

# Membrane-anchored and Soluble Forms of Betaglycan, a Polymorphic Proteoglycan that Binds Transforming Growth Factor- $\beta$

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**Abstract.** Transforming growth factors  $\beta$ 1 and  $\beta$ 2 bind with high affinity to the core protein of a 250–350-kD cell surface proteoglycan. This proteoglycan (formerly referred to as the type III TGF- $\beta$  receptor) coexists in many cells with the receptor implicated in TGF- $\beta$  signal transduction (type I TGF- $\beta$  receptor), but its function is not known. We report here that soluble TGF- $\beta$ -binding proteoglycans are released by several cell types into the culture media, and can be found in serum and extracellular matrices. As has been shown for the membrane-bound form, the soluble proteoglycans have a heterogeneous core protein of 100–120 kD that carries chondroitin sulfate and/or heparan sulfate

glycosaminoglycan chains and a small amount of N-linked carbohydrate. The membrane-bound form of this proteoglycan is hydrophobic and associates with liposomes, whereas the soluble forms lack a membrane anchor and do not associate with liposomes. Differences in the electrophoretic migration of the soluble and membrane forms of this proteoglycan suggest additional structural differences in their core proteins and glycosaminoglycan chains. These soluble and membrane-bound proteoglycans, for which we propose the name “betaglycans,” might play distinct roles in pericellular retention, delivery, or clearance of activated TGF- $\beta$ .

THE cell–cell communication events that guide morphogenesis and tissue repair in pluricellular organisms are mediated by cell surface receptors. Some receptors bind diffusible factors whereas others bind adhesion molecules located on the surface of other cells or in the extracellular matrix. Although many membrane receptors are glycoproteins, several receptors have been recently shown to be proteoglycans. These include syndecan (19), CD44/Hermes antigen (11, 32), and the invariant chain associated with murine class II major histocompatibility complex (26), which have been implicated, respectively, in epithelial cell adhesion to extracellular matrix components, lymphocyte homing to lymph nodes, and antigen presentation. The *Drosophila* gene *Toll*, required to establish dorsoventral polarity during embryogenesis, encodes a predicted integral membrane protein that has amino acid sequence similarity to a human fibroblast proteoglycan core protein (13), and the membrane precursor for Alzheimer’s disease  $\beta$ -amyloid protein appears to be associated with a heparan sulfate proteoglycan (29). These two putative proteoglycans may also be involved in intercellular recognition or communication.

An additional member of this group of receptor-like molecules is a 250–350-kD integral membrane proteoglycan that is expressed by many mammalian and avian cells, and has the unique property of avidly binding transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>1</sup> (6, 7, 8, 24, 30). TGF- $\beta$ , which is the prototype of a large family of differentiation, morphogene-

sis, and growth suppression factors (23, 34), binds to a site located in the 100–120-kD core of this proteoglycan. The glycosaminoglycan chains, generally consisting of both chondroitin sulfate and heparan sulfate (7, 30), are attached to a domain of the core protein distinct from the TGF- $\beta$ -binding domain, and are not required for either surface expression or TGF- $\beta$  binding activity of this receptor (4). This TGF- $\beta$  binding proteoglycan, which was formerly referred to as the type III TGF- $\beta$  receptor, coexists in most cells with the TGF- $\beta$  receptor types I and II, two TGF- $\beta$ -binding glycoproteins of 53 and 73 kD, respectively (6). The function of this proteoglycan is not known. It is not detectable in certain cell types that respond normally to TGF- $\beta$  *in vitro* (25, 27). Selection of chemically induced cell mutants resistant to TGF- $\beta$  action results with high frequency in the isolation of clones that have selectively lost the type I TGF- $\beta$  receptor, with no apparent effect on the expression of the TGF- $\beta$ -binding proteoglycan (2). This evidence implicates the type I TGF- $\beta$  receptor as the mediator of TGF- $\beta$  action, but leaves unresolved the question of the potential role of the other TGF- $\beta$  binding proteins.

We report here the existence of forms of the TGF- $\beta$ -binding proteoglycan that lack the membrane anchor. These forms are released by many cell lines and can be found in cell culture fluids, serum, and extracellular matrices. Based on these findings, we propose the name “betaglycans” to designate the members of this group of TGF- $\beta$ -binding proteoglycans. These findings provide new clues about possible roles for these molecules in the biology of TGF- $\beta$ .

1. *Abbreviation used in this paper:* TGF- $\beta$ , transforming growth factor-beta.

## Materials and Methods

### Source of Soluble and Membrane-derived Betaglycan

The source, culture conditions, and differentiating conditions (where applicable) for 3T3-L1, Mv1Lu, WI-38, and L<sub>6</sub>E<sub>9</sub> cells were as previously described (5, 16, 22, 25). CCL-39 and Rat-1 cells were grown in DME supplemented with 10% FCS. Serum-free washes of cell monolayers and conditioning of serum-free medium were done using Waymouth's medium (Gibco Laboratories, Grand Island, NY). Media used as a source of soluble betaglycan had typically been conditioned for 3 d by cell monolayers as they reached confluency, or by confluent 3T3-L1 adipocyte cultures. Immediately after collection, the conditioned media were supplemented with 0.2 mM PMSF, and cleared of loose cells and debris by centrifugation at 2,000 g for 15 min at 4°C, followed by ultracentrifugation (200,000 g for 40 min at 4°C). Mycoplasma-free FCS was from Gibco Laboratories (lot No. 35K3772).

To obtain cellular matrix extracts and detergent-soluble cell extracts, cell monolayers were first incubated for 30 min at 37°C with a Tris- or Hepes-buffered (10 mM, pH 7.4) isotonic saline solution. Extracellular matrix extracts, prepared by scraping cell cultures into a solution containing 1 M urea, 10 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 10 mM Tris, pH 7.0, as previously described (1, 17), were clarified by centrifugation at 150,000 g for 45 min at 4°C. Detergent-soluble cell extracts were obtained by scraping untreated or affinity-labeled cell monolayers into a solution containing 0.25 M sucrose, 10 mM Tris, 1 mM EDTA, 0.1 mM PMSF, pH 7.0. The cell pellet, obtained by centrifugation at 10,000 g for 10 min, was solubilized by incubation for 40 min at 4°C with a solution containing 1% Triton X-100, 10 mM Tris, 1 mM EDTA, and a mixture of protease inhibitors (22), and was then clarified by centrifugation at 10,000 g for 15 min or at 100,000 g for 40 min.

### TGF- $\beta$ Binding and Affinity Labeling Assays for Soluble Betaglycan

These assays were based on the ability of betaglycan to bind to DEAE-Sepharose under high salt and acidic pH conditions (7). Samples of conditioned media, detergent extracts of cells, or extracellular matrix extracts were adjusted to contain 0.25 M NaCl and brought to pH 6.0 by addition of 50  $\mu$ l/ml of 1 M bis-Tris, pH 6.0. Serum samples were diluted with 2 vol of a solution containing 225 mM NaCl, 75 mM BIS-Tris, pH 6.0. These samples were then passed through a minicolumn (0.5 ml) of DEAE-Sepharose Fast Flow (Sigma Chemical Co., St. Louis, MO) that had been equilibrated with 0.35 M NaCl, 10 mM BIS-Tris, pH 6.0 (plus 0.5% Triton X-100 for detergent-soluble extracts). The column was washed with 3 ml of this buffer and was thoroughly drained, unpacked, and resuspended in 1 ml of binding buffer (50 mM NaCl, 10 mM MgCl<sub>2</sub>, 5 mM KCl, 25 mM Hepes, pH 7.6) (22) containing 1 mg/ml of BSA and 150 pM <sup>125</sup>I-TGF- $\beta$ 1 alone or with unlabeled TGF- $\beta$ 1 or TGF- $\beta$ 2 prepared from bovine bone (31). After incubation for 3.5 h at 4°C with continued mixing, the beads were washed five times with cold binding buffer (without albumin). For binding assays, the beads were loaded into a minicolumn, drained, and eluted with two column volumes of 0.7 M NaCl, 50 mM BIS-Tris, pH 6.0. The radioactivity in the eluate was then determined in a gamma counter. For affinity-labeling assays, the washed beads were incubated for 15 min at 4°C with 1 ml of albumin-free binding buffer containing 0.16 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL). The cross-linking reaction was stopped by addition of 10  $\mu$ l of 2 M Tris, pH 7.0. The beads were then packed into a minicolumn, drained, and eluted with 0.7 M NaCl. Triton X-100 (1%) was present in all the steps in assays of detergent-solubilized cell extracts. The eluate from the beads was heated with electrophoresis sample buffer, run on a 6% polyacrylamide-SDS gel, and subjected to autoradiography with film (X-Omat; Eastman Kodak Co., Rochester, NY) and an intensifying screen (Cronex; Dupont Co., Diagnostic & Bioresearch Systems, Wilmington, DE).

Affinity labeling of cell monolayers with <sup>125</sup>I-TGF- $\beta$  was performed as previously described (22). Detergent-soluble extracts from cell monolayers were prepared in the presence of protease inhibitors as described above.

### Enzymatic Deglycosylations

Treatment of cell monolayers with chondroitinase ABC and heparitinase was performed as previously described (7). Removal of glycosaminoglycan chains from cellular or medium proteoglycans affinity-labeled on DEAE-Sepharose beads was carried out by diluting the high salt eluates from these

beads with 3 vol of 50 mM Tris, pH 6.8, containing 0.1 mg/ml of BSA and the cocktail of protease inhibitors mentioned above. This sample was then incubated overnight at 37°C with 0.2 U/ml of chondroitinase ABC (ICN K & K Laboratories Inc., Plainview, NY) or  $2 \times 10^{-3}$  IU/ml of heparitinase (ICN K & K Laboratories, Inc.). Proteins were precipitated with either 2 vol of acetone at -20°C for at least 4 h in preparation for gel electrophoresis, or with 10% TCA in preparation for digestion with endoglycosidase F (Boehringer Mannheim Biochemicals, Indianapolis, IN) as previously described (7). Samples were heated at 100°C with sample buffer, and subjected to electrophoresis and autoradiography.

### Liposome Formation

For these experiments, cell monolayers were affinity labeled, solubilized, and clarified by ultracentrifugation as described above, with the exception that the detergent used was 70 mM of octylglucoside (Boehringer Mannheim Biochemicals). The solubilized material was bound to and eluted from DEAE-Sepharose as described above, with 50 mM of octylglucoside present in all buffers. The resulting high salt eluate was diluted with 4 vol of a 50-mM octylglucoside solution. A high salt eluate containing affinity-labeled betaglycan prepared from conditioned medium was also diluted with 4 vol of the 50-mM octylglucoside solution. These samples were each mixed with 0.5 mg of egg phosphatidylcholine (Type III-E; Sigma Chemical Co.) dried in the bottom of a glass test tube together with 1  $\mu$ Ci of <sup>3</sup>H-dipalmitoyl phosphatidylcholine (Dupont Co. Diagnostic and Research Systems; New England Nuclear, Boston, MA). Liposomes were formed by dialysis of these samples against 200 vol of 10 mM Na phosphate, pH 7.0, 150 mM NaCl, and 0.1 mM PMSF for 24 h at 4°C, with a buffer change after 12 h. Dialyzed samples were mixed with 3 vol of 60% (wt/wt) sucrose solution in 10 mM Tris, 1 mM EDTA, pH 7, and 2 ml of this sample was placed in the bottom of a rotor tube (SW50.1 Beckman Instruments, Inc., Palo Alto, CA), overlaid with 2 ml of 30% sucrose solution and 1.3 ml of 10% sucrose solution made in the same buffer. Where indicated, 1% Triton X-100 was added to the three sucrose solutions. The samples were centrifuged for 22 h at 45,000 rpm at 4°C and fractionated from top to bottom into seven 0.75-ml fractions. 20- $\mu$ l aliquots were taken from each fraction and <sup>3</sup>H radioactivity was quantitated by scintillation counting. The level of <sup>125</sup>I radioactivity in these samples was negligible compared with the level of <sup>3</sup>H radioactivity and did not interfere with counting. The rest of the fraction was counted for <sup>125</sup>I radioactivity, diluted with water, precipitated with acetone, and the precipitate was subjected to electrophoresis and autoradiography.

In some experiments, the fractions were incubated with chondroitinase and heparitinase before electrophoresis. Samples were diluted with 2.5 vol of a solution containing 50 mM Tris, pH 7.0, 1 mg/ml of BSA, and protease inhibitors, and processed for enzymatic deglycosylation as described above.

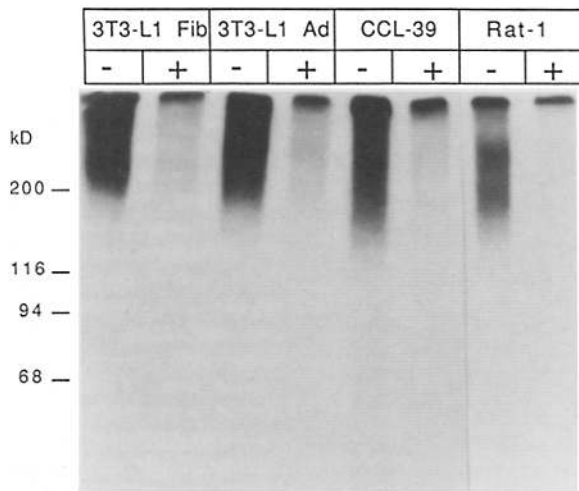
### Hydrophobic Chromatography

DEAE-Sepharose eluates containing affinity-labeled samples from cells and medium prepared as described for the liposome reconstitution experiments were diluted with 0.7 M NaCl and 10 mM Hepes, pH 7.0, to lower the octylglucoside concentration to 5 mM. These samples were loaded on a 3-ml column of octyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.7 M NaCl, 10 mM Hepes, and 5 mM octylglucoside, pH 7.0. The column was washed with this buffer and then eluted with a linear gradient of octylglucoside. The <sup>125</sup>I radioactivity in the resulting fractions was determined by gamma counting. The fractions were then acetone precipitated and subjected to electrophoresis and autoradiography.

## Results

### Soluble Forms of Betaglycan

To examine whether cells release proteoglycans into the medium that bind TGF- $\beta$ , clarified samples of medium conditioned by various cell lines were chromatographed over DEAE-Sepharose under conditions of high salt and low pH. Under these conditions, most of the material that binds to DEAE should be strongly anionic and should include any sulfated proteoglycans present in the sample. Material bound to DEAE-Sepharose was affinity labeled with <sup>125</sup>I-TGF- $\beta$ 1 using disuccinimidyl suberate as the cross-linking agent.



**Figure 1.** Anionic TGF- $\beta$ -binding components in conditioned media from various cell lines. Highly anionic material from 12-ml aliquots of media conditioned by the indicated cell lines was bound to DEAE-Sepharose in the presence of 0.35 M NaCl, and was affinity labeled with  $^{125}\text{I}$ -TGF- $\beta$ 1 alone (-) or in the presence of 15 nM TGF- $\beta$ 1 (+). The affinity-labeled material was eluted from the Sepharose beads and displayed by electrophoresis and autoradiography.

The affinity-labeled material was eluted with 0.7 M NaCl and visualized by electrophoresis and autoradiography. When the conditioned media from 3T3-L1 mouse preadipocytic fibroblasts, differentiated 3T3-L1 adipocytes, CCL-39 Chinese hamster lung fibroblasts, and Rat-1 rat embryo fibroblasts were assayed in this manner, strongly labeled species that migrated in denaturing electrophoresis gels as broad bands of high (>200 kD) molecular mass were detected (Fig. 1). The affinity-labeled material from 3T3-L1 cells frequently resolved into two broad bands of 200–300 kD and ~400 kD, respectively (see also Figs. 2, 4, and 6). Labeling of these species was specific and reflected high affinity binding as determined by inhibition of labeling with the presence of a moderate excess (15 nM) of unlabeled TGF- $\beta$ 1 (Fig. 1) or TGF- $\beta$ 2 (not shown) during the incubation with  $^{125}\text{I}$ -TGF- $\beta$ 1. Lower but significant levels of labeled species with similar characteristics were obtained with samples of media conditioned by MvLu mink lung epithelial cells or WI-38 human lung fibroblasts; the signal obtained with medium conditioned by L<sub>6</sub>E<sub>9</sub> rat myoblasts (not shown) was not significantly above a low background signal given by fresh medium containing 20% FCS (see Fig. 5). The level of the TGF- $\beta$  binding species in the various conditioned media was roughly proportional to the level of cell surface betaglycan in each of the cell lines surveyed as judged by the intensity of labeling with  $^{125}\text{I}$ -TGF- $\beta$ 1 (Table I).

Experiments with serum-free medium were performed in order to assess the cellular origin of the TGF- $\beta$ -binding species in the medium. Of the cell lines with a high yield of soluble TGF- $\beta$ -binding species, only Rat-1 cells survived well in long-term culture under serum-free conditions. Confluent monolayers of Rat-1 cells were rinsed twice with serum-free medium and incubated for 4 h with serum-free medium to allow dissociation of serum components from the cell monolayer. They were then allowed to condition serum-

free medium for 3 d. These media had a level of TGF- $\beta$ -binding species that was ~65% of the level present in serum-containing media conditioned by parallel cultures of the same cell line, as determined by the intensity of labeling with  $^{125}\text{I}$ -TGF- $\beta$ 1 (not shown). These results indicated that a large proportion of the TGF- $\beta$ -binding species in the medium were cell products rather than serum components. Their lower level in serum-free conditioned medium is attributable, at least in part, to depressed cellular activity in the absence of serum. Since medium conditioned by 3T3-L1 fibroblasts was the richest source of TGF- $\beta$ -binding material, most of the subsequent experiments were done with this medium.

The proteoglycan nature of the TGF- $\beta$ -binding species from conditioned medium and their relationship to cell surface betaglycan was further assessed by treatment with glycosaminoglycan-digesting enzymes. Treatment of the affinity-labeled soluble product from 3T3-L1 cells with either chondroitinase ABC which digests chondroitin sulfate chains, or heparitinase which digests heparan sulfate chains, led to a partial conversion of the labeled material into core products of 120–130 kD (~110–120 kD after subtracting the molecular mass of one cross-linked TGF- $\beta$  monomer) (Fig. 2 A). Both enzymes acting together led to a virtually complete conversion of the labeled material into 120–130-kD core products (Fig. 2 A). The results shown in Fig. 2 A suggest that the 200–300-kD soluble TGF- $\beta$ -binding species consisted of molecules containing either heparan sulfate or chondroitin sulfate glycosaminoglycan chains, whereas the ~400-kD form appeared to be a chondroitin sulfate proteoglycan. Thus, these TGF- $\beta$ -binding species are proteoglycans with properties similar to those of cell surface betaglycan from 3T3-L1 fibroblasts and other cell lines (7, 30).

However, the core components of the soluble and membrane-bound forms of betaglycan devoid of glycosaminoglycan chains showed significant differences in their electrophoretic mobility. Membrane betaglycan yielded three distinct labeled core products of 120, 130, and 140 kD, respectively

**Table I. Relative Levels of Soluble and Cell Surface Betaglycan**

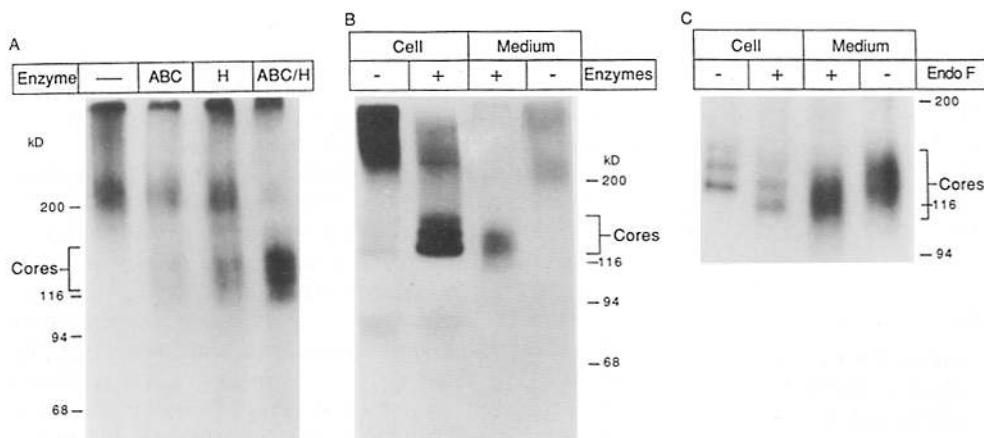
Source*	Betaglycan in medium	Cell surface betaglycan <sup>§</sup>
	cpm <sup>‡</sup>	Binding sites/cell $\times 10^{-3}$
3T3-L1 mouse embryo fibroblast	12,600	120
3T3-L1 adipocyte	15,160	120
CCL-39 hamster lung fibroblast	5,940	20
Rat-1 rat embryo fibroblast	2,620	18
MvLu mink lung epithelial	950	5
WI-38 human lung fibroblast	240	1
MG-63 human osteosarcoma	220	1
L <sub>6</sub> E <sub>9</sub> rat skeletal muscle myoblast	250	<0.31
FCS (10% in medium)	170	—
Calf serum (10% in medium)	80	—

\* All cell lines were grown with 10% FCS or 10% calf serum, except for L<sub>6</sub>E<sub>9</sub> cells which were grown with 20% FCS.

<sup>‡</sup>  $^{125}\text{I}$ -TGF- $\beta$ 1 cpm associated with the affinity-labeled betaglycan band corresponding to 12-ml aliquots of conditioned media.

<sup>§</sup> TGF- $\beta$ -binding sites per cell, as determined by saturation binding assays and affinity-labeling assays.

<sup>||</sup> Below the detection limit of 300 sites/cell.

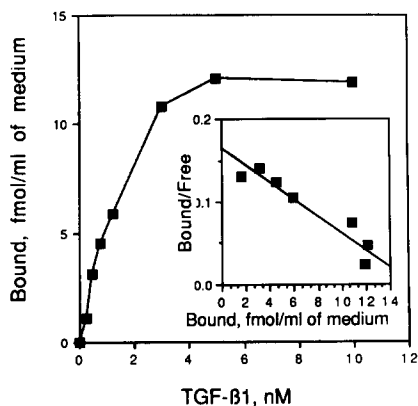


**Figure 2.** Core proteins from soluble and membrane-bound TGF- $\beta$ -binding proteoglycans. (A) Affinity-labeled anionic components from 3T3-L1 fibroblast conditioned medium were incubated with chondroitinase ABC (ABC), heparitinase (H), both enzymes (ABC/H), or buffer alone (-). (B) 3T3-L1 cell monolayers (Cell) treated with chondroitinase plus heparitinase (+) or with buffer alone (-) were affinity labeled with  $^{125}\text{I}$ -TGF- $\beta$ 1, extracted with detergent, and electrophoresed in parallel with affinity-labeled secreted proteoglycans that had been treated with these enzymes or with buffer alone. A small amount of betaglycan core proteins lacking glycosaminoglycan chains is typically observed in nondigested 3T3-L1 cells (reference 7). (C) 3T3-L1 conditioned media and detergent extracts of 3T3-L1 cells, affinity-labeled on DEAE-Sephacrose, were treated with chondroitinase and heparitinase followed by incubation with endoglycosidase F (+) or buffer alone (-). This material was then displayed by electrophoresis and autoradiography.

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(Fig. 2 B and reference 7), whereas soluble betaglycan yielded a broadly migrating labeled core of 120–130-kD (Fig. 2 B), which in some experiments was poorly resolved into two bands. The diffuse appearance of the soluble betaglycan core relative to the membrane betaglycan core products was not an artifact of the labeling procedure performed with DEAE-Sephacrose-bound material, since the detergent-solubilized membrane betaglycan affinity labeled on DEAE-Sephacrose still yielded the distinct pattern of three core components after removal of the glycosaminoglycan chains (Fig. 2 C). Similar results were obtained with membrane and soluble betaglycans from Rat-1 cells (not shown). As seen in Fig. 2 and as previously noted (5, 7), 3T3-L1 cells expressed a low level of membrane betaglycan cores devoid of glycosaminoglycan chains.

Treatment with endoglycosidase F, an enzyme that re-

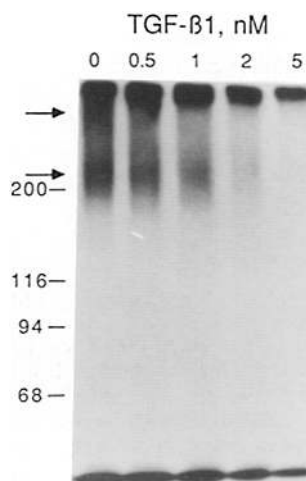


**Figure 3.**  $^{125}\text{I}$ -TGF- $\beta$ 1 binding to 3T3-L1 medium betaglycan. Equilibrium binding saturation curve obtained by determining the amount of TGF- $\beta$ 1 specifically bound to soluble proteoglycan from 3T3-L1 cells using 50 pM  $^{125}\text{I}$ -TGF- $\beta$ 1 and sufficient TGF- $\beta$ 1 to achieve the indicated final concentrations. Nonspecific  $^{125}\text{I}$ -TGF- $\beta$ 1 binding determined in the presence of 20 nM TGF- $\beta$ 1 was 0.04 fmol/ml and has been subtracted from all data points. (Inset) Scatchard plot of the same data.

moves many types of N-linked glycan chains from glycoproteins, decreased the apparent molecular mass of the core products from membrane-bound and soluble betaglycans by  $\sim 10$  kD, but did not alter the different appearance of the deglycosylated core products from both betaglycan forms (Fig. 2 C). Differences were also apparent between the fully glycosylated membrane-derived and secreted betaglycan forms. The soluble forms resolved on gels into two broad bands whereas the membrane form migrated as a single broad band (see Figs. 2 B, 4, and 6 B).

#### Ligand Binding Properties of Secreted Betaglycan

Equilibrium binding assays performed with the DEAE-Sephacrose-bound fraction prepared from media conditioned for 3 d by a confluent monolayer of 3T3-L1 fibroblasts indicated that the anionic fraction from these media contained a TGF- $\beta$ 1-binding capacity of 16 fmol/ml with a  $K_d$  of 1.9 nM, as determined by Scatchard analysis of the binding data (Fig. 3). Based on these results, it is calculated that a near-confluent ( $8\text{--}9 \times 10^4$  cells/cm $^2$ ) monolayer of 3T3-L1 cells, which contain  $\sim 1.2 \times 10^5$  type III (betaglycan) TGF- $\beta$ -binding



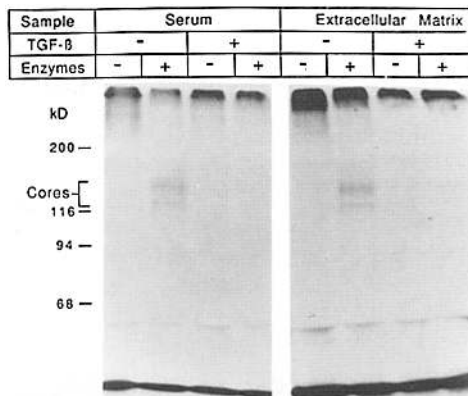
**Figure 4.** Affinity-labeling competition assay of soluble betaglycan. Soluble proteoglycans released by 3T3-L1 cells were bound to DEAE-Sephacrose and affinity labeled with 150 pM  $^{125}\text{I}$ -TGF- $\beta$ 1 in the presence of the indicated concentrations of TGF- $\beta$ 1. (Arrows) The two major labeled betaglycan species whose labeling was inhibited by TGF- $\beta$ 1.

sites per cell (16), accumulated in 3 d a level of soluble betaglycan in the medium that was at least 16–19% of the level of membrane-bound betaglycan. This calculation does not assume any losses of betaglycan that might occur during the chromatography steps and the binding assay. Competition affinity-labeling assays (Fig. 4) indicated that half-maximal inhibition of soluble betaglycan labeling was obtained with  $\sim 1$  nM TGF- $\beta 1$ , which is in good agreement with the results of the equilibrium binding assays.

### Betaglycan in Serum and Extracellular Matrix

The DEAE-Sepharose-bound fraction of fresh culture medium containing 10% FBS was used as a control during the survey of media conditioned by cell lines described above. Affinity labeling of this fraction with  $^{125}\text{I}$ -TGF- $\beta 1$  yielded a low but measurable amount of labeled high molecular weight components. To confirm the possible relationship of these components to betaglycan, samples of FBS were subjected to DEAE-Sepharose chromatography, and the bound material was affinity labeled with  $^{125}\text{I}$ -TGF- $\beta 1$  followed by incubation with or without heparitinase and chondroitinase ABC. Electrophoretic analysis of these samples revealed the presence of specific TGF- $\beta$ -binding proteoglycan(s) that yielded labeled core products of 120–130 kD after treatment with glycosaminoglycan-degrading enzymes (Fig. 5).

Secreted proteoglycans can be deposited in extracellular matrices (14), and certain membrane proteoglycans can bind to extracellular matrix collagen and fibronectin (19). We therefore investigated whether soluble betaglycan is present in the extracellular matrix of 3T3-L1 cells. Cell monolayers, washed extensively with serum-free medium, were extracted with a urea-containing buffer which has previously been used to extract extracellular matrix components including matrix-associated proteoglycans (1). When subjected to the same experimental protocol described above for the identification of betaglycan in serum, the extracellular matrix extracts from 3T3-L1 cell monolayers yielded affinity-labeled



**Figure 5.** Betaglycan in FCS and 3T3-L1 fibroblast extracellular matrix. Highly anionic material from FCS or from the extracellular matrix of confluent 3T3-L1 cells was bound to DEAE-Sepharose and affinity labeled with  $^{125}\text{I}$ -TGF- $\beta 1$  alone or in the presence of an excess of TGF- $\beta 1$ . After elution from the beads, the affinity-labeled material was incubated with chondroitinase ABC plus heparitinase (Enzymes +) or with buffer alone, and was then subjected to electrophoresis and autoradiography. The material loaded per lane was derived from 8 ml of serum or from  $6 \times 10^6$  cells.

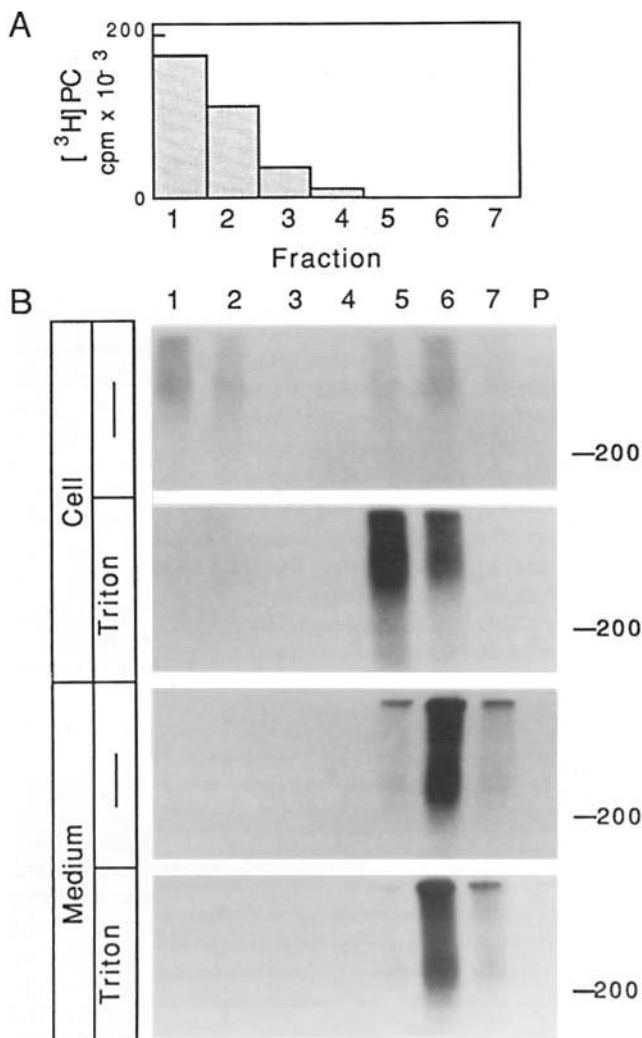
high molecular weight material that poorly penetrated the electrophoresis gels, but whose labeling with  $^{125}\text{I}$ -TGF- $\beta 1$  was partially inhibited by the presence of an excess of TGF- $\beta 1$ . More importantly, treatment of these affinity-labeled samples with heparitinase and chondroitinase ABC resulted in the generation of specifically labeled 120–130-kD products characteristic of betaglycan (Fig. 5). The level of betaglycan detectable in extracellular matrix extracts from 3T3-L1 cells was  $\sim 2$ –5% of that seen in the medium conditioned by an equivalent number of cells.

The core products from serum-derived and extracellular matrix betaglycans appear in the experiments shown in Fig. 5 as a doublet containing a diffuse major band and a sharper lower molecular weight band. From examination of the Coomassie blue-stained gels (not shown) it was evident that the lower band is the result of artifactual disruption and sharpening of the labeled band by an abundant 120-kD protein contaminant present in the samples. This contaminant originated in the albumin preparation used during enzyme treatment of the samples. The reason for the appearance of this artifact only in extracellular matrix or serum-derived samples is that a relatively large amount of sample with a large amount of albumin had to be loaded on the gels in order to visualize the labeled products.

### Evidence for a Hydrophobic Membrane Anchor in Membrane Betaglycan but Not in Soluble Betaglycan

The presence of a membrane anchor in cell surface betaglycan has been inferred from the ability of detergents but not chaotropic agents or glycosaminoglycan-digesting enzymes to release membrane-associated betaglycan (7, 24, 30). In the present studies, we determined the intrinsic ability of membrane-derived and soluble betaglycans to become intercalated into liposomes. For this purpose, 3T3-L1 cell surface betaglycan was affinity labeled with  $^{125}\text{I}$ -TGF- $\beta 1$ , solubilized with octylglucoside, and partially purified by chromatography over DEAE-Sepharose. This material was mixed with phosphatidylcholine in the presence of octylglucoside. The liposomes formed by dialysis of this mixture against buffer lacking detergent were separated from the unincorporated material by centrifugation through a sucrose density gradient. Approximately half of the membrane-derived betaglycan associated with the liposomes and was found at the top of the gradient (Fig. 6 B), consistent with the presence of a membrane anchor in this form of betaglycan. Furthermore, all of the membrane betaglycan remained near the bottom of the gradient if liposomes were disrupted with Triton X-100 before centrifugation (Fig. 6 B). In contrast to the ability of membrane betaglycan to intercalate into liposomes, affinity-labeled soluble betaglycan did not associate with liposomes and remained in the bottom of the sucrose gradients (Fig. 6 B). Disruption of liposomes with detergent before centrifugation did not alter the position of soluble betaglycan in the gradients. The failure of a portion of the membrane-derived betaglycan to bind to liposomes (Fig. 6 B) might be due to inefficiency of the technique, proteolysis during the liposome reconstitution process, or contamination of the cell samples with soluble material.

Interestingly, the membrane betaglycan core products obtained by enzyme digestion of the liposome-containing fractions from sucrose density gradients included the lowest mo-



**Figure 6.** Liposome intercalation of betaglycans. Phosphatidylcholine liposomes were formed in the presence of affinity-labeled betaglycans from 3T3-L1 cells or 3T3-L1 conditioned medium. Each liposome suspension was divided in two aliquots, one of which received 1% Triton X-100 to dissolve the liposomes. Each sample was loaded in the bottom of a sucrose gradient. After centrifugation, the gradients were fractionated from top (*Fraction 1*) to bottom. The macromolecular material from these fractions and the pellets from each gradient were displayed by electrophoresis and autoradiography. One-third of each fraction from the sample corresponding to cell material without detergent was devoted to an experiment similar to the one described in Fig. 7. *B* shows the relevant portion of the resulting autoradiograms. *A* shows the distribution of the liposome marker, [<sup>3</sup>H] dipalmitoyl phosphatidylcholine, as determined by counting small aliquots from the top set of gradient fractions shown in *B*.

lecular mass (120-kD) labeled core species (Fig. 7). Since the apparent molecular weight of this core component was not significantly larger than the molecular weight of the core products derived from soluble betaglycan (see Fig. 2), these results suggest that the betaglycan membrane anchor and, if present, the cytoplasmic domain have a relatively small mass.

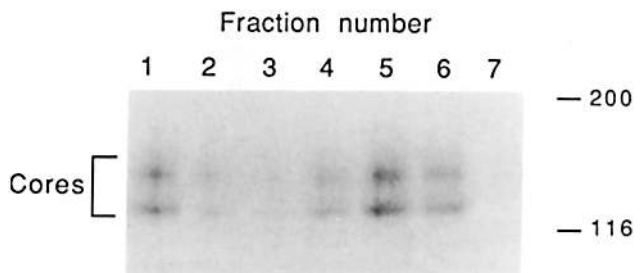
In a complementary set of experiments, the hydrophobic character of soluble and membrane-derived betaglycans was

determined by subjecting them to hydrophobic chromatography on octyl-Sepharose and elution with a gradient of octylglucoside. Although all betaglycan forms bound to octyl-Sepharose, the interaction of soluble betaglycan with octyl-Sepharose was weak. This form of betaglycan began to elute at a concentration of octylglucoside (~10 mM) well below the critical micelle concentration (23 mM) of this detergent (Fig. 8). Electrophoretic analysis of the peak fractions showed that the split appearance of the soluble betaglycan peak in Fig. 8 was due to elution of nonspecifically labeled high molecular weight material (see Fig. 1) with the late fractions of the betaglycan peak. Membrane-derived betaglycan had stronger affinity for octyl-Sepharose, and eluted with ~35 mM octylglucoside. Taken together, these results provide strong evidence for the presence of a hydrophobic membrane anchor in membrane betaglycan that is absent in the soluble betaglycan.

### Discussion

The finding of soluble forms of betaglycan (formerly, the type III TGF- $\beta$  receptor) coexisting with the membrane-bound forms provides new insight into the structural properties and potential biological role of these TGF- $\beta$ -binding proteoglycans. The soluble proteoglycans identified in this report have the general structural and functional properties that characterize membrane-bound betaglycan except that the soluble forms lack a membrane anchor. Thus, betaglycans are defined as membrane bound or soluble proteoglycans with a core protein of 100–120 kD that contains a high affinity binding site for TGF- $\beta$ , and carries ~10 kD of N-linked glycan chains and between 100 and 300 kD of heparan sulfate and/or chondroitin sulfate glycosaminoglycan chains, as determined by SDS-PAGE.

The membrane and soluble forms of betaglycan differ in their hydrophobic properties and their ability to associate with liposomes. Previous studies have shown that anchorage of betaglycan to the cell surface is via the core protein (4, 7, 30). The absence of a hydrophobic membrane anchor in soluble betaglycan does not increase the mobility of the core protein on electrophoresis gels when compared to the membrane betaglycan core protein; the apparent molecular weight of the secreted betaglycan core is not smaller than the small-



**Figure 7.** Core proteins from liposome-associated betaglycan. After liposome intercalation and fractionation on a sucrose density gradient as described in Fig. 6, affinity-labeled cell surface betaglycan from 3T3-L1 cells was treated with chondroitinase ABC plus heparitinase, and subjected to electrophoresis and autoradiography to display the core proteins of the betaglycan in each fraction of the gradient.

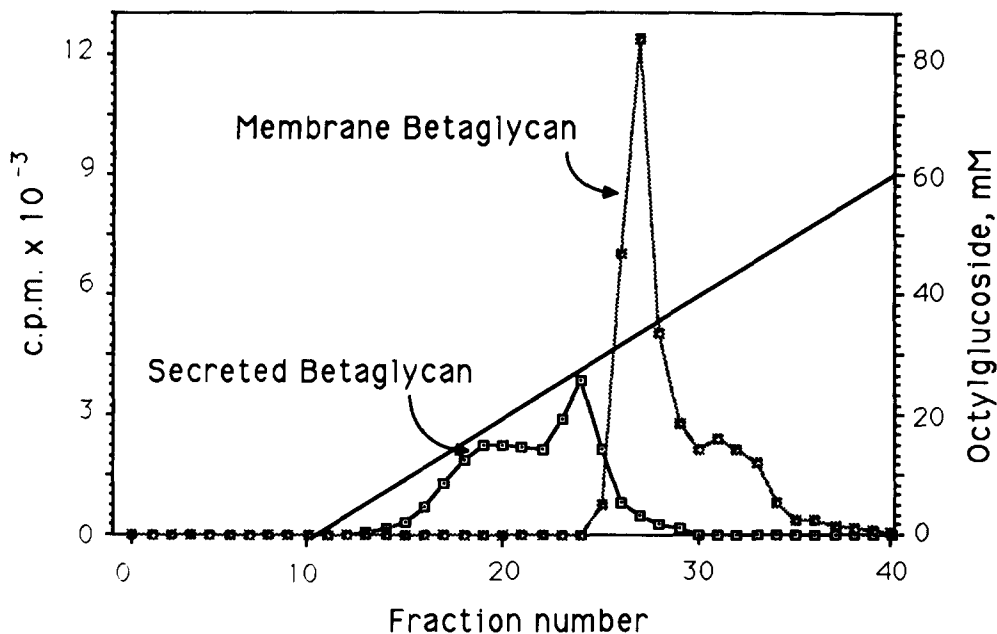


Figure 8. Octyl-Sepharose chromatography of membrane-derived and soluble betaglycans. DEAE-Sepharose fractions containing affinity-labeled betaglycans from 3T3-L1 cells and from conditioned medium were chromatographed over an octyl-Sepharose column eluted with a linear gradient of octylglucoside. The <sup>125</sup>I radioactivity in the resulting fractions was then determined.

est membrane betaglycan core that associates with liposomes. This result suggests that the betaglycan membrane anchor and, if present, the cytoplasmic domain might be small. We have been unable to release membrane-bound betaglycan by hydrolysis with the phosphoinositol-specific phospholipase C (21) that cleaves glycosyl-phosphatidylinositol anchors present in certain membrane proteins (data not shown). Attempts to release membrane betaglycan included treatment of cells with this phospholipase either before or after affinity-labeling cells with TGF- $\beta$ , with or without previous enzymatic removal of glycosaminoglycans, or followed by rinsing cells with a 1 M urea solution. These results suggest the presence of a polypeptide membrane anchor in betaglycan, and are consistent with the domain structure model proposed on the basis of tryptic fragmentation analysis of membrane betaglycan (7).

Additional differences between the membrane and soluble forms of betaglycan from 3T3-L1 cells are suggested by their pattern of migration on electrophoresis gels. Membrane betaglycan migrates on electrophoresis gels as a broad band of 250–350 kD whereas soluble betaglycan migrates as two broad bands between the 200-kD mark and the top of the gels. These differences may be attributable to differences in the number or type of glycosaminoglycan chains attached to each proteoglycan. The core proteins from membrane and soluble betaglycans also show differences: membrane betaglycan core proteins resolve as three distinct species of 100, 110, and 120 kD (after subtracting the mass of N-linked glycans and cross-linked TGF- $\beta$  monomer) whereas soluble betaglycan core protein(s) migrate as a broad 100–110-kD band.

Do cells constitutively release a portion of the membrane betaglycan that they synthesize? Soluble betaglycan is produced by several types of fibroblasts, lung epithelial cells, and adipocytes derived from five different mammalian species; it is produced by proliferating cells as well as quiescent, terminally differentiated adipocytes; and, the level of betaglycan in the medium of all cell lines examined is propor-

tional to the level of membrane-bound betaglycan. Thus, the available evidence is, in part, compatible with the hypothesis that soluble betaglycan might be constitutively released by a hydrolytic event that cleaves the hydrophobic anchor of membrane betaglycan. However, the differences in electrophoretic mobility observed between membrane betaglycan and soluble betaglycan tend to support the alternative hypothesis that the various forms of betaglycan are synthesized or post-translationally modified as separate products. Precedent for this possibility exists. For example, alternative mRNA splicing events lead to the coexpression of membrane-bound and secreted forms of the intercellular recognition molecules, neutral adhesion molecular N-CAM (12), major histocompatibility complex Qa-2 antigen (33), and lymphocyte cell surface molecule CD8 (10). Clearly, more work is needed to determine the origin of soluble betaglycan and the potential regulation of its expression.

Betaglycan in serum added to cell cultures represents only a small fraction (1–6%, Table I) of the total soluble betaglycan in the 3T3-L1, Rat-1, and CCL-39 conditioned media. The results of control experiments described above clearly indicate that most of the betaglycan in conditioned media is produced by the cells in culture. On the other hand, the finding of soluble betaglycan in fetal serum is significant because it indicates that release of betaglycan is not an event restricted to cells in culture. It will be of interest to determine if betaglycan is present in circulating plasma, whether its presence in serum is due to events that occur during the blood clotting process, or whether its role in circulation is related to that of  $\alpha$ 2-macroglobulin which can also bind TGF- $\beta$  (34).

The ability of betaglycan to bind TGF- $\beta$ , its proteoglycan structure, and its polymorphic nature suggests that betaglycan might be a multifunctional molecule. Its potential roles include mediation of TGF- $\beta$  action, mediation of cell adhesion, and a role as a pericellular TGF- $\beta$  chelator. With regards to the former possibility, genetic evidence (2) and pharmacological evidence (27) indicate that the type I TGF- $\beta$

receptor is directly involved in mediating cellular responses to TGF- $\beta$ , and that it can act in the absence of betaglycan expression. This evidence, the structural features of betaglycan revealed by the present studies, and release of betaglycan into the medium do not support the initial proposal (6, 24) of a direct role for this molecule in transmembrane signaling. More attractive is the suggested possibility that membrane betaglycan might participate in cell-to-cell or cell-to-extracellular matrix adhesion (7). This potential role of betaglycan has a precedent in other well characterized membrane proteoglycans, including syndecan from mammary epithelial cells (19) and the CD44/Hermes lymphocyte homing receptor (11, 32). Interestingly, syndecan is also found in soluble and membrane-bound forms (35).

Betglycans might be involved in TGF- $\beta$  action by intervening in ligand presentation to the other TGF- $\beta$  receptor types, or by serving as pericellular reservoirs of bioactive TGF- $\beta$  (2). A role for betaglycan as a pericellular reservoir of TGF- $\beta$  becomes particularly plausible with our finding of soluble betaglycan in the medium and the extracellular matrix of cultured fibroblasts. Interstitial collagens, fibronectin, and other extracellular matrix components have glycosaminoglycan-binding domains that allow the assembly of soluble proteoglycans into extracellular matrices (14). Similar interactions might occur with soluble betaglycan. Since the glycosaminoglycan chains are not required for expression, membrane anchorage or TGF- $\beta$ -binding activity of betaglycan (4), they may be available for interaction with extracellular matrix components. Only low levels of betaglycan, however, are detectable in the extracellular matrix of 3T3-L1 cells. This could be due to competition between betaglycan and the abundant 3T3-L1 secreted proteoglycans, biglycan (PG I), and decorin (PG II) (1, 9, 19, 20), for binding to a relatively poorly structured extracellular matrix. In vivo, the accumulation of soluble betaglycan in extracellular matrices might be quantitatively more important than in vitro. It is worth noting in this regard that TGF- $\beta$  is present in abundance in tissues that have a high content of extracellular matrix, such as bone (31). Immunohistochemical evidence indicates that TGF- $\beta$ , which itself is a major regulator of extracellular matrix deposition (17, 28) and cell adhesion (18), indeed accumulates in extracellular matrices of bone and other tissues (3, 15, 31). It will be of interest to determine how betaglycan as a component of extracellular matrices may participate in such important functions as localized retention, delivery, receptor presentation, and clearance of activated TGF- $\beta$ .

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