT and Caco-2-T cells (42 380 ± 5600 and 30 240 ± 4570 respectively), than in control Caco-2-H cells (151 760 ± 14 115).

To compare $^{35}$S-sulphate distribution in the HS and CS side-chains of syndecan-1 in the three cell lines, labelled syndecan-1 ectodomains were isolated from Caco-2-H, Caco-2-MT and Caco-2-T cells and subjected to chondroitin ABC lyase or nitrous acid degradation, and the remaining HS or
CS chains were examined for their specific radioactivity. In the three cell lines, the syndecan-1 ectodomain consisted mainly of HS chains but also possessed some CS chains (Table II). In control Caco-2-H cells, most of the total 35S-sulphate incorporated into the syndecan-1 ectodomain (i.e. 87.9 ± 2.8%) was degraded with nitrous acid, indicating that it consisted of HS chains, whereas CS chains only accounted for 12.1 ± 2.8%, as determined by sensitivity to chondroitin ABC lyase. In contrast, in oncogene-transfected Caco-2-MT and Caco-2-T cells, the proportions of 35S-sulphate incorporated into HS chains decreased to 74.1 ± 3.6% and 64.6 ± 5.8% of total 35S-labelling respectively, whereas the respective proportions of 35S-sulphate incorporated into CS chains rose to 25.9 ± 3.6% and 35.4 ± 5.8%. These data provide clear evidence that the functional insertion of Ha-ras or Py-MT oncogenes in Caco-2 cells markedly lowered the specific activity of the syndecan-1 ectodomain, and concomitantly reduced the HS/CS specific activity ratio.

Polyanionic properties of GAG side-chains in the syndecan-1 ectodomain

To establish the basis of the post-translational modifications of the syndecan-1 ectodomain in oncogene-transfected Caco-2-MT and Caco-2-T cells, we examined two major characteristics of their GAG side-chains: the degree of sulphation and molecular mass.

We first determined the extent of sulphation of the syndecan-1 GAG side-chains to see if it was modified by oncogene transfection. For this purpose, the polyanionic properties of the 35S-labelled GAGs released from the syndecan-1 ectodomain by alkaline borohydride were investigated by ion-exchange chromatography on DEAE-Sepharose (Figure 3). GAGs from Caco-2-MT and Caco-2-T cells (Figure 3b and 3c) bound less tightly to the column than GAGs from control Caco-2-H cells (Figure 3a), as indicated by their elution at lower sodium chloride concentrations than those required for elution of GAGs from control Caco-2-H cells (0.47 M and 0.40 M vs 0.56 M). These results indicate a lower degree of sulphation of syndecan-1 GAG side-chains in oncogene-transfected cells than of the corresponding chains in control cells.

Size analysis of syndecan-1 ectodomain

35S-labelled syndecan-1 ectodomains isolated from control and oncogene-transfected Caco-2 cells were analysed on sepharose CL-4B column (Figure 4). Syndecan-1 from control Caco-2-H cells eluted as a peak with a Kav of 0.50 (Figure 4a), whereas syndecan-1 from transfected Caco-2-MT and Caco-2-T cells eluted as a peak with Kav values of 0.62 (Figure 4b) and 0.66 (Figure 4c) respectively. These results show that the ectodomain of syndecan-1 synthesised by Ha-ras or Py-MT oncogene-transfected Caco-2 cells exhibited smaller hydrodynamic sizes than that of syndecan-1 from control Caco-2-H cells.

GAG chain length of syndecan-1 ectodomain

To determine whether the lower relative molecular mass of syndecan-1 ectodomain in transfected Caco-2 cells was due to a reduction in its GAG chain length, 35S-labelled ectodomains were subjected to alkaline β-elimination and then to degradation with either chondroitin ABC lyase or nitrous acid. The resulting HS or CS chains were chromatographed on Sepharose CL-6B and their respective Kav values in Caco-2-MT and Caco-2-T cells were 0.60 and 0.65 for HS chains, and 0.53 and 0.56 for CS chains (Table III). In control Caco-2-H cells, the Kav value was 0.45 for HS chains and 0.50 for CS chains. Thus, the syndecan-1 synthesised by oncogene-transfected Caco-2 cells contained HS and CS chains of a smaller hydrodynamic size than the syndecan-1 synthesised by control Caco-2-H cells (Table III).

![Figure 3](image-url) Ion-exchange chromatography on DEAE-Sepharose of GAG chains from syndecan-1. 35S-labelled GAGs released from syndecan-1 ectodomain of control Caco-2-H (a) and oncogene-transfected Caco-2-MT (b) or Caco-2-T (c) cells by alkaline borohydride were applied to a DEAE-Sepharose column (1 x 2 cm). After extensive washing of the column with a buffer containing 50 mM Tris–50 mM sodium chloride (pH 6.8), the elution was performed with a linear gradient of 50 mM–800 mM sodium chloride in the same buffer. The resulting 2 ml fractions were collected and analysed for radioactivity.

![Figure 4](image-url) Size analysis of syndecan-1 ectodomain. (a) Caco-2-H (b) Caco-2-MT (c) Caco-2-T. The syndecan-1 ectodomain was analysed on sepharose CL-4B column (Figure 4). Syndecan-1 from control Caco-2-H cells eluted as a peak with a Kav of 0.50 (Figure 4a), whereas syndecan-1 from transfected Caco-2-MT and Caco-2-T cells eluted as a peak with Kav values of 0.62 (Figure 4b) and 0.66 (Figure 4c) respectively. These results show that the ectodomain of syndecan-1 synthesised by Ha-ras or Py-MT oncogene-transfected Caco-2 cells exhibited smaller hydrodynamic sizes than that of syndecan-1 from control Caco-2-H cells.

| Cell line          | Syndecan 1 ectodomain (d.p.m. mg⁻¹ of protein) | HS (d.p.m. mg⁻¹ of protein) | CS (d.p.m. mg⁻¹ of protein) |
|--------------------|-----------------------------------------------|-----------------------------|-----------------------------|
| Caco-2-H           | 151 760 ± 11 115                               | 133 550 ± 13 620            | 18 210 ± 2 385              |
| Caco-2-MT          | 42 375 ± 5 600                                 | 31 400 ± 4 430              | 10 975 ± 1 700              |
| Caco-2-T           | 30 230 ± 4 570                                 | 19 530 ± 3 280              | 10 700 ± 1 840              |
|                    | (100%)                                        | (100%)                      | (100%)                      |
|                    | (87.9 ± 2.8%)                                  | (74.1 ± 3.6%)               | (64.6 ± 5.8%)               |
|                    | (12.1 ± 2.8%)                                  | (25.9 ± 3.6%)               | (35.4 ± 5.8%)               |
|                    | (100%)                                        | (100%)                      | (100%)                      |

Cells were metabolically labelled for 24 h with 35S-sulphate. The syndecan-1 ectodomain from control Caco-2-H and oncogene-transfected Caco-2-MT or Caco-2-T cells were collected by mild trypaninisation. The radioactivity in the syndecan-1 ectodomain was quantified by binding to Zeta-Probe membranes as described in Materials and methods. Radiolabel in HS and CS was determined by specific susceptibility to nitrous acid or chondroitin ABC lyase respectively, and was quantified as for the intact syndecan-1 ectodomain. Results are expressed as d.p.m. mg⁻¹ of protein. Numbers in brackets represent percentage of total 35S-sulphate incorporation in the syndecan ectodomain. Each value represents the mean ± s.d. of five experiments.
Heparanase degradative activity in oncogene-transfected Caco-2-MT and Caco-2-T cells

The reduced hydrodynamic size of the HS chains of the syndecan-1 ectodomain in oncogene-transfected Caco-2 cells led us to suspect HS degradation by active heparanase in these cells. To test this hypothesis, intact control Caco-2-H cells and oncogene-transfected Caco-2-MT and Caco-2-T cells, as well as cell extracts from all three cell lines, were compared for heparanase degradative activity using 35S-labelled HS extracted from control Caco-2-H cells as a substrate (see Materials and methods).

Incubations were carried out at 37°C for 3, 6, 18 and 30 h. The degradation products were analysed by gel chromatography on Sepharose CI-6B and monitored by scintillation counting. The first set of experiments, carried out with intact Caco-2 cells, showed that neither Caco-2-H cells nor oncogene-transfected Caco-2-MT and Caco-2-T cells contained significant heparanase activity, whatever the incubation time studied (data not shown). With cell extracts of the three cell lines, no appreciable degradation was observed after short incubation periods (3 h or 6 h). By contrast, after 18 h incubation we detected heparanase activity which degraded the HS in the cell extracts from the oncogene-transfected Caco-2 cells. The presence of this activity was indicated by an incompletely resolved, slowly eluting peak of lower molecular weight species than in the control Caco-2-H cells, which displayed no heparanase activity. The elution patterns of Caco-2-MT and Caco-2-T cells after incubation for 18 h are compared in Figure 5b and 5c with the pattern obtained for the control cell extracts (Figure 5a). However, after an even longer incubation period (30 h), no further degradation was observed (data not shown). These results indicate the presence of heparanase degradative activity in cell extracts from oncogene-transfected Caco-2 cells.

**Figure 4** Sepharose CI-4B chromatography of syndecan-1. 35S-sulphate labelled syndecan-1 ectodomains from control Caco-2-H (a) and oncogene-transfected Caco-2-MT (b) or Caco-2-T (c) cells were applied to a Sepharose CI-4B column (0.9 x 60 cm). Elution was performed with a 4 M Gdn HCl - 50 mM sodium acetate buffer (pH 5.8) containing 0.5% (w/v) CHAPS and protease inhibitors. Fractions of 0.5 ml were collected and analysed for radioactivity.

**Figure 5** Heparanase degradative activity in oncogene-transfected Caco-2 cells. After a 24h labelling, 35S-labelled HS from control Caco-2-H cells was prepared by alkaline borohydride degradation. This 35S-HS (3 x 10^6 d.p.m) was incubated for 18 h in the presence of Caco-2-H (a), Caco-2-MT (b) and Caco-2-T (c) cell extracts (3 mg proteins) and then fractionated on a 0.9 x 60 cm Sepharose CI-6B equilibrated with 4 M Gdn HCl - 50 mM sodium acetate (pH 7.0), at a flow rate of 9 ml h^-1.
Table III  Approximate molecular mass of GAG side chains of the syndecan-1 from control and oncogene-transfected Caco-2 cells

| Cell line | HS Kav | kDa | CS Kav | kDa |
|-----------|--------|-----|--------|-----|
| Caco-2-H  | 0.45   | 28.0| 0.50   | 24  |
| Caco-2-MT | 0.60   | 12.5| 0.53   | 19  |
| Caco-2-T  | 0.65   | 9.0 | 0.56   | 16  |

*a* Kav values displayed are for the peak elution fractions on Sepharose CL-6B columns. *b* The approximate kDa of HS and CS chains was calculated on the basis of a standard curve according to the method of Wasteson (1971).

Discussion

The present study was designed to examine the alterations in syndecan-1 during the tumorigenic progression induced by oncogenic p21ras and pp60c-src in human colonic epithelial cells. To this end, we used Caco-2 cells transfected either with an activated (Val 12) human Ha-ras gene or with the cDNA encoding the Py-MT antigen, a constitutive activator of the tyrosine kinase of pp60c-src.

One important observation made in our study is that both oncogene-transfected Caco-2 cell lines displayed similar alterations in their syndecan-1, although each expressed a specific oncoprotein. In a recent report, we provided clear evidence that the Py-MT oncogene caused a constitutive increase in the activity of p21ras in Caco-2-MT cells, which was almost identical to that caused by the oncogenic activation of p21ras in Caco-2-T cells (Baron-Delage et al., 1994). Therefore, in human colonic Caco-2 cells, p21ras lies downstream of Py-MT/pp60c-src in the same signal transduction pathway, as reported for another cell type (Pickett and Gutierrez-Hartmann, 1994).

Here, we show that oncogenic p21ras and Py-MT/pp60c-src induced a decrease in syndecan-1 expression at the protein but not the mRNA level. A similar finding was yet reported by others for the Ha-ras-transformed epithelial NOG-8 cell line and was suggested to result from decreased mRNA translation in oncogene-transformed cells (Kirjavainen et al., 1994). Along with decreased syndecan-1 expression, ras- and PY-MT-transfected Caco-2 cells exhibited a dramatic activation of their tumorigenic potential in nude mice but failed to become invasive as previously determined by using an in vitro invasion assay (Chastrette et al., 1993). In fact, several arguments support the notion that the invasiveness of transformed cells is correlated with a complete down-regulation of syndecan-1 expression: (1) In vivo studies of syndecan-1 expression in transformed keratinocyte cell lines have shown that when cells were injected into nude mice, poorly differentiated areas, i.e. the most invasive areas within the tumour, were devoid of syndecan-1 expression at the protein and mRNA levels (Inki et al., 1992); (2) S115 mouse mammary tumour cells that exhibited malignant growth behaviour lacked syndecan-1 gene expression but lost their malignant growth behaviour after the re-expression of syndecan-1 (Leppä et al., 1992); (3) In vitro studies using B lymphoid cells have shown that syndecan-1 must be lost before cells can invade the extracellular matrix (Liebersbach and Sanderson, 1994). In the light of these findings which point to an inverse relationship between syndecan-1 expression and the invasive potential of transformed cells, one may wonder whether the failure of ras or Py-MT oncogenes to endow Caco-2 cells with the invasive phenotype may be connected with their inability to repress completely syndecan-1 expression in these cells.

Initial studies provided evidence for a close correlation between syndecan mRNA and its immunoreactive protein expression in healing wounds or during tooth organogenesis, thus suggesting that the regulation of syndecan expression occurs at the transcriptional level (Elenius et al., 1991; Vainio et al., 1991). However, more recent papers provided experimental arguments indicating that syndecan-1 expression is regulated at the translational level (Sanderson et al., 1992; Vainio et al., 1992; Kirjavainen et al., 1993). In the present study, we provide clear evidence that Ha-ras and PY-MT oncogenes alter syndecan-1 expression at both the translational and post-translational levels. Caco-2-MT and Caco-2-T cells indeed showed a decrease in syndecan-1 protein but not mRNA expression and also exhibited alterations in syndecan-1 ectodomain. These alterations consisted of a decrease in the length and degree of sulphation of the GAG chains attached to the protein core together with modification of the GAG composition. Although the main GAG of the syndecan-1 ectodomain in control and oncogene-transfected Caco-2 cells consists of HS, a higher proportion of CS was observed in Caco-2-MT and Caco-2-T cells. In agreement with our results, another group of authors reported that GAGs isolated from colon tumours contain larger amounts of CS than those from normal colonic mucosa (Iozzo and Wight, 1982; Iozzo et al., 1989).

We present here, as far as we know, the first evidence for altered glycosylation of syndecan-1 associated with malignant progression in human colonic epithelial cells. Other studies have shown that the interactions of syndecans with extracellular matrix components and their ability to undergo glycosylation or sulphation (Sanderson et al., 1994), strongly suggesting a central role for syndecan in cell–matrix adhesion. In the light of these findings, our results are significant, and the post-translational modifications of syndecan-1 in Ha-ras- and Py-MT-transfected Caco-2 cells might provide Caco-2-MT and Caco-2-T cells with a mechanism that would allow the loosening of their attachment to the extracellular matrix and maintain their growth.

The first step in the development of cancer is uncontrolled cell growth. The next critical step is the transition of the tumour from mere growth to invasion and metastasis. During this transition, a series of genetic and molecular events causes tumour cells to increase their production and activation of the enzymes that cleave the extracellular matrix. Activity that degrades ECM components is mediated by a variety of proteases and endoglycosidases, including the plasmin/plasminogen activator family of serine proteases, the matrix metalloproteinases and the heparanases, which are specific HS PG-degrading endoglycosidases (Stetler-Stevenson et al., 1993). The present results show that oncogene-transfected Caco-2 cells were able to degrade purified HS, thus reflecting the presence of heparanase activity in these cells. However, the activity fails to be seen in vivo and shows only on HS degradation. Therefore, the decrease in HS chain size of syndecan-1 cannot be solely accounted for by the presence of the heparanase activity and probably also reflects the synthesis of the oncogene-transfected Caco-2 cells of smaller HS chains on the same core protein.

Recent advances in the understanding of HS PGs implicate them as important participants in cell signalling. At the cell surface, syndecan may transduce ECM–cytoskeletal actin-mediated signals essential for cell growth. The conserved cytoplasmic domain of syndecans could transmit signalling, directly or indirectly, by specifically interacting with the membrane and cytoplasmic transduction systems. In addition, syndecan may also mediate signalling through interaction with cytoskeletal proteins (Rapraeger, 1993; Carey et al., 1994). In this connection, Rapraeger speculated that the formation of the syndecan/FGF/tyrosine kinase receptor complex results in phosphorylation of the syndecan itself, thus influencing its interaction with the cytoskeleton or with other receptors such as the cadherins, and thereby directly affecting cell shape and behaviour (Rapraeger, 1993).

In conclusion, our results demonstrate that the dramatic activation of the tumorigenic potential induced by oncogenic p21ras or PY-MT/pp60c-src in Caco-2 cells altered syndecan-1 expression at the protein but not the mRNA level, and induced marked alterations in the syndecan-1 ectodomain.
thus indicating that these oncoproteins influence the translational regulation and the post-translational machinery responsible for syndecan-1 processing. These alterations may be critical for altered cell–matrix adhesion properties and for the malignant growth of the onconeplasty-transfected Caco-2 cells.

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