Effect of transforming growth factor-β1 and basic fibroblast growth factor on the expression of cell surface proteoglycans in human lung fibroblasts

Enhanced glycanation and fibronectin-binding of CD44 proteoglycan, and down-regulation of glypican

Manuel ROMARÍS,* Anna BASSOLS† and Guido DAVID*‡

*Center for Human Genetics, University of Leuven, B-3000 Leuven, Belgium, and †Departament de Bioquímica i Biologia Molecular, Facultat de Veterinària, Universitat Autonòma de Barcelona, 08193-Bellaterra, Spain

We have tested the effects of transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor (bFGF) and TGF-β1 + bFGF on the expression of the cell surface proteoglycans (CD44, syndecans and glypican) in cultures of human lung fibroblasts (HLF). Cell surface proteoglycan expression was monitored by quantitative immunoprecipitation from metabolically labelled cells, Western and Northern blotting and evaluation of the glycanation of the proteoglycans. Stimulation of the cells with TGF-β1 increased the length of the chondroitin sulphate (CS) chains on CD44 ($\sim$ 1.6-fold). bFGF, administered solely, also increased the length of the CS chains on CD44 ($\sim$ 1.4-fold), whereas the combination of TGF-β1 + bFGF nearly doubled both the length and the number of the CS chains on CD44. None of these treatments lead to changes in CD44 message or core-protein expression. This enhanced glycanation of CD44 after the TGF-β1, bFGF and combined treatments correlated with a 2-fold increase in the affinity of the proteoglycan for fibronectin but had no influence on the binding to type I collagen. TGF-β1, alone or in combination with bFGF, also stimulated the CS content of syndecan-1, but none of the other syndecans was significantly affected by any of the factors or combinations tested. The expression of glypican however was significantly decreased (nearly halved) by the combination of TGF-β1 + bFGF, less so by TGF-β1 and not at all by bFGF. This decrease occurred both at the level of the message and of the core protein. These data demonstrate specific and differential effects of TGF-β1 and bFGF on the structure, expression and interactions of the cell surface proteoglycans of HLF.

INTRODUCTION

The cell surface proteoglycans represent a heterogeneous group of receptors for cell adhesion molecules, growth factors, matrix components, enzymes and enzyme inhibitors that are found, in varying combinations, at the surfaces of virtually all types of cells [1–4]. Their functions appear auxiliary in the sense that, generally, their ligands in addition bind to other receptor structures at the cell surface, often with unique specificities or with specificities that by far exceed those that characterize the interactions of the proteoglycans. The interactions of these proteoglycans are not accessory, however, as in certain cases they seem required for the activation or stability of the ligand. In that sense the cell surface proteoglycans function as essential modulators of processes that control the shape, growth and migration of cells [5,6].

The common denominator which groups these otherwise unique membrane proteins is their substitution with glycosaminoglycan (GAG) chains. These consist of heparan sulphate (HS) or chondroitin sulphate (CS) chains, sometimes both together, which in large part determine the types and affinities of the proteoglycan interactions. Some cell surface proteoglycans share, in addition, structural motifs in their protein moieties, or feature structural elements that have also been encountered in other proteoglycans or glycoproteins with defined functions. Syndecan (syndecan-1), fibroglycan (syndecan-2), *N*-syndecan (syndecan-3) and amphiglycan or ryudocan (syndecan-4) have transmembrane and cytoplasmic domains that are highly similar and that may interact with the same or similar intracellular structures (reviewed in [7]). These four proteins are entirely distinct from glypican [8] and cerebroglycan [9], membrane HS proteoglycans that are linked to the cell surface through glycosylphosphatidylinositol moieties, and from CD44, a polymorphic membrane protein (reviewed in [10]) with various degrees of glycanation [11] that bears a resemblance to hyaluronan-binding proteins and proteoglycans of the extracellular matrix [12,13] and that functions as a cell surface receptor for hyaluronate [14], fibronectin [15] and type I collagen [16].

On the other hand, various cytokines, and TGF-β1 in particular, influence cellular behaviour and differentiation by affecting the production, composition and turnover of the extracellular matrix and of the cellular receptors for the matrix, whereby the matrix also influences the activities of the cytokine itself [17]. Effects of transforming growth factor-β1 (TGF-β1) on secreted matrix GAGs and proteoglycans include stimulation of the production of hyaluronate [18], biglycan [19,20] and versican [20,21], changes in the glycanation of these molecules [22] and enhanced expression of the basement membrane proteoglycan perlecan [23]. In mouse mammary epithelial cells TGF-β1 has also been shown to enhance the incorporation of CS in syndecan-1, while leaving the amount of HS on this hybrid molecule relatively unaffected [24]. In combination with basic fibroblast growth factor (bFGF), TGF-β1 stimulates the production of syndecan-1 message and protein in 3T3 cells, but not in mouse mammary epithelial cells, suggesting synergistic effects of different cytokines and differences in responsiveness amongst cells [25]. Interestingly, TGF-β1 in turn binds to the core protein of decorin [26], biglycan and fibromodulin [27], and to the core protein of betaglycan, a polymorphic hybrid proteoglycan that occurs both at the cell surface [28–31] and in the extracellular matrix [32].

Cultured human lung fibroblasts (HLF), in particular, respond to TGF-β1 by an increased production of collagen [33], fibronectin [34] and biglycan [19] and by enhanced expression of integrins at their cell surfaces [34,35], primarily through tran-

Abbreviations used: TGF-β, transforming growth factor-β; bFGF, basic fibroblast growth factor; HLF, human lung fibroblasts; CS, chondroitin sulphate; HS, heparan sulphate; GAG, glycosaminoglycan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody.

scriptional mechanisms. These cells express all four syndecans [36], glypican [8] and two forms of cell surface CS [37] that in concert with the integrins may act as receptors for matrix components. In the current paper we have examined the effects of TGF-β1 and bFGF on the expression of these cell surface proteoglycans. TGF-β1 was found to significantly stimulate the amount of GAG on the cell surface CS proteoglycan that was identified as a glycanated form of the CD44/hyaluronate receptor, with little or no stimulatory effects on the HS proteoglycans. TGF-β1 increased the length of the CS chains on CD44, without affecting the RNA messages or the levels of the glycoprotein and proteoglycan forms of this protein. The combination of TGF-β1 and bFGF, which has been shown to stimulate syndecan-1 at the mRNA and protein level in 3T3 cells [25], did not stimulate the accumulation of CD44 or syndecan messages and core proteins in HLF. Yet, this combination markedly enhanced the glycanation of CD44, by increasing both the length and the number of CS chains on this proteoglycan, and decreased the expression of glypican. The effect on the glycanation of CD44 correlated with an increase in the affinity of this proteoglycan for fibronectin. TGF-β1 and bFGF, in this sense, behave as specific modulators of the expression and properties of the cell surface proteoglycans in HLF.

MATERIALS AND METHODS

Materials

TGF-β1 from human platelets was purchased from British Biotechnology Ltd. (Oxford, U.K.), bFGF and fibronectin were from GIBCO BRL (Gent, Belgium). Heparitinase (heparin sulphate lyase, EC 4.2.2.8), heparinase (heparin lyase, EC 4.2.2.7) and chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) were from Seikagaku Kogyo (Tokyo, Japan). Endopeptidase K (Proteinase K; EC 3.4.21.64) was from Merck (Darmstadt, Germany). HS and CS were obtained from Sigma (St. Louis, MO, U.S.A.). The monoclonal antibodies (mAbs) Hermes-1 and Hermes-3 were gifts from Dr. S. Jalkanen (National Public Health Institute, Turku, Finland). Polyclonal 'anti-CS stub' antibodies 36/4 and 36/6, raised against chondroitinase ABC-digested aggrecan and directed against the desaturated uronates on the residual CS stubs of the proteoglycan, were gifts from Dr. J. R. Couchman (University of Alabama, Birmingham, AL, U.S.A.). All other antibodies have been described before [37–39]).

Cell culture

Normal HLF, between passages 11 and 18, were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. Subconfluent cultures were grown in serum-free medium for 8 h before each experiment. Then, TGF-β1 (2.5 ng/ml), bFGF (10 ng/ml) or a combination of TGF-β1 + bFGF were added and the cells were incubated further for an additional 16 h. At the same time, when needed, labelling with 100 mCi/ml of carrier-free $H_2{}^{35}SO_4$ (New England Nuclear) or with 50 μCi/ml of both $H_2{}^{35}SO_4$ and [^{3}H]glucosamine in low-sulphate medium (0.08 mM) was initiated. No effects on cell proliferation were noticeable after the 16 h treatments, for any of the cytokines tested.

For the time-course experiments with TGF-β1 the cells were treated for 0, 6, 12, 24 and 48 h with 2.5 ng/ml of TGF-β1. Medium and cytokine were changed after 24 h. Labelling with $^{35}SO_4$ was carried out during the last 6 h of the treatment. For the dose–response experiment the cells were stimulated for 16 h at various concentrations of TGF-β1 [19].

Proteoglycan isolation

The media were removed and the monolayers were washed three times with cold PBS. The cell surface proteoglycans were extracted in Triton X-100 buffer as described before [40] and absorbed on DEAE-Trisacryl M beads. The beads were rinsed with 0.2 M NaCl/20 mM Tris/HCl, pH 7.4/0.5% Triton X-100 and the proteoglycans eluted with 0.9 M NaCl in the same buffer (DEAE eluate). The eluates were used for immunopurifications or analysed by Western blotting as described below.

Immunopurification

The anti-core-protein mAbs, 2E9 (which reacts with both syndecan-1 and syndecan-3), 6G12 and 10H4 (anti-syndecan-2), 1C7 (anti-syndecan-3), 8G3 (anti-syndecan-4), S1 and 1G11 (anti-glypican) and 7D8 (anti-CD44) were immobilized on CNBr-activated Sepharose 4B [38]. The identification of mAb 7D8 [37] as an anti-CD44 antibody was based on the cross-reactivity of the 7D8-immunopurified proteoglycan with the Hermes-1 and Hermes-3 antibodies. The expression of the different proteoglycans was measured by incubating DEAE eluates from $^{35}SO_4$-labelled cells overnight at 4 °C with the different immunobeads in 0.1% Triton X-100/150 mM NaCl/50 mM Tris/HCl, pH 7.4. Bound proteoglycans were eluted from the various immunobeads with 0.1% Triton X-100/4 M guanidinium chloride/50 mM Tris/HCl, pH 8.0. Eluted proteoglycan was quantified by β-counting or analysed by electrophoresis in SDS/4% NuSieve agarose or 6–20% polyacrylamide gradient gels and detected by autoradiography [37]. In some cases, after the immunopurifications, the proteoglycans were digested with chondroitinase ABC, heparitinase or both enzymes to study the effects on CS and HS synthesis.

Western blotting

The DEAE eluates were incubated for 3 h at 37 °C with 50 m-units/ml of chondroitinase ABC, with 10 m-units/ml of heparitinase or with both chondroitinase ABC and heparitinase in 100 mM NaCl, 1 mM $CaCl_2$, 50 mM sodium-Hepes (pH 7.0) and 50 μg/ml BSA. The digests were resolved by SDS/PAGE (6–20% acrylamide gradients) under non-reducing conditions, transferred to Z-Probe membranes (Bio-Rad) and incubated with mAbs (anti-core-protein, anti-'HS stub') and polyclonal anti-'CS stub' antibodies in PBS/0.5% casein buffer as described [38,39]. The binding of the antibodies was detected by chemiluminescence using alkaline phosphatase-conjugated rabbit anti-(mouse immunoglobulin) or goat anti-(rabbit immunoglobulin). Quantification of the band intensities was performed with a Personal Densitometer using ImageQuant software (Molecular Dynamics). The readings represented the averages of four independent experiments and were taken from exposures falling within the linear range of the film response.

Northern blotting

CD44 cDNA was amplified from total RNA extracts from HLF using the RNA-PCR-Kit from Perkin-Elmer Cetus. Sense (5′-GCTGTCGACACCATGGACAAGTTTTGGTGG) and antisense (5′-ATGGTCGACGTGTTACACCCCAATCTTCAT)

primers were synthesized according to published CD44 sequences [12,13] and used during 35 thermal cycles (1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C). The single PCR product (approximately 1100 bp) was restricted with *Pst*I and *Bam*HI and subcloned into pGEM3Z (Promega) for sequence verification. A probe for mouse syndecan-3 was produced using the primer combination and a mouse lung cDNA library as template source for the amplification, as described by others [41].

RNA from control and cytokine-treated HLF was extracted using the acid/guanidinium thiocyanate/phenol/chloroform protocol [42]. Aliquots of 10 μg of total RNA were fractionated in a 1.2 % agarose gel in the presence of formaldehyde, transferred to Hybond-N membranes (Amersham) and hybridized with ^{32}P-labelled cDNA probes for mouse syndecan-3 and human syndecan-1 (HUSYN), fibroglycan (48K5), amphiglycan (35K17), glypican (64K1) and CD44, as described before [8,36,40,43].

Chromatography

$^{35}SO_4$-labelled CD44 was purified from control, TGF-β1-, bFGF-and TGF-β1 + bFGF-treated cells using antibody 7D8. To isolate protein-free CS chains the proteoglycan was digested with 100 μg/ml of proteinase K and then treated with 10 m-units/ml of heparinase and 10 m-units/ml of heparitinase or, alternatively, with nitrous acid at pH 1.5 [44]. The presence of only CS chains in the residue was confirmed by further digestion with 50 m-units/ml of chondroitinase ABC, precipitating the sample with 95 % ethanol/1 % sodium acetate/1 μg/ml of carrier CS, and counting the label in the pellets and supernatants.

The [^{35}S]CS chains were sized by chromatography over Sepharose CL6B (60 × 1 cm) equilibrated with 0.1 % SDS and 0.1 M Tris/HCl, pH 8.0. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min. Purified CS chains from $^{35}SO_4$-labelled CD44 were loaded on a DEAE-Trisacryl M column (4 ml of beads) and eluted with a linear (0–2 M) NaCl gradient in 0.5 % Triton X-100/6 M urea/50 mM Tris/HCl, pH 8.0, at a flow rate of 0.5 ml/min [38]. Fractions of 0.6 ml were collected and counted in an LKB β-counter.

Doubly $^{35}SO_4$- and [^{3}H]glucosamine-labelled CD44 was isolated from control and TGF-β1-stimulated cells as described above. Doubly labelled CS chains were isolated from CD44 and from non-CD44 proteoglycan fractions by proteinase K digestion, nitrous acid degradation at pH 1.5 and reabsorption on DEAE. The non-CD44-derived CS from stimulated and non-stimulated cells was digested with chondroitinase ABC, the ΔDi-4S products present in the digests were isolated by TLC in n-butanol/glacial acetic acid/1 M ammonia (2:3:1) and the ^{35}S/^{3}H ratio was measured [45].

Binding experiments

Unlabelled or ^{35}S-labelled CD44 proteoglycan was purified from control, TGF-β1-, bFGF- or TGF-β1 + bFGF-treated cells as described above. Unlabelled CD44 was radio-iodinated using chloramine T as described before [38]. Both [^{35}S]CD44 and ^{125}I-CD44 were analysed for binding to fibronectin that had been coupled to CNBr-Sepharose beads. Serial dilutions of the labelled proteoglycans were incubated with 1 μg of immobilized fibronectin in 1 ml of 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.5 % Triton X-100 and 0.01 % casein. After overnight incubation at 4 °C the fibronectin beads were pelleted by centrifugation. Both the supernatants and the pellets were analysed in a β- or γ-counter. The CD44–fibronectin binding experiments were also done after the digestion of the proteoglycan with chondroitinase ABC, heparitinase or chondroitinase ABC + heparitinase. For the calculation of the K_d value we assumed two CS chains per CD44 core in the TGF-β-treated cells. In those particluar experiments 1 d.p.m. of incorporated ^{35}S corresponded to 1 fmol of incorporated sulphate or 0.008 fmol of CD44.

^{125}I-CD44 was incubated with 20 μg of rat tail collagen either during or after fibrillogenesis in 1 ml of PBS/0.5 % Triton X-100 at 37 °C for 24 h, as described before [46]. After the incubation, the samples were centrifuged at 15000 g for 10 min, the pellets were washed three times with PBS and counted in a γ-counter. The collagen-binding experiments were done both in the absence or presence of 1 μg/ml of CS, HS, BSA or casein, and both before and after digestions with chondroitinase ABC, heparitinase or both enzymes. Samples incubated without collagen were included as controls.

All the Figures represent typical results from at least two independent experiments.

RESULTS

Effects on cell surface proteoglycan metabolic labelling

We initially tested the effects of TGF-β1, bFGF and the combination of TGF-β1 + bFGF on the metabolic $^{35}SO_3$-labelling of the four members of the family of syndecan-like proteoglycans, glypican and CD44 (Table 1). We observed a ~ 2.4-fold increase in the amount of ^{35}S-label immunoprecipitated with mAb 7D8 (anti-CD44) when the cells were treated with TGF-β1. Cells treated with bFGF showed only a moderate, ~ 1.4-fold increase, while cells treated with both TGF-β1 and bFGF showed a near 4-fold increase in CD44-associated ^{35}S-label. No major effects were observed for the other proteoglycans analysed, except for glypican where the combination of TGF-β1 and bFGF resulted in a clear decrease in incorporated label. In time-course experiments, whereby the cells were treated with only TGF-β1 for 0–48 h, the effect of the growth factor on the incorporation of label in CD44 was maximal (~ 3-fold stimulation) after 12 h of incubation, decreasing slightly (~ 2-fold increase only) after a treatment of 24 h or longer (results not shown). In dose–response experiments (0.1–10 ng/ml), a progressive increase was observed with near maximal effects (~ 3-fold increase) at concentrations of TGF-β1 $\geqslant$ 5 ng/ml (results not shown). In all these instances the effect of TGF-β1 remained

Table 1 Effect of cytokines on the incorporation of ^{35}S into proteoglycans

Effects of TGF-β1, bFGF and bFGF + TGF-β1 on the incorporation of ^{35}S in different cell surface proteoglycans, expressed as percentages of the respective control values (amounts of ^{35}S immunoprecipitated from non-treated cells). The numbers represent means and S.D.s (in parenthesis) for five independent experiments.

Proteoglycan fraction	Incorporation of ^{35}S (%)		
	TGF-β1	bFGF	bFGF + TGF-β1
Syndecan-1 + 3*	122 (24)	105 (7)	162 (39)
Syndecan-2	138 (41)	109 (18)	144 (10)
Syndecan-3	90 (19)	78 (21)	83 (22)
Syndecan-4	116 (21)	98 (16)	101 (8)
Glypican	86 (14)	99 (20)	63 (19)
CD44	242 (67)	143 (24)	414 (87)

* The mAb 2E9 reacts with both syndecan-1 and syndecan-3. These samples were further processed to isolate the CS chains which, in contrast to the HS in the sample, stemmed only from syndecan-1. Exposure to TGF-β1 resulted in a 2–3-fold stimulation of this syndecan-1-associated CS (see the Discussion section).

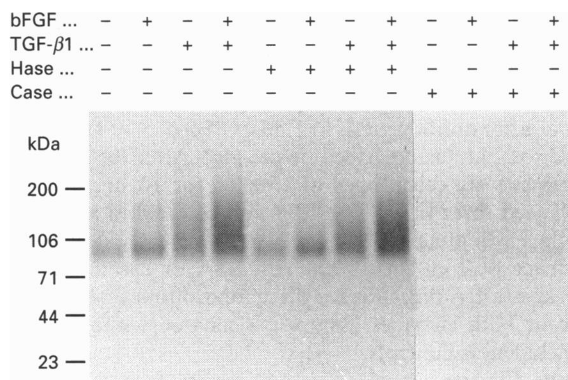

Figure 1 Sizing and enzyme susceptibility of [^{35}S]CD44 proteoglycan

[^{35}S]CD44 proteoglycan was isolated from unstimulated (−) cells and from cells stimulated (+) with bFGF, TGF-β1 or bFGF+TGF-β1 by absorption on DEAE-trisacryl M and immunoprecipitation with mAb 7D8. The immunopurified samples were analysed by electrophoresis in SDS/4% NuSieve agarose without digestion (−) or after digestion (+) with either chondroitinase ABC (Case) or heparitinase (Hase), and traced by autoradiography.

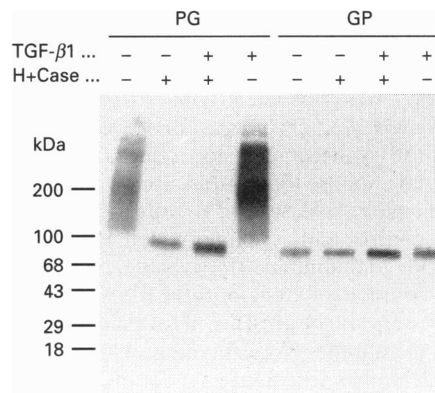

Figure 2 CD44 isolated from doubly labelled cells

CD44 was isolated, by immunoprecipitation with mAb 7D8, from doubly, ^{35}S- and [^{3}H]glucosamine-labelled unstimulated (−) and TGF-β1-stimulated (+) cells, both from the proteoglycan (PG) fractions that bound and from the glycoprotein (GP) fractions that failed to bind to DEAE-Trisacryl M, and subjected to SDS/PAGE and fluorography, either without (−) or after (+) digestion with both heparitinase and chondroitinase ABC (H+Case).

inferior to that of the combined administration of TGF-β1 and bFGF.

The effects of these factors on CD44 were further investigated by SDS/agarose gel electrophoresis, blotting and autoradiography of the immunopurified ^{35}S-labelled proteoglycan (Figure 1). In agarose gels, the [^{35}S]CD44 samples isolated from control and bFGF-treated cells migrated as fuzzy streaking bands ($\sim$ 100 kDa), whereas samples from cells exposed to TGF-β1 or the combination of TGF-β1 and bFGF migrated as broad smears with average apparent molecular masses of 150–180 kDa. Little or no effects on these migrations were observed when the CD44 samples were incubated with heparitinase, indicating that, at most, traces of HS-CD44 were produced. Digestion with chondroitinase ABC, in contrast, removed virtually all ^{35}S label from all samples, indicating that most of this label was present in CS. The small amounts of residual label were not further affected by heparitinase (results not shown), suggesting incomplete ABCase digestions or trace contamination of the immunoprecipitates with sulphoprotein.

To further investigate the composition of the immunoprecipitates and the effect of TGF-β1 (see also below), CD44 was immunoprecipitated from extracts of TGF-β1-stimulated and unstimulated cells that were doubly metabolically labelled with [^{3}H]glucosamine and $^{35}SO_4$ (Figure 2). Analysis of the immunoprecipitates from the DEAE eluates (proteoglycan fractions) by SDS/PAGE and autoradiography, without and after digestion with heparitinase and chondroitinase ABC, revealed only a single core in control and in stimulated cells, and more intensely labelled CD44 proteoglycans and CD44 proteoglycan cores in stimulated than in control cells (lanes 1–4). The CD44 molecules in the extracts that failed to bind to DEAE (glycoprotein fractions) were not sensitive to heparitinase or chondroitinase ABC and were also more intensely labelled in stimulated than in unstimulated cells (lanes 5 and 6). Yet, for both stimulated and unstimulated cells, the amount of ^{3}H-label precipitated as CD44-glycoprotein represented $\sim$ 80–85% of the total enzyme-resistant ^{3}H-label incorporated into CD44 (glycoprotein + proteoglycan core), suggesting that TGF-β1 had no influence on the proportion of the CD44 molecules that became substituted with GAG chains.

Effects on the proteoglycan cores

Possible effects of TGF-β1, bFGF and TGF-β1+bFGF on the expression and substitution of the core proteins of the different cell surface proteoglycans were also studied by dot blotting and Western blotting.

For CD44 more specifically, titration of the 7D8 epitope in the unfractionated detergent extracts or in the fall-through fractions and eluates from the DEAE columns revealed no differences between stimulated and non-stimulated cells, neither in the total amount of epitope nor in the fractions recovered as proteoglycan, which confirmed that the growth factors did not augment the conversion of CD44 into proteoglycan (results not shown). In stimulated and unstimulated situations there was 2–4 times more 7D8 epitope (CD44) in the glycoprotein than in the proteoglycan fractions, without noticeable effects of the growth factors on the total amounts of these materials. The proteoglycan fractions were also analysd by SDS/PAGE and Western blotting using the antibodies 7D8 and Hermes-3. The 7D8 antibody recognized the $\sim$ 85 kDa protein core of a proteoglycan that was primarily substituted with CS, as evident from separate and combined enzyme digestions. In the absence of any enzyme treatment or after only heparitinase, 7D8-reactive materials were barely transferable from the gels, whereas prominent and equally stained $\sim$ 85 kDa bands were detected after chondroitinase ABC or combined enzyme digestions (results for controls and cells stimulated with TGF-β1 are shown in Figure 3). The Hermes antibody yielded similar banding patterns and staining differences as mAb 7D8, confirming the specificity of mAb 7D8 and providing an independent assessment of the expression of this core protein (results not shown). On Western blots from total proteoglycan extracts the 7D8 antibody did not detect significant changes in the expression of CD44 proteoglycan after TGF-β1 stimulation, stimulation with bFGF or combined stimulation (results not shown). Likewise, neither TGF-β1 nor bFGF, nor the combination of TGF-β1+bFGF, increased the amount of CD44 core protein present in 7D8 immunoprecipitates from total cell extracts (results not shown) or in immunoprecipitates from the proteoglycan (DEAE-binding) fractions of these extracts (Figure

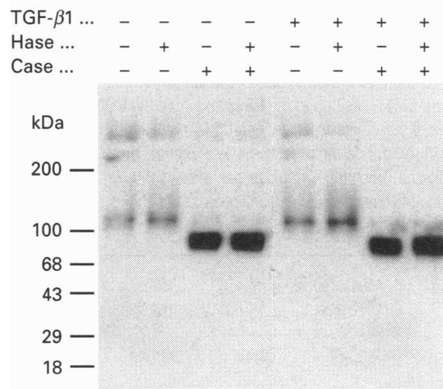

Figure 3 Effect of TGF-β1 on the CD44 proteoglycan core protein

Triton X-100 extracts from unstimulated (—) and TGF-β1-treated (+) cells were concentrated on DEAE-Trisacryl M, left untreated (—) or treated (+) with heparitinase (Hase), with chondroitinase ABC (Case), or with both enzymes, and fractionated in a 6–20% polyacrylamide gel gradient in the presence of SDS before transfer to Z-probe and staining with mAb 7D8.

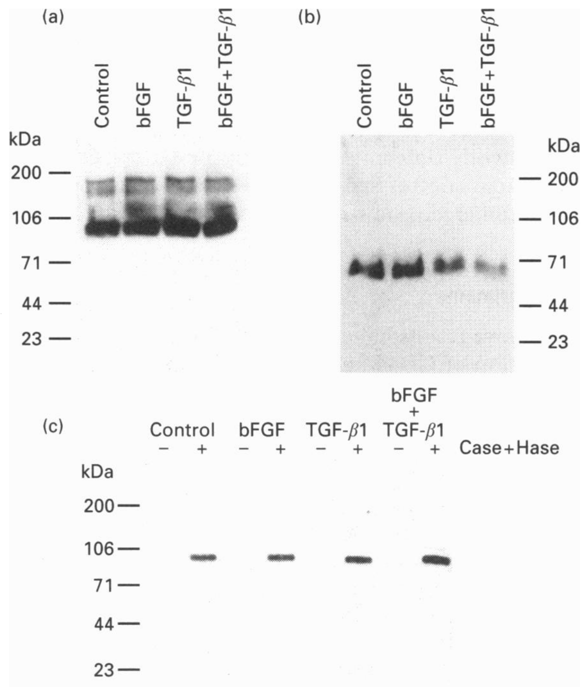

Figure 4 Effects of TGF-β1 and bFGF on the glycanation of CD44 and the expression of glypican

CD44 was immunopurified (**a** and **c**) from control, bFGF-, TGF-β1- and bFGF+TGF-β1-stimulated cells. Immunopurified CD44 samples were digested with both chondroitinase ABC and heparitinase (in **a**) or were compared with (+) or without (—) these enzyme digestions (in **c**). The products were applied on a 6–20% polyacrylamide gel gradient, transferred to a Z-probe filter and stained with mAb 7D8 (**a**) or with a polyclonal anti-CS-stub antibody (**c**). Total proteoglycan extracts (**b**) from these cells were digested similarly with both enzymes, fractionated by electrophoresis, blotted and stained with a mixture of the anti-glypican mAbs S1 and 1G11.

4a). In contrast, we observed a decrease in glypican core protein in the proteoglycan fractions after TGF-β1 treatment ($\sim$ 30%) and after TGF-β1 + bFGF treatment ($\sim$ 54%), while an incubation with bFGF alone had no effect (Figure 4b). No significant effects were observed in similar tests with the anti-(syndecan core protein) antibodies (results not shown).

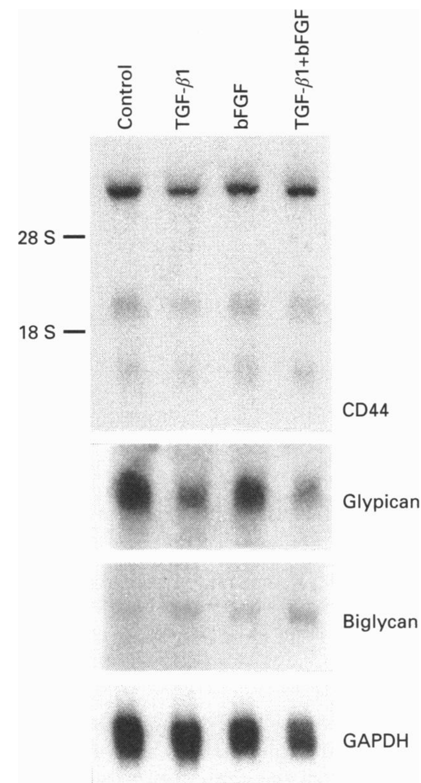

Figure 5 Northern blot analyses of the messages for CD44 and glypican

Total RNA samples from control HLF and from cells treated with TGF-β1, bFGF or TGF-β1 + bFGF were fractionated in a denaturating formaldehyde agarose gel, blotted to nylon filters and analysed by hybridization to specific probes for CD44, glypican, biglycan and GAPDH.

Effects on proteoglycan messages

In further studies we investigated the effects of TGF-β1, bFGF and TGF-β1 + bFGF on the mRNA levels for syndecan-1, syndecan-2, syndecan-3, syndecan-4, glypican and CD44 in HLF. For CD44, three different messages (1.5, 2.2, and 4.5 kb) were detected under high-stringency conditions of hybridization (Figure 5). None of the three bands showed significant changes (the average of three separate experiments showing maximally 20% deviation from control) after the treatments with TGF-β1 (lane 2), bFGF (lane 3) or TGF-β1 + bFGF (lane 4). TGF-β1 and TGF-β1 + bFGF, in contrast, decreased the levels of the glypican mRNA [relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message in the same sample] by $\sim$ 35 and $\sim$ 55% respectively (Figure 5). No significant effects were observed on the various syndecan messages (not shown). A biglycan probe, used as a positive control, showed an $\sim$ 5-fold increase in message in TGF-β1- and TGF-β1 + bFGF-stimulated cells (Figure 5).

Effects on glycanation of the proteoglycans

The above results suggested that for CD44 the main effects of the cytokines were on the post-translational modifications of the proteoglycan fraction of this protein. Proteinase K, nitrous acid and chondroitinase ABC treatments (see Materials and methods

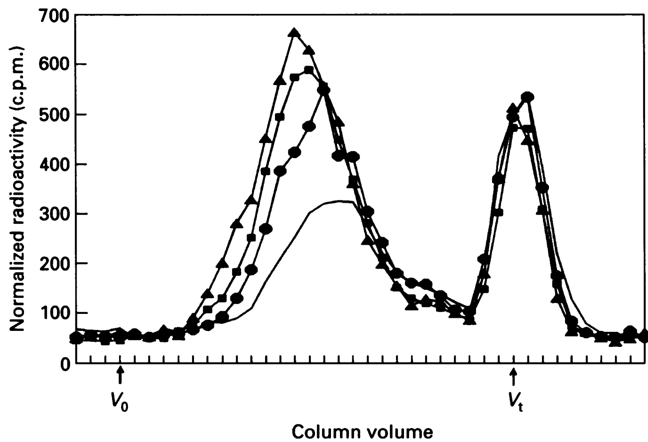

Figure 6 Sizing of the CD44-associated CS chains

[^{35}S]CD44 was immunopurified with mAb 7D8 from control (———) and from TGF-β1- (●), bFGF- (■) and TGF-β1 + bFGF- (▲) treated HLF, digested with proteinase K, heparitinase and heparinase, normalized by c.p.m. number (except for the control sample) and loaded on a Sepharose CL 6B column. V_0, void volume; V_t, total volume.

Table 2 Effect of TGF-β1 on the sulphation of CD44-CS

^{35}S- and [^{3}H]glucosamine-labelled CS chains were isolated from the CD44 and non-CD44 (all other cell surface proteoglycans) proteoglycan fractions of TGF-β1-stimulated and control cells. The ^{35}S/^{3}H label ratio was measured in the CS chains from the CD44 fractions and in the ΔDi-4S spot obtained from the digestion of the non-CD44 proteoglycans with chondroitinase ABC. Since the sulphate/disaccharide molar ratio in these chondroitinase ABC digestion products is equal to 1, the average amount of sulphate per disaccharide in the CD44 CS chains was calculated by dividing the label ratios obtained for the CD44 chains by the label ratios measured in the ΔDi-4S spots from the corresponding cultures.

	Measured label ratio (^{35}S/^{3}H)		Calculated molar ratio (SO_3/disaccharide)
TGF-β1	ΔDi-4S	CD44	CD44
−	1.277	1.233	0.966
+	0.864	0.789	0.913

section) confirmed that virtually all ^{35}S label in CD44 was incorporated into CS and that TGF-β1 and bFGF, in the various combinations, stimulated the amount of this [^{35}S]CS by a factor of ~ 2.5 (TGF-β1) to 4 (TGF-β1 + bFGF).

When sized by gel-filtration chromatography over Sepharose CL 6B and compared with controls, the CS chains isolated from CD44 in TGF-β1-treated cells were displaced towards the void volume of the column, indicating a greater length than in control cells (Figure 6). This change was calculated to correspond to a ~ 1.6-fold increase in the molecular mass of the chains (from ~ 16 to ~ 26 kDa). Increases in the sizes of the CS chains were also observed for CD44 immunoprecipitated from bFGF- (~ 23 kDa) and from TGF-β1 + bFGF-treated cells (~ 30 kDa). No differences in chain length were observed when similar tests were done for the HS chains isolated from syndecan-1, syndecan-2, syndecan-3, syndecan-4 and glypican after treatment of the cells with TGF-β1, bFGF or both together (not shown).

Staining of blots of heparitinase- and chondroitinase ABC-digested proteoglycan fractions with the anti-'HS stub' antibody 3G10 (which reacts with the desaturated uronates that terminate the heparitinase-resistant linkage regions of the chains) indicated that similar numbers of HS chains were initiated on the different syndecan cores under all conditions tested and that none were initiated on CD44 (not shown). When immunopurified CD44 proteoglycan was digested with chondroitinase ABC and heparitinase and stained with an anti-'CS stub' antibody, however, a clear enhancement of the staining of the core was observed when the cells were stimulated with TGF-β1 + bFGF (~ 2.2-fold increase versus controls), indicating that this combination did increase the number of (at least a particular kind of) CS chains in CD44 (Figure 4c). No effect on chain number was measured after incubation with only bFGF (~ 0.9-fold increase) or only TGF-β1 (~ 1.1-fold increase). Since all four CD44 bands stained identically for the 7D8 epitope, this indicated a doubling of the number of CS chains per CD44 core after TGF-β1 + bFGF stimulation in comparison to the control and single stimulations.

To assess whether TGF-β1 might affect the sulphation of the CS chains we isolated CD44 from doubly $^{35}SO_4$- and [^{3}H]glucosamine-labelled cells (see Figure 2). Measurement of the ^{35}S/^{3}H ratios in whole CD44-CS chains and in reference ΔDi-4S spots obtained from the digestion of CS chains from the corresponding cultures indicated that the average sulphation of the CD44-CS chains was close to one sulphate per disaccharide in control cells and was virtually unaltered by TGF-β1 (Table 2). Possible effects of the growth factors and their combinations on the sulphation of the CS chains were also studied by ion-exchange chromatography over DEAE. All profiles obtained were very similar, with only slightly different elution positions for CD44-derived CS chains from control and cytokine-treated cells, revealing, at most, slight differences in negative charge densities (not shown).

Binding experiments

Since the above results indicated that the growth factors altered the glycanation of CD44 and therefore could modify the interactions and associations of this proteoglycan, binding studies were undertaken using known ligands for CD44 proteoglycan. Both [^{35}S]CD44 and ^{125}I-CD44 preparations were used as ligands for binding to fibronectin-coated beads, with similar results. Increasing concentrations of soluble proteoglycan from both stimulated and unstimulated cells were mixed with fixed amounts of immobilized fibronectin. When these data were represented as Scatchard plots, CD44-associated label isolated from TGF-β1-, bFGF- and TGF-β1 + bFGF- treated fibroblasts showed a nearly 2-fold higher affinity for fibronectin than CD44 from control cells (Figure 7). Both TGF-β1 and bFGF, alone or in combination, decreased the K_d value by ~ 44 ± 5 % to an estimated value of ~ 50 pM (average of three separate experiments) both for the ^{35}S-labelled and for the ^{125}I-labelled CD44. The total number of binding sites for CD44-associated label on fibronectin appeared similar for all the growth factors tested. The fibronectin–CD44 binding interaction depended on the CS chains of the proteoglycan, since after chondroitinase ABC treatment of ^{125}I-CD44 or the addition of free heparin only background binding was observed (not shown). Casein, in contrast, had no effect on this binding.

CD44 bound also to type I collagen purified from rat tail, both during and after fibrillogenesis. Some 30–40 % of the ^{125}I-CD44 isolated from control cells bound to collagen and a similar extent of binding was observed when CD44 was isolated from cells stimulated with TGF-β1, bFGF or TGF-β1 + bFGF. The binding to collagen was inhibited by treating the proteoglycan with chondroitinase ABC and by the addition of CS or HS (not shown).

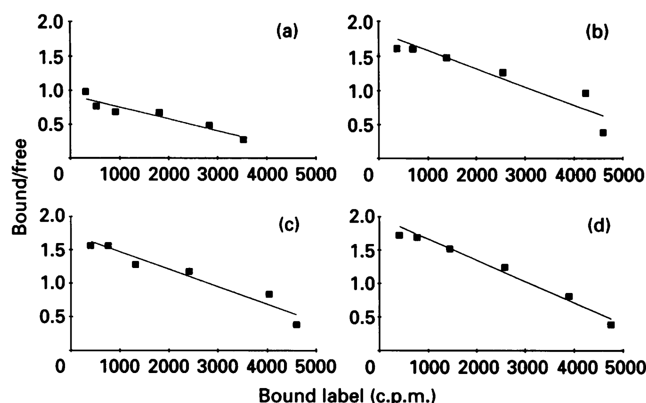

Figure 7 Binding of CD44 to fibronectin

[^{35}S]CD44 proteoglycan was immunopurified from (a) control, (b) bFGF-, (c) TGF-β1- and (d) bFGF + TGF-β1-treated HLF and incubated overnight at various concentrations with fibronectin beads. The beads were separated by centrifugation and analysed in a β-counter. Scatchard plots of these binding experiments are shown. The abscissa shows bound label in c.p.m., the ordinate the ratios of bound and free label.

DISCUSSION

We have investigated the effects of TGF-β1, bFGF and TGF-β1+bFGF on the synthesis of cell surface proteoglycans by HLF. TGF-β1 alone, and more so in combination with bFGF, reduced the amount of glypican mRNA and core protein in these cells and significantly enhanced the amount of CD44-associated CS. No major effects of TGF-β1, alone or in combination and at the levels that were tested, were observed on the expression of the syndecans in these cells.

The results indicate that TGF-β1 and bFGF affect the structure and potential interactions of the CS proteoglycan that represents the glycanated form of the CD44/Hermes homing or hyaluronate receptor. TGF-β1 increases the length and, at least in combination with bFGF, also the number of CS chains that are implanted on the proteoglycan form of this protein, without significantly affecting the levels of the protein or increasing the fraction that is processed into proteoglycan. This enhanced glycanation allows the proteoglycan form of CD44 to bind with an almost 2-fold greater affinity to fibronectin but does not affect the binding of CD44 to type I collagen.

Exposing the cells to bFGF only, led to a ~ 40 % increase in the average length of the CD44-associated CS chains without detectable effect on the number of chains implanted on the CD44 core protein itself, consistent with the observed ~ 43 % increase in CD44-associated immunoprecipitated radiosulphate. In the presence of both bFGF and TGF-β1, the average size of the CD44-associated CS chains and the number of these chains (traced as immunoreactive CS-stubs after a chondroitinase ABC digestion) were almost double what was measured for the chains associated with CD44 in control cells, which appears consistent with the near 4-fold increase in immunoprecipitated radiosulphate and CS in that situation. The effects of TGF-β1, added as single factor, on the number of CS GAG chains per CD44-core, as detected by the anti-CS stub antibody (no or little effect), and on the length (1.6-fold increase) of these chains, in contrast, do not seem to account for the ~ 2.4-fold increases in total CD44-associated radiosulphate and [^{35}S]CS that were observed. The reason for this discrepancy is not clear. It did not pertain to the incorporation of radiosulphate into HS or into the non-GAG moiety of the proteoglycan (see Figure 2), to a change in the degree of sulfation of the chains (Table 2) or to the contamination of the immunoprecipitates by other (e.g. CD44-associated) CS-substituted molecules (see Figure 2 and Figure 4c), unless these CS chains were present on cores that were not labelled or not retained on the blotting membranes. Confirmatory immunoprecipitations from radio-iodinated proteoglycan extracts yielded also only a single ~ 85 kDa core (not shown). One alternative possibility could be that the anti-CS antibody does not recognize all the residual chondroitinase ABC-resistant stubs on the enzyme-treated cores, for steric reasons or because of the potential heterogeneity of these stubs in terms of structure and epitope content. In that case the results may indicate that TGF-β1 alone also enhances the number of chains in the CD44 proteoglycan, but with superimposed effects on the sequence microheterogeneity of the chains (and of the chain stubs produced). Attempts to obtain further direct evidence for this interpretation using currently available mAbs that differentially react with various chondroitinase digestion products were however unsuccessful (not shown). On the other hand, the observation that treatment of the CD44 proteoglycan with chondroitinase ABC (which always leaves a residual and potentially sulphated CS-disaccharide on the chain stubs) left ~ 2 times more residual $^{35}SO_4$ on the CD44 core when the cells were exposed to TGF-β1 than under the control conditions (not shown) is potentially consistent with this interpretation.

The TGF-β1 effect on CD44 seems to confirm a general enhancement of CS production by this cytokine. These effects on the glycanation of CD44 in human fibroblasts are indeed similar to the changes in the structure of syndecan-1 previously reported for mouse mammary epithelial cells exposed to this cytokine [47]. TGF-β1 in these cells had only moderate effects on the substitution of syndecan with HS (which accounts for the bulk of the GAG in this proteoglycan) but markedly stimulated its CS content by tripling the number of these chains and nearly doubling their length [6]. A closer comparison of the immunoprecipitates obtained with mAb 2E9, which in fibroblasts reacts with syndecan-1 but also with a second proteoglycan (syndecan-3) that carries only HS, also revealed a 2–3-fold stimulation of the amount of CS in this fraction, suggesting that TGF-β1 also modulates the structure of syndecan-1 in fibroblasts (not shown). However, there was nearly 20 times less CS present on syndecan-1 than on CD44 in HLF, so the changes in syndecan, although of similar relative magnitude, represented only minor changes in absolute amounts of GAG in fibroblasts. TGF-β1 has also been shown to increase the length of the CS chains present on the small proteoglycans biglycan and decorin [20,22] and on versican [21], indicating that it generally affects the elongation and termination of this type of GAG.

Serine-glycine sequences that occur in acidic contexts are proposed consensus structures for the attachment of GAG chains [48–50]. Sequences of the type acidic-XSG-acidic, of which there are three examples in syndecan-1, have been shown to function for the attachment of CS in this proteoglycan [51], and glycanation sites of this sequence have also been identified in proteoglycans that carry only CS [52]. The amino acid determinants that drive the expression of HS are more complex [50]. Splice variants of CD44 have been reported (CD44E) that also carry HS [53–55], but enzyme digestions of the metabolically labelled proteoglycans and the use of the anti-HS stub mAb 3G10 indicated that these forms are not expressed in HLF, irrespective of the cytokines used. The form of CD44 that fibroblasts express (a contention supported by the obtention of only a single PCR product in these cells) probably features four SG sequences. Yet, only two of these are preceded by a cluster of acidic residues and may therefore be amenable to substitution with GAG chains.

The increase in CS chain numbers after TGF-β1 + bFGF treatment in any case implies that these SG sequences are not always fully substituted in the proteoglycan form of the molecule. The doubling that is observed would be consistent with a two acceptor-site model, whereby under basal conditions only part of the CD44 population becomes substituted and probably carries only one chain, whereas the treatment with TGF-β1 + bFGF does not affect the fraction that becomes substituted but increases the proportion of the proteoglycan fraction that becomes fully substituted. The data also suggest that in fibroblasts only a specific fraction of CD44 can be confronted with the CS synthesis machinery and that the cytokines augment the efficiency of the machinery but not the way it is fed with substrate.

In HLF, the combination of TGF-β1 and bFGF had no effect on the syndecan-1 mRNA levels or core protein levels (only on its CS moiety, see above), in contrast with what occurs in 3T3 cells where both factors together lead to a several-fold increase in the syndecan-1 message and proteoglycan [25], suggesting different syndecan-1 regulatory mechanisms for HLF. For CD44 too, in fibroblasts, the only increase observed after TGF-β1 + bFGF treatment was in the CS moiety, without changes in message or protein levels. The message and protein levels for the other syndecans were also not significantly affected by TGF-β1 or the combination with bFGF. On the other hand, TGF-β1 and especially TGF-β1 + bFGF down-regulated the expression of glypican mRNA, correlated with similar decreases at the core-protein and proteoglycan levels. This glypican decrease contrasts with the effects of TGF-β1 in human fibroblasts on the expression of integrins and of several extracellular matrix components, including type I collagen, fibronectin, versican and biglycan. For all of these components 5–20-fold increments in the levels of transcription have been observed, representing a major mechanism by which TGF-β affects the accumulation of these proteins [56]. Further experiments will be needed to clarify whether glypican belongs to a category of components that are negatively transcriptionally regulated by TGF-β1 or whether the regulation occurs at post-transcriptional levels.

The significance of the TGF-β1- and bFGF-induced structural changes of CD44 for the fates or functions of this proteoglycan and for the biological actions of these cytokines still has to be established. CD44 has been described as a receptor for hyaluronate [14], type I collagen [16] and fibronectin [15]. The binding to fibronectin occurs for the CS-containing form of the molecule. The affinity of this binding is quite high and the increase of the CS moiety in CD44 even further enhances this binding, as the present study shows. Cytokine-modulated binding affinities could be part of a complex regulation of the extracellular matrix organization and recognition by cells in diseases where these growth factors are overexpressed, as is the case for TGF-β1 in glomerulonephritis [57]. It has been reported (while this work was in progress) that TGF-β1 also has an effect on the CS substitution of CD44 in mouse melanoma cells [58] and it has been suggested that this increase stimulates the motility of these cells on type I collagen gels, implying possible differences in affinity for this substrate. In our experiments, in HLF the binding with type I collagen was not altered in CD44 isolated from TGF-β1-treated cells, possibly because the interaction with collagen does not rely exclusively on the CS moiety of the proteoglycan [59]. Interestingly, mouse melanoma cells also rely on a phosphatidylinositol-anchored HS proteoglycan for their adhesive interactions with fibronectin-coated substrates [60]. In competition for the same ligands, the opposite regulation of glypican (which is also a collagen- and fibronectin-binding proteoglycan) and of CD44 in human fibroblasts by the combination of TGF-β1 and bFGF may have synergistic effects on the 'throwing of switches' in cell–matrix interactions. Such adhesive switches may modulate signalling pathways that may be activated as a result of these interactions and that translate into, for example, cytoskeletal reorganizations and enhanced motility of cells. At the minimum our results imply that CD44- and glypican-supported cellular interactions with the matrix may be regulated by cytokines.

We thank Helga Ceulemans, Christien Coomans, Eef Meyen and An Rayé for their expert technical assistance. We also thank Dr. Sirpa Jalkanen for the gift of the Hermes antibodies and Dr. John R. Couchman for the anti-CS-stubs antibodies. These investigations were supported by grant 3.0073.91 from the 'Nationaal Fonds voor Wetenschappelijk Onderzoek' of Belgium, by a grant 'Geconcerteerde Onderzoeks Acties' from the Belgian Government, by the Interuniversity Network for Fundamental Research (1991-1996) and by the EEC Biomed I Concerted Action Contract BMH1 CT92 1766. M.R. was supported by an EMBO short-term fellowship and a Human Capital and Mobility Program (EU) fellowship (ERBCHBI-CT 92-0224). G.D. is a research director of the 'Nationaal Fonds voor Wetenschappelijk Onderzoek' of Belgium.

REFERENCES

1 Kjellén, L. and Lindahl, U. (1991) Annu. Rev. Biochem. **60**, 443–475
2 Esko, J. D. (1991) Curr. Opin. Cell Biol. **3**, 805–816
3 Yanagishita, M. and Hascall, V. (1992) J. Biol. Chem. **267**, 9451–9454
4 David, G. (1993) FASEB J. **7**, 1023–1030
5 Gallagher, J. T. (1989) Curr. Opin. Cell Biol. **1**, 1201–1218
6 Rapraeger, A. C. (1993) Curr. Opin. Cell Biol. **5**, 844–853
7 Bernfield, M., Kokenyesi, R., Kato, M. et al. (1992) Annu. Rev. Cell Biol. **8**, 365–369
8 David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J.-J. and Van den Berghe, H. (1990) J. Cell Biol. **111**, 3165–3176
9 Stipp, C. S., Litwack, E. D. and Lander, A. D. (1994) J. Cell Biol. **124**, 149–160
10 Lesley, J., Hyman, R. and Kincade, P. W. (1993) Adv. Immunol. **54**, 271–335
11 Jalkanen, S., Jalkanen, M., Bargatze, R., Tammi, M. and Butcher, E. C. (1988) J. Immunol. **141**, 1615–1623
12 Goldstein, L. A., Zhou, D. F. H., Picker, L. J. et al. (1989) Cell **56**, 1063–1072
13 Stamenkovic, I., Amiot, M., Pesando, J. M. and Seed, B. (1989) Cell **56**, 1057–1062
14 Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B. and Seed, B. (1990) Cell **61**, 1303–1313
15 Jalkanen, S. and Jalkanen, M. (1992) J. Cell Biol. **116**, 817–825
16 Faassen, A. E., Schrager, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R. and McCarthy, J. B. (1992) J. Cell Biol. **116**, 521–531
17 Nathan, C. and Sporn, M. (1991) J. Cell Biol. **113**, 981–986
18 Salustri, A., Ulisse, S., Yanagishita, M. and Hascall, V. C. (1990) J. Biol. Chem. **265**, 19517–19523
19 Romarís, M., Heredia, A., Molist, A. and Bassols, A. (1991) Biochim. Biophys. Acta **1093**, 229–233
20 Kähäri, V. M., Larjava, H. and Uitto, J. (1991) J. Biol. Chem. **266**, 10608–10615
21 Schönherr, E., Järveläinen, H. T., Sandell, L. J. and Wight, T. N. (1991) J. Biol. Chem. **266**, 17640–17647
22 Bassols, A. and Massagué, J. (1988) J. Biol. Chem. **263**, 3039–3045
23 Dodge, G. R., Kovalszky, I., Hassel, J. R. and Iozzo, R. V. (1990) J. Biol. Chem. **265**, 18023–18029
24 Rapraeger, A. (1989) J. Cell Biol. **109**, 2509–2518
25 Elenius, K., Määttä, A., Salmivirta, M. and Jalkanen, M. (1992) J. Biol. Chem. **267**, 6435–6441
26 Yamaguchi, Y., Mann, D. M. and Ruoslahti, E. (1990) Nature (London) **346**, 281–283
27 Hildebrand, A., Romaris, M., Rasmussen, L. M. et al. (1994) Biochem. J. **302**, 527–534
28 Cheifetz, S., Andres, J. L. and Massagué, J. (1988) J. Biol. Chem. **263**, 16984–16991
29 Segarini, P. and Seyedin, S. M. (1988) J. Biol. Chem. **263**, 8366–8370
30 López-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S. and Massagué, J. (1991) Cell **67**, 785–795
31 Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F. and Weinberg, R. A. (1991) Cell **67**, 797–805
32 Andres, J. L., Stanley, K., Cheifetz, S. and Massagué, J. (1989) J. Cell Biol. **109**, 3137–3145
33 Ignotz, R. A. and Massagué, J. (1986) J. Biol. Chem. **261**, 4337–4345
34 Roberts, C. J., Birkenmeier, T. M., McQuillan, J. J. et al. (1988) J. Biol. Chem. **263**, 4586–4592
35 Heino, J., Ignotz, R. A., Hemler, M. E., Crouse, C. and Massagué, J. (1989) J. Biol. Chem. **264**, 380–388

36 Lories, V., Cassiman, J.-J., Van den Berghe, H. and David, G. (1992) J. Biol. Chem. **267**, 1116–1122
37 David, G., Lories, V., Heremans, A., Van Der Schueren, B., Cassiman, J.-J. and Van den Berghe, H. (1989) J. Cell Biol. **108**, 1165–1175
38 Lories, V., Cassiman, J.-J., Van den Berghe, H. and David, G. (1989) J. Biol. Chem. **264**, 7009–7016
39 David, G., Van Der Schueren, B., Marynen, P., Cassiman, J.-J. and Van den Berghe, H. (1992) J. Cell Biol. **118**, 961–969
40 Lories, V., David, G., Cassiman, J.-J. and Van den Berghe, H. (1986) Eur. J. Biochem. **158**, 351–360
41 Kim, C. W., Goldberger, O. A., Gallo, R. L. and Bernfield, M. (1994) Mol. Biol. Cell **5**, 797–805
42 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
43 Marynen, P., Zhang, J., Cassiman, J.-J., Van den Berghe, H. and David, G. (1989) J. Biol. Chem. **264**, 7017–7024
44 Shively, J. E. and Conrad, H. E. (1976) Biochemistry **15**, 3932–3942
45 Wasserman, L., Ber, A. and Allalouf, D. (1977) J. Chromatogr. **136**, 342–347
46 Brown, D.C and Vogel, K. G. (1989) Matrix **9**, 468–478
47 Rasmussen, S. and Rapraeger, A. (1988) J. Cell Biol. **107**, 1959–1967
48 Bourdon, M. A., Krusius, T., Campbell, S., Schwartz, N. B. and Ruoslahti, E. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3194–3198
49 Mann, D. M., Yamaguchi, Y., Bourdon, M. A. and Ruoslahti, E. (1990) J. Biol. Chem. **265**, 5317–5323
50 Zhang, L. and Esko, J. D. (1994) J. Biol. Chem. **269**, 19295–19299
51 Kokenyesi, R. and Bernfield M. (1994) J. Biol. Chem. **269**, 12304–12309
52 Zimmerman, D. R. and Ruoslahti, E. (1989) EMBO J. **8**, 2975–2981
53 Brown, T. A., Bouchard, T., St. John, T., Wayner, E. and Carter, W. G. (1991) J. Cell Biol. **113**, 207–221
54 Kugelman, L. C., Gaguly, S., Haggerty, J. G., Weismann, S. M. and Milstone, L. M. (1992) J. Invest. Dermatol. **99**, 381–385
55 Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Shields, J. and Whittle, N. (1995) J. Cell Biol. **128**, 673–685
56 Massagué, J. (1990) Annu. Rev. Cell Biol. **6**, 597–641
57 Border, W. A. and Ruoslahti, E. (1992) J. Clin. Invest. **90**, 1–7
58 Faassen, A. E., Moorodian, D. L., Tranquillo, R. T. et al. (1993) J. Cell Sci. **105**, 501–511
59 Carter, W. G. and Wayner, E. A. (1988) J. Biol. Chem. **263**, 4193–4201
60 Drake, S. L., Klein, D. J., Mickelson, D. J., Oegema, T. R., Furcht, L. T. and McCarthy, J. B. (1992) J. Cell Biol. **117**, 1331–1341

Received 24 November 1994/6 April 1995; accepted 12 April 1995