Transforming Growth Factor (Type β) Promotes the Addition of Chondroitin Sulfate Chains to The Cell Surface Proteoglycan (Syndecan) of Mouse Mammary Epithelia

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Abstract. Cultured monolayers of NMuMG mouse mammary epithelial cells have augmented amounts of cell surface chondroitin sulfate glycosaminoglycan (GAG) when cultured in transforming growth factor-β (TGF-β), presumably because of increased synthesis on their cell surface proteoglycan (named syndecan), previously shown to contain chondroitin sulfate and heparan sulfate GAG. This increase occurs throughout the monolayer as shown using soluble thrombospondin as a binding probe. However, comparison of staining intensity of the GAG chains and syndecan core protein suggests variability among cells in the attachment of GAG chains to the core protein. Characterization of purified syndecan confirms the enhanced addition of chondroitin sulfate in TGF-β: (a) radiosulfate incorporation into chondroitin sulfate is increased 6.2-fold in this proteoglycan fraction and heparan sulfate is increased 1.8-fold, despite no apparent increase in amount of core protein per cell, and (b) the size and density of the proteoglycan are increased, but reduced by removal of chondroitin sulfate. This is shown in part by treatment of the cells with 0.5 mM xyloside that blocks the chondroitin sulfate addition without affecting heparan sulfate. Higher xyloside concentrations block heparan sulfate as well and syndecan appears at the cell surface as core protein without GAG chains. The enhanced amount of GAG on syndecan is partly attributed to an increase in chain length. Whereas this accounts for the additional heparan sulfate synthesis, it is insufficient to explain the total increase in chondroitin sulfate; an approximately three-fold increase in chondroitin sulfate chain addition occurs as well, confirmed by assessing chondroitin sulfate ABC lyase (ABCCase)-generated chondroitin sulfate linkage stubs on the core protein. One of the effects of TGF-β during embryonic tissue interactions is likely to be the enhanced synthesis of chondroitin sulfate chains on this cell surface proteoglycan.

Transforming growth factor-β (TGF-β) is a 25-kD polypeptide dimer that elicits various cellular behaviors, including reduced proliferation and enhanced differentiation of epithelial cells (for reviews, see references 16 and 33). Platelets are a rich source of the growth factor (1, 37), and porcine platelets contain two types, termed β1 and β2, which occur primarily as homodimers. These factors are equivalent to the cartilage-inducing factors (32). More recently, the potential biological effects of these factors have been extended to amphibian development where a protein similar to TGF-β2 is reported to induce mesodermal structures at the time of gastrulation (25).

The mechanisms whereby TGF-β affects cells are not completely understood. The factor binds to at least three proteins at the surfaces of most cells (3, 31), and the cellular response to the growth factor is reported to vary with the identity of the occupied receptor. A cellular response that has been studied in increasing detail is the enhanced deposition of matrix components and the augmented expression of their receptors. For example, the expression of collagen and fibronectin (FN) is increased in a variety of cell types, the latter via regulation of mRNA levels (11, 13).

Synthesis of chondroitin sulfate proteoglycan (PG) is also augmented (2, 5), due in part if not entirely, to enhanced expression of their core proteins (2). In addition, preadipocytes and human fibroblasts treated with TGF-β show an enhanced expression of integrin, an FN receptor, via regulation at the pre- and posttranslational processing steps (12, 24). Thus, the ability of cells to deposit and recognize their extracellular matrix is dramatically altered, often correlated with a change in their shape.

Previous work (22) identified an integral membrane PG polarized to the basolateral cell surface of mouse mammary epithelia. Subsequent examination of its tissue distribution

1. Abbreviations used in this paper: ABCCase, chondroitin sulfate ABC lyase; CM-PBS, calcium- and magnesium-containing PBS; FN, fibronectin; GAG, glycosaminoglycan; PG, proteoglycan; TGF-β, transforming growth factor (type β); TSP, thrombospondin.
shows that it is not restricted to the mammary epithelium, but is detected in abundance on most epithelia (9). Recently, the PG has been further identified by deduction of its primary amino acid sequence from DNA cloning data and has been named syndecan (29). On NMuMG mammary epithelial cells, syndecan has been shown to bear primarily heparan sulfate GAG chains, in addition to a minor proportion of chondroitin sulfate (18). The heparan sulfate chains mediate binding to components of the extracellular matrix, including FN (28), interstitial collagens (15), and thrombospondin (TSP) (34). The PG is thus proposed to be a matrix receptor with a role in cell adhesion to the extracellular matrix, similar to roles suggested for cell surface heparan sulfate PGs of other cells (for review, see reference 26). However, the potential regulation and role of chondroitin sulfate on syndecan remains unknown. In an attempt to determine if the expression of syndecan is affected by TGF-β, we recently examined the amount of cell surface GAG on these cells in the presence of the growth factor and reported that cell surface GAG increased ~2.5-fold due in part to an increased synthesis of chondroitin sulfate, but that the amount of PG core protein remained relatively unchanged. This suggested that the structure of syndecan was altered by an increase in its proportion of chondroitin sulfate. This report specifically examines the possibility that additional chondroitin sulfate chains are initiated on this PG core protein when the cells are treated with TGF-β.

**Materials and Methods**

**Cell Culture and Radiolabeling**

NMuMG mouse mammary epithelial cells (passages 13–25) were maintained in bicarbonate-buffered DME (Gibco Laboratories, Grand Island, NY) containing 10% FBS (Tissue Culture Biologicals, Tulare, CA) and laminin (ICN Biomedicals, Inc., Costa Mesa, CA) and supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). Cells on coverslips were fixed for 24 h in calcium- and magnesium-free 35% ethanol containing 0.5 mM EDTA on ice and centrifuged (200 g). The cells were initially extracted with isotonic, 1% Triton X-100 at pH 5.0 to release soluble contents, then syndecan was released by a subsequent treatment in an identical solution containing 0.5 M KCl (21). DNA in the remaining cell pellet was measured as described previously (23). Syndecan was purified on an antibody (mAb 281.2) affinity column specific for its core protein (14) followed by binding to an HPLC AX-300 anion exchange column (Iscour, Inc., Lincoln, NE) in 8 M urea and elution at 1.0 M NaCl in a salt gradient. Trypsin treatment of cell surfaces to remove intact core protein was performed as described previously (18).

**Structural Analysis**

**Quantification of Radiolabel in Chondroitin Sulfate and Heparan Sulfate GAG.** Purified PG or GAG chains were dialyzed into ABCase digestion buffer containing 0.1% Triton X-100 (21), incubated with or without 50 mU/ml ABCase for 60 min and spotted via a dot-blot apparatus (V & P Scientific Inc., San Diego, CA) onto cationic nylon (Zeta-Probe; Bio-Rad Laboratories, Cambridge, MA) which retains PG and intact GAG chains (19). Dots were dried and assayed by scintillation counting (LS-5800 detector; Beckman Instruments, Palo Alto, CA) in BioSafe (Research Products International Corp., Prospect, IL) cocktail to determine 35SO4 incorporation. Alternatively, the dot blots were subjected to autoradiography (X-Omat film; Eastman Kodak Co., Rochester, NY). The difference between non-digested and enzyme-treated samples reflected incorporation into chondroitin sulfate. To assess heparan sulfate, selected samples on Zeta-Probe dots were digested to nitrous acid and acid digestion in 3.6 M acetic acid containing 0.24 M sodium nitrite for 90 min, repeated for an additional 90 min, then washed in 0.7 M NaCl buffered at pH 8.0 with 10 mM Tris to remove heparan sulfate fragments (19). ABCase digestion followed by the nitrous acid treatment removes >95% of the radiolabel from syndecan.

**Size of Syndecan and Its GAG Chains.** Chromatographic procedures and size estimates of syndecan from nontreated NMuMG cells have been published previously (15, 20). Briefly, cells were extracted directly in sample buffer, electrophoresed on 3–15% polyacrylamide gels as described by Koda et al. (15). The PG was electrophoretically transferred to Zeta-Probe and spotted using the method of Towbin et al. (36) using one-quarter strength transfer buffer at 100 mA for 3 h. Core protein was detected by mAb 281.2 immunostaining as described previously (23) and below. To assess GAG chain size, purified PG was treated overnight with 0.1 N NaOH in 1 M sodium borohydride to release the chains, which were then chromatographed on Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) in 10 mM Tris (pH 8.0), 8 M urea and 1% Triton X-100. Incorporation into chondroitin sulfate or heparan sulfate was determined for each column fraction spotted on cationic nylon as described above. Size estimates of the GAG chains derived from published data using chondroitin sulfate standards (20).

**Core Protein and Chondroitin Sulfate Linkage Sites.** Purified syndecan was incubated with or without ABCase and spotted in twofold serial dilutions on cationic nylon. To quantify relative amounts of core protein, the PGs were incubated with monoclonal antibody (mAb 281.2, 50 μg/ml) as described previously (23). Alternatively, identical blots were incubated with a 1:10 dilution of a rabbit serum antibody (R44, kindly supplied by Dr. Jardine Couchman, University of Alabama) directed against the unsaturated uronic acid products of ABCase digestion (6). Unlike certain monoclonal antibodies that show specificity for chondroitin sulfate type, this antibody recognizes equally the unsaturated uronic acid remaining after ABCase digestion of chondroitin 4-sulfate, -6-sulfate, and nonsulfated chondroitin (J. Couchman, personal communication). The blots were then incubated with secondary horseradish peroxidase-conjugated anti-rat or anti-rabbit antibodies (Jackson Immuno Research Laboratories Inc., Avondale, PA), respectively, and detected by incubation in 30 mM sodium acetate, 0.2 mM thimerosal, 4 mM succinic acid, 1 mM 3-amin-9-ethylcarbazole, and 0.01% hydrogen peroxide. Blots were photographed (P/N 55 film; Polaroid Corp., Cambridge, MA).

**Density Analysis.** Purified PG (0.5 ml) was treated with or without 50 μU/ml ABCase or 0.01 μU/ml heparitinase and subjected to nitrous acid digestion in 3.6 M acetic acid containing 0.24 M sodium nitrite for 90 min, repeated for an additional 90 min, then washed in 0.7 M NaCl buffered at pH 8.0 with 10 mM Tris to remove heparan sulfate fragments (19). ABCase digestion followed by the nitrous acid treatment removes >95% of the radiolabel from syndecan.

**Isolation and Purification of Syndecan**

After a 24-h incubation in radiolabeled with or without TGF-β, the cells were recovered by scraping into calcium- and magnesium-free–PBS containing 0.5 mM EDTA on ice and centrifuging (200 g). The cells were initially extracted with isotonic, 1% Triton X-100 at pH 5.0 to release soluble contents, then syndecan was released by a subsequent treatment in an identical solution containing 0.5 M KCl (21). DNA in the remaining cell pellet was measured as described previously (23). Syndecan was purified on an antibody (mAb 281.2) affinity column specific for its core protein (14) followed by binding to an HPLC AX-300 anion exchange column (Iscour, Inc., Lincoln, NE) in 8 M urea and elution at 1.0 M NaCl in a salt gradient. Trypsin treatment of cell surfaces to remove intact core protein was performed as described previously (18).
Table I. Change in the Amount of Chondroitin Sulfate and Heparan Sulfate on the Cell Surface PG from TGF-β-Treated Cells

|                  | No TGF-β | + TGF-β    |
|------------------|----------|------------|
| [35]SO₄, DPM/μg DNA* | 1 (2.70 (0.49)) |
| Percent degraded by ABCase | 20.2 (2.9) | 46.3 (1.6) |
| Percent degraded by HNO₂ | 78.2 (6.0) | 51.9 (1.3) |

* Expressed as a proportion of the incorporation in no TGF-β.
† Number in parentheses is SEM, n = 6 different experiments.

Results

Incorporation of Radiosulfate into Cell Surface Chondroitin Sulfate and Heparan Sulfate GAG in the Presence of TGF-β

Previous work suggested that NMuMG cells show increased GAG at their surfaces when cultured in TGF-β₁ or TGF-β₂ and that the majority of the increase was in chondroitin sulfate. To quantify this increase more specifically, the cell surface PG (syndecan) that has been characterized on these cells (22) was isolated after a 24-h radiosulfate label in the presence or absence of the growth factor, purified on an antibody affinity column specific for its core protein, and degraded with ABCase and/or nitrous acid to quantify [35]SO₄ incorporation into chondroitin sulfate and/or heparan sulfate, respectively (Table I). In the nontreated cells, the bulk of the incorporation (78.2%) is into heparan sulfate, whereas chondroitin sulfate accounts for a minor proportion (20.2%). This confirms earlier work that demonstrated syndecan to be a heparan sulfate-rich hybrid PG (8, 20).

In the presence of TGF-β, combined radiosulfate incorporation into these two GAGs increases an average of 2.7-fold/cell compared to nontreated cells (Table I). This incorporation occurs nearly equally into the two GAG types; heparan sulfate accounts for 51.9% of the incorporation, whereas 46.3% is in chondroitin sulfate. The chondroitin sulfate is also susceptible to chondroitin AC lyase, indicating that the increase is in chondroitin-4-sulfate and chondroitin-6-sul-
fate, rather than in dermatan sulfate. Taking into account the augmented incorporation per microgram DNA in the presence of TGF-β, these proportions indicate that heparan sulfate is increased ~1.8-fold and chondroitin sulfate 6.2-fold.

**Specific Localization of Core Protein, Heparan Sulfate, and Chondroitin Sulfate on the Cell Surface**

To determine if the augmented chondroitin sulfate synthesis is a response of all the cells in the monolayer, or limited to a subset of the cells, a procedure was used that visualized the distribution of the GAG chains at the cell surface. This was based on the observation that TSP binds to heparan sulfate and chondroitin sulfate (17). The binding to heparan sulfate has been confirmed using the NMuMG cell surface PG (34). In addition, chondroitin-rich syndecan from TGF-β-treated cells binds to TSP affinity columns via either GAG chain (Rapraeger, A., unpublished observations). Thus, incubation of fixed NMuMG cell monolayers with either mAb 281.2, specific for the PG core protein, or with exogenous TSP, which binds GAG chains, allows the colocalization of these structural components. In untreated NMuMG monolayers, the antibodies and TSP are restricted to the apical cell surface due to impassable lateral cell junctions (21). At this surface, the PG is seen on those cells which have yet to completely polarize their basolateral antigens (Fig. 1A). These same cells bind exogenous TSP and the binding is abolished if cell surface GAG is removed with a combined treatment with ABCase and heparitinase (reference 34; Fig. 1D). In addition, specific enzyme digestion of one GAG type, leaving the other, can be used to demonstrate the binding attributable to each type; binding is primarily via heparan sulfate (Fig. 1B), whereas only rare cells bind TSP via chondroitin sulfate (Fig. 1C).

In contrast to untreated cells, almost all of the cells fail to polarize the PG to the basolateral cell surface when cultured in TGF-β (23). This widespread apical distribution of the cell surface PG is confirmed by localization of its core protein (Fig. 1E). It is also demonstrated by the binding of TSP to the apical surface of all cells in monolayers cultured in TGF-β. TSP binding to the cells is via cell surface GAG as treatment with ABCase and heparitinase abolishes the TSP staining (Fig. 1H). In addition, these cells bind TSP via chondroitin sulfate (Fig. 1G) as well as heparan sulfate chains (Fig. 1F) that allows visualization of both GAG types throughout the monolayer, and demonstrates that the increased synthesis of chondroitin sulfate occurs on most, if not all, of the cells.

However, staining for core protein and TSP binding at the apical cell surface is not uniform. Cells that fail to stain intensely for core protein are nonetheless often seen to bind TSP (Fig. 2). In contrast, some cells stain intensely for core protein but bind very little TSP. This lack of uniformity may indicate variability among cells in the numbers and/or types of chains decorating the cell surface core protein. In addition, the possibility exists that a cell surface PG(s) in addition to syndecan is being regulated by TGF-β.

**TGF-β Promotes Synthesis of More GAG on Syndecan**

An increase in the density and size of syndecan supports the conclusion that the enhanced cell surface chondroitin sulfate is on this PG.

**Cell Surface PG Has an Increased Density.** Purified syndecan from cells treated with or without TGF-β was centrifuged to equilibrium in cesium trifluoroacetate density gradients. The PG distribution on the gradients was determined by quantification of 35SO4 label and detection of mAb 281-recognized core protein on immunoblots. The PG from nontreated cells distributes in a single peak with a modal density of 1.55 g/ml (Fig. 3A). Staining for core protein shows a similar distribution, although PG at the higher densities shows less core protein staining per dpm than at lower densities, suggesting that more GAG is attached per core protein at higher densities. This confirms the heterogeneity described previously for this molecule (20) and may explain the variable core protein versus TSP staining described above.

PG from cells treated with TGF-β also distributes as a single peak in the density gradient, although at a greater modal density than the control. In addition, the shift in density of the PG is duplicated by a shift of the core protein recognized by mAb 281. The amount of core protein on the gradient is approximately the same, as PG from equal numbers of cells was loaded, but the total radiosulfate in the PG sample from TGF-β-treated cells is approximately twofold greater and this accounts for the shift of the core protein to a greater density.

Removal of chondroitin sulfate via ABCase digestion removes 24% of the label from the control PG preparation and 49% from the PG of treated cells. This has a slight, if any, effect on the density of the control PG, whereas the density

![Figure 2. Comparison of chondroitin sulfate and core protein distribution. Monolayers in 3 ng/ml of TGF-β were treated with heparitinase and stained with mAb 281.2 and TSP binding, as in Fig. 1, E and G, to localize core protein (A) and chondroitin sulfate (B), respectively. Note the dissimilarities in core protein and GAG distribution. Bar, 10 μm.](image)
of the PG from TGF-β-treated cells is reduced to that of the control (Fig. 3 B).

Digestion with heparitinase generates two peaks in the gradient (Fig. 3 C). A peak at ~1.6 g/ml is seen as a shoulder on peaks containing PG from both control and TGF-β-treated cells and corresponds to the density of heparan sulfate fragments that are generated by the digestion and that are retained on the cationic nylon blot. In control samples, the core protein retaining chondroitin sulfate is found at ~1.47 g/ml and contains ~30% of the initial radioactivity. This proportion is slightly higher than expected (20%) and may reflect some heparan sulfate remnants attached to the core protein. In contrast to controls, the chondroitin sulfate-decorated core protein from TGF-β-treated cells retains ~60% of the radiolabel and is at a density of 1.52 g/ml. Thus, the core protein bears more radiolabeled GAG than the control PG preparation shown in Fig. 3 A and more than either preparation shown in Fig. 3 B, yet bands at a lower density, again suggesting that this chondroitin sulfate-rich configuration is less dense than the core protein bearing heparan sulfate.  

**PG Has an Increased Size That Is Prevented by Treatment with Xyloside.** The synthesis of heparan sulfate and chondroitin sulfate on the core protein is affected by culture of NMuMG cells in methylumbelliferyl-β-D-xylopyranoside (xyloside), which competes with xylose attached to the core protein for initiation and synthesis of GAG linkage regions. The xyloside differentially affects chondroitin sulfate compared to heparan sulfate synthesis, as shown by analysis of PG derived from cells cultured in TGF-β in the presence of various concentrations of the inhibitor (Fig. 4). In the absence of xyloside, 44% of the radiolabel in the PG is chondroitin sulfate and 56% is in heparan sulfate. In xyloside concentrations up to 0.5 mM, however, the incorporation into chondroitin sulfate is reduced by up to 80% with only a slight reduction in heparan sulfate. In contrast, an increase in xyloside concentration to 5 mM also affects heparan sulfate, which is reduced by 77% compared to a 90% reduction in chondroitin sulfate.

Examination of the size of the PG derived from cells cultured in the presence of xyloside shows a differential reduction in its size as the attachment of chondroitin sulfate or both GAG types is prevented. In cells cultured in the absence of TGF-β, the PG size distribution is broad with a median size of 190 kD (Fig. 5). Culture in the presence of up to 0.5 mM xyloside reduces the size distribution only slightly, consistent with chondroitin sulfate having only a minor contribution to its overall size, and fails to reduce the heterogeneity in its distribution. However, 5 mM xyloside, which affects heparan sulfate addition as well, reduces the size of the PG essentially to a single protein band of 69 kD (Fig. 5).  

PG derived from cells cultured in TGF-β is increased in size (Fig. 5). It migrates with a median size of 280 kD on gradient PAGE. This is confirmed by chromatography on Sepharose CL-4B where it elutes at a K_v of 0.25 compared to the control at a K_v of 0.33. This size increase is largely because of additional chondroitin sulfate synthesis, as it is abolished if the cells are cultured in 0.5 mM xyloside (Fig. 5). At this concentration, the PG bearing primarily heparan sulfate chains is nearly of the same size and modal distribution as nontreated cells. In 5 mM xyloside, the TGF-β-treated cells express syndecan as a single protein band of 69 kD, similar to cells in the absence of the growth factor. Despite the failure to acquire its GAG chains, this protein still
Figure 6. Trypsin treatment of cells in 5 mM xyloside: susceptibility of syndecan core protein. Cells cultured in TGF-β and 5 mM xyloside were scraped and treated with or without trypsin. Cell extracts were then assessed for syndecan core protein as in Fig. 5. Arrow shows position of core protein.

Figure 5. PAGE analysis of the size of syndecan from cells treated with various concentrations of xyloside. Monolayers were cultured in xyloside (either 0, 0.5, or 5 mM) in the absence or presence of TGF-β, and then were scraped and extracted directly in PAGE sample buffer. After PAGE chromatography and transfer to cationic nylon, the distribution of syndecan core protein was assessed by staining with mAb 281.2. Molecular sizes were determined by comparison with known molecular mass standards.

Increased Size of the PG Is because of Increased GAG Chain Length and Numbers of Chondroitin Sulfate Chains

Examination of the size of the heparan sulfate and chondroitin sulfate GAG chains released from the cell surface PG by alkaline borohydride treatment shows that they are both increased in length in cells cultured in TGF-β. The heparan sulfate chains elute from Sepharose CL-6B with a modal $K_v$ of 0.28 compared to the control at 0.37 (Fig. 7 A). Estimates of the chain sizes of 61 and 37 kD, respectively, indicate an overall increase in size of 1.7-fold. This increase in size closely parallels the 1.8-fold increase in synthesis of this GAG type (cf., Table I).

The chondroitin sulfate chains are also increased in size, although they remain shorter than the heparan sulfate chains. Chains from cells cultured in the presence or absence of TGF-β elute from Sepharose CL-6B with $K_v$ of 0.41 and 0.54 (Fig. 7 B), respectively, and are estimated to be 31 and 17 kD. However, this 1.8-fold increase in length cannot account for the entire 6.2-fold increase in this GAG type.

To achieve the total increase in chondroitin sulfate synthesis on syndecan, an additional threefold increase must be accounted for. One possible explanation is that more chains are initiated on the core protein. However, it is also possible that the number of chains initiated on the core protein is not changed, and, rather, that syndecan from untreated cells has some very short chondroitin sulfate chains which escape detection and these chains become much longer in TGF-β. To rule out the latter possibility, we examined the number of linkage stubs remaining on the core protein after ABCase digestion. This procedure trims the chondroitin sulfate chain into unsaturated disaccharides and generates an unsaturated uronic acid capping the linkage region that remains attached to the core protein. The remaining stub would be irrelevant to original chain length and is recognized by a specific antibody (R44, courtesy of J. Couchman; reference 6) that equally recognizes chondroitin-4-sulfate or 6-sulfate or nonsulfated chondroitin.

To test the efficacy of the procedure, PG with or without ABCase-generated linkage stubs was immobilized on cationic nylon and stained with antibody R44. This demonstrates that PG containing intact chondroitin sulfate and heparan sulfate chains is not stained by the antibody (Fig. 8 A). Scintillation counting of the dots verified that the PG is quantitatively retained on the blot. In addition, the antibody fails to stain after ABCase treatment if the linkage region is released from the core protein by alkali (Fig. 8 B). The unsaturated disaccharides generated by ABCase are not retained on the blot (19), and the released linkage region presumably fails to bind as well. However, the antibody does recognize native PG treated with ABCase (Fig. 8 C), presumably via the unsaturated uronic acid that remains on the chondroitin sulfate GAG linkage region and the staining intensity is reduced with twofold decrements of bound PG.
This staining procedure shows that more chondroitin sulfate chains are initiated on syndecan of cells in TGF-β (Fig. 9). Comparison of [35S]sulfate-labeled syndecan from cells cultured in the presence or absence of the growth factor shows that the relative amounts of core protein per cell are the same, as shown previously (23). Nonetheless, autoradiography confirms that the PG from TGF-β-treated cells contains at least twofold more radioactivity and that this difference is largely reduced by ABCase treatment. In addition, reaction of the ABCase-treated core protein with antichondroitin sulfate antibody shows an approximately threefold increase in the number of unsaturated uronic acid residues retained on the core protein. This suggests that the augmented incorporation of radiolabel into chondroitin sulfate is because of (a) increased length and (b) the initiation of approximately threefold more chains.

Discussion

Syndecan is an integral membrane PG found primarily on epithelia. Among the interesting features of this molecule is its complement of both chondroitin sulfate and heparan sulfate GAG, which has prompted investigation into the regulation and significance of GAG attachment to the core protein. The hybrid nature of the molecule was first shown on NMuMG mouse mammary epithelial cells in which 15–24% of the GAG was reported to be in chondroitin sulfate and the remainder in heparan sulfate (20). Although it was proposed at that time that the proportions of the two GAG types might change with cell behavior, it was not clear what behavior would elicit the change. However, a recent report (23) demonstrates that treatment with TGF-β promotes FN accumulation, reduced proliferation and shape changes in these cells, as it does with other epithelia. In addition, it was shown that an increased amount of chondroitin sulfate was present at the cell surface despite unchanged amounts of syndecan core protein. This suggests either that the amount of chondroitin sulfate on syndecan is altered, or that the GAG is on a different PG whose appearance is regulated by the growth factor.

Several approaches demonstrate that the increase in cell surface chondroitin sulfate is because of an augmented proportion on syndecan. Analysis of the molecule on density gradients shows an alteration in the density of the PG. Secondly, the size of syndecan is increased, which is attributed to additional chondroitin sulfate as it is blocked by treatment with xyloside. Xylosides have previously been shown to affect chondroitin sulfate addition (30), although the concentrations required to affect heparan sulfate addition are less well understood. In the case of syndecan, the attachment of both GAG types can be blocked, although chondroitin sulfate addition is affected at 10-fold lower concentrations than heparan sulfate. Blockage of both types of chains allows comparison of core protein sizes between treated and nontreated cells; the identical sizes suggest that additional chain addition cannot be attributed to increased core protein size, as might occur as a result of splicing mechanisms during synthesis, or cross-linking to another PG. Thus, the data support the addition of more chondroitin sulfate to the syndecan core protein. This suggests that the amino acid sequence alone is not sufficient to designate numbers and/or types of chains and implicates an additional control mechanism.

The change in structure of syndecan is not limited to chon-
chondroitin sulfate. Heparan sulfate is increased 1.8-fold, and this is accounted for by an increase in its length. Although chondroitin sulfate shows a similar increase in length, the sixfold increase in this GAG requires at least a threefold increase in chain number. This is confirmed by quantification of relative numbers of linkage sites. Treatment of syndecan with ABCase cleaves all chondroitin sulfate chains, leaving a terminal unsaturated saccharide at the nonreducing end. Regardless of initial chain length, these would be one per chain. Detection of these sites indicates an approximately threefold increase in syndecan from TGF-β-treated cells.

The means by which TGF-β promotes the accumulation of additional chondroitin sulfate is not clear. The possibility that TGF-β selects a group of cells that are predisposed to express syndecan with a high chondroitin sulfate content is ruled out as the cells do not proliferate in the growth factor (23) and thus cannot generate a clonal population in response to the growth factor. Furthermore, TGF-β appears to promote a net increase in the amount of chondroitin sulfate on most, if not all, cells rather than increasing the numbers of cells that are already expressing a PG rich in chondroitin sulfate. This is shown by indirect staining with TSP, which detects only cells with the augmented amount of chondroitin sulfate; it fails to bind to chondroitin sulfate on nontreated cells, despite the presence of syndecan with ~20% chondroitin sulfate (34) but binds to chondroitin sulfate on most cells treated with TGF-β.

Despite the overall increase in GAG of TGF-β-treated cells, heterogeneity is observed on a single cell basis. This heterogeneity is likely to be responsible for the large size distribution of syndecan reported earlier (20). TSP staining shows that some cells express core protein that binds little TSP whereas others contain core protein, presumably richer in GAG, that readily binds the matrix component. The staining method shows that both chondroitin sulfate and heparan sulfate vary, but does not allow simultaneous comparison of both GAG types on the same cell. The biochemical data, therefore, reflect averages of this heterogeneity.

Five ser-gly sequences (e.g., potential GAG attachment sites) in the syndecan core protein have been deduced from the cloned gene (29). It is not known how many of these sites are actually used. If all five are used, the size and proportions of the GAG chains described here would dictate two heparan sulfate chains regardless of the presence of TGF-β and an increase from one chondroitin sulfate chain to three in the presence of TGF-β (Table II).

Table II. Model of Syndecan

|                    | No TGF-β | +TGF-β |
|--------------------|----------|--------|
| ChonSO₄ chains     |          |        |
| Number             | 1        | 3      |
| Size               | 17 kD    | 31 kD  |
| Total mass         | 17 kD    | 93 kD  |
| Percent of total GAG| 20%     | 43%    |
| HepsO₄ chains      |          |        |
| Number             | 2        | 2      |
| Size               | 36 kD    | 61 kD  |
| Total mass         | 72 kD    | 122 kD |
| Percent of total GAG| 80%     | 57%    |
| Core protein*      | 33 kD    | 33 kD  |
| Total kilodaltons  | 122 kD   | 248 kD |

* Based on sequence data (29). Actual core protein migrates anomalously on SDS-PAGE (29).

The role of chondroitin sulfate on syndecan remains unclear. The heparan sulfate chains serve to bind the PG to fibronectin (28), to collagen types I, III and V (15) and to TSP (34). As shown here, either heparan sulfate or the enhanced amount of chondroitin sulfate binds TSP to cell surfaces.

Although it provides an additional mechanism for binding TSP, the chondroitin sulfate may interfere with other cell adhesion mechanisms (for review, see Ruoslahti [26]). Recently, Yamagata et al. (38) have demonstrated that chondroitin sulfate PG, but not isolated chains, blocks the binding of BHK cells to FN, laminin, vitronectin, and type I collagen; the authors postulate that the chains must be anchored to the core protein to generate a multivalent ligand that binds and may alter the conformation of cell surface receptors. Alternatively, the core protein may position the GAG chains such that they block binding sites on the matrix ligand. Extending these findings to syndecan, the enhanced attachment of chondroitin sulfate to this PG may orient the GAG such that it modifies the function of other cell surface matrix receptors; e.g., integrin-mediated binding to FN.

Alternatively, it is speculated that chondroitin sulfate on the same core protein with heparan sulfate may block or
otherwise modify the heparan sulfate-mediated anchorage to the extracellular matrix. Preliminary data (Rapraeger, unpublished observations) show that chondroitin sulfate-rich syndecan isolated from TGF-β-treated cells does bind FN affinity columns, although only via heparan sulfate. However, it remains to be determined if the PG oriented by anchorage in the plasma membrane is an equally effective receptor. It is intriguing that two potential GAG anchorage sites on syndecan are near the plasma membrane whereas the remaining three are near the distal end of the ectodomain. As discussed by Saunders et al. (29), the amino acid sequence of the distal sites are characteristic of chondroitin sulfate attachment. However, it remains to be confirmed (a) that TGF-β treatment promotes attachment of chondroitin sulfate to these sites, and (b) that the proximal sites (e.g., near the membrane) are exclusively decorated with heparan sulfate. Indeed, the possibility that heparan sulfate is abolished on the core in some cell behaviors, leaving solely chondroitin sulfate, has not been ruled out.

In mouse tissues, syndecan is restricted primarily to epithelia (9). Variation is noted in the number of chondroitin sulfate and heparan sulfate chains that it contains in simple compared to stratified epithelia (27), suggesting variability in its molecular form in vivo. Examination of its distribution during early development of the tooth suggests that it is expressed at sites of epithelial/mesenchymal interactions (35). As TGF-β is localized to the mesenchyme of similar sites (10) and is believed to affect the epithelium, it is likely that the PG at these sites is rich in chondroitin sulfate. Treatment with xyloside, which blocks the addition of chondroitin sulfate and heparan sulfate chains that it contains in simple glycosaminoglycan synthesis. J. Biol. Chem. 263:16984-16991.

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