A 60-kD Protein Mediates the Binding of Transforming Growth Factor-β to Cell Surface and Extracellular Matrix Proteoglycans

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Abstract. The biological activity of many cytokines is regulated by binding proteins present at the cell surface, in extracellular matrices or in soluble phase. We describe here a TGF-β binding protein that is both an extracellular matrix and a cell surface protein. When intact extracellular matrices of HEP-G2 cells were affinity cross-linked with ^{125}I-TGF–β1, two major binding components were seen: a 250-kD, proteoglycan-like molecule, presumed to be betaglycan, and a 60-kD protein. The 60-kD TGF-β–binding protein was also present at the cell surface. It could be released from the cell surface by treating cells with high salt, heparin, chondroitin sulfate, heparitinase, or chondroitinase, indicating that it is bound to heparan sulfate and chondroitin sulfate proteoglycans. The 60-kD protein bound TGF-β1 with an apparent dissociation constant of 1.6 nM, and there were 30,000 binding sites per cell at the cell surface. In addition to the HEP-G2 cells and another hepatoma cell line, the 60-kD protein was also found in a human colon carcinoma (HT-29) cell line but not in rat kidney (NRK-49F) or human fibroblast (HUT-12) cell lines. The 60-kD protein could be extracted from cells containing it and transferred to the surface of previously negative cells. The 60-kD protein may serve to regulate the binding of TGF-β to its signal transducing receptors by targeting TGF-β to appropriate locations in the microenvironment of cells.

TGF-β's play an important role in the regulation of cell proliferation and in tissue repair (for reviews see Roberts and Sporn, 1990; Massagué, 1990). The TGF-β family consists of three known isoforms, TGF-β1, 2, and 3, that are structurally and functionally closely related to one another.

TGF-β is a potent growth suppressor for epithelial, myeloid, and lymphoid cells. This together with the observation that many transformed cells are resistant to the growth suppressing effects of TGF-β suggests that TGF-β functions as a general homeostatic factor suppressing undesired cell proliferation. Consequently, abnormalities in TGF-β function have been implicated in cellular transformation and malignancy (Masui et al., 1986; Shipley et al., 1986; Knabbe et al., 1987) and in a lymphoproliferative disorder seen in mice lacking a functioning TGF-β1 gene (Shull et al., 1992; Kulkarni et al., 1993). A prominent feature among TGF-β activities is the ability of this growth factor to enhance the deposition of extracellular matrix and stabilize such matrices (Sporn et al., 1983; Roberts et al., 1986). This is accomplished by increased production of extracellular matrix pro-

teins (Balza et al., 1988; Ignotz and Massagué, 1986; Bassols and Massagué, 1988; Pearson et al., 1988) and their cellular receptors (Ignotz and Massagué, 1987) as well as decreased proteolysis (Edwards et al., 1987; Laiho et al., 1987). These activities of TGF-β are likely to be important in tissue repair.

TGF-β induces its own production in many cells (van Obberghen-Schilling et al., 1988; Kim et al., 1989), and it has been hypothesized that sometimes this self-amplifying cascade would lead to excessive and destructive fibrous tissue formation (Border and Ruoslahti, 1992). The fact that TGF-β is the driving force in many fibrotic disorders is suggested by elevated TGF-β expression at the site of excessive matrix formation and by the ability of neutralizing anti-TGF-β antibodies to hinder the development and progression of such disorders (Border and Ruoslahti, 1992). Considering the potency of TGF-β in eliciting both beneficial and potentially harmful responses, an effective regulatory system balancing TGF-β activity would seem to be a necessity.

One of the regulators of TGF-β activity is the binding of TGF-β to cell surface components other than signal transducing receptors and to extracellular matrices (Ruoslahti and Yamaguchi, 1991). A cell surface proteoglycan, betaglycan (also known as TGF-β receptor type III), binds TGF-β at the cell surface (Massagué, 1992) apparently enhancing the binding of TGF-β to one of the signal transducing receptors (Wang et al., 1991). A number of extracellular matrix

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(ECM)[1] components have also been shown to bind TGF-β. These include fibronectin (Fava and McClure, 1987), thrombospondin (Murphy-Ullrich et al., 1992), collagen type IV (Paralkar et al., 1991) and the core proteins of small interstitial proteoglycans of the decorin family (Yamaguchi et al., 1990). The binding of TGF-β to the decorin-type proteoglycans neutralizes the activity of the growth factor (Yamaguchi et al., 1990; Border et al., 1992). While betaglycan and the decorin-type proteoglycans bind TGF-β through the core protein, TGF-β can also bind to glycosaminoglycan chains of proteoglycans (McCaffrey et al., 1992). Immunohistochemical stainings have shown that, indeed, TGF-β can be found in the extracellular matrix in vivo (Flanders et al., 1989; Heine et al., 1990; Silberstein et al., 1992), but the binding proteins involved in this localization are not known.

In addition to regulating the receptor binding of TGF-β, cell surface and ECM binding proteins influence TGF-β activity by sequestering the growth factor at the location of the binding protein. We have identified a 60-kD protein that in addition to binding TGF-β can bind to heparin/heparan sulfate and chondroitin sulfate proteoglycans and hence may target TGF-β to cell surfaces and ECMs.

Materials and Methods

Materials

Hep-G2, Hep-3B, HT-29, and NRK-49F cells were obtained from American Type Culture Collection (HB 8065, HB 8064, HTB 38, CRL 1570, respectively). HUT-12 cells were kindly provided by Dr. J. Leavitt (Leavitt et al., 1987). FCS was purchased from Tissue Culture Biologicals (Tulare, CA) and L-glutamine and antibiotics from Irvine Scientific (Santa Ana, CA). DME was from GIBCO BRL (Gaithersburg, MD), carrier-free Na^{125}I from Dupont-NEN (Burbank, CA), IODO-GEN and disuccinimidyl suberate from Pierce (Rockford, IL). SDS-PAGE precast gels were from Novex (San Diego, CA). Molecular weight standards came from GIBCO BRL and Centricon micro-concentrator from Amicon (Danvers, MA). V$_8$ protease (EC 3.4.21.19, Staphylococcus aureus endoproteinase Glu-C) was from Boehringer Mannheim Corp. (Indianapolis, IN). Vydac 218TP54 column (4.6 × 250 mm) was from Vydac Separations Group (Hesperia, CA). All other chromatographic materials including pre-packed PD-10 columns were from Pharmacia (Uppsala, Sweden). Heparitinase I and chondroitinase ABC were from Seikagaku (Tokyo, Japan). Other chemicals came from Sigma Immunochemicals (St. Louis, MO).

Cell Cultures

Cells were grown at 37°C in a humidified 10% CO_2 in air atmosphere in DME containing 10% FCS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Confluent cultures were detached by treatment for 5 min with PBS containing 0.2 mg of EDTA and 0.5 mg of trypsin per ml and replated at least 18 h before the start of each experiment.

TGF-β1

TGF-β1 was expressed in CHO cells and purified as described earlier (Gentry et al., 1987). Before use, the purity of each stock was verified by reversed-phase HPLC on a Vydac 218TP54 column. The sample was dissolved in 0.06% trifluoroacetic acid (TFA) and injected onto the column equilibrated with 0.06% TFA. A linear gradient of acetonitrile (0–60%) containing 0.06% TFA was used to elute the protein. Iodination of TGF-β1 was done using Iodo-Gen according to the manufacturer's instructions. The specific activity of the label ranged from 80 to 150 μCi/μg.

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1. Abbreviations used in this paper: ECM, extracellular matrix; TFA, trifluoroacetic acid.

Ligand Binding and Affinity Labeling

Confluent monolayers of cells were used for affinity labeling experiments. The conditions used were essentially as described earlier (Massagué, 1987). The following buffers were used: 25 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM MgSO$_4$, 5 mM KCl, 1 mM CaCl$_2$, 2 mg/ml BSA ("binding buffer"); 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 ("solubilization buffer"); 10 μg/ml antipain, 10 μg/ml leupeptin, 2 mM benzamidine, 1 mM EDTA ("protease inhibitor cocktail," final concentrations). A fresh stock of disuccinimidyl suberate (DSS) (10 mg/ml in DMSO) was prepared for each experiment and a 1:200 dilution in binding buffer without BSA was used in cross-linking for 15 min on ice. The reaction was quenched by 10 mM Tris-HCl, pH 7.4, 150 mM NaCl and the samples were treated as indicated.

Electrophoresis, Autoradiography, and Densitometric Scanning

SDS-PAGE was performed according to Laemmli (1970). For autoradiography the gels were fixed after electrophoresis in 10% isopropanol, 10% acetic acid for 15 min and dried. Kodak X-O-MAT/AR film (Eastman Kodak Co., Rochester, NY) with a Dupont Cronex enhancing screen was used for autoradiography. Densitometric scans of the autoradiograms were performed on a laser densitometer (Ultroscan XL, LKB 222-020).

Peptide Maps

To obtain peptide maps of the 60-kD TGFβ binding protein and the type I TGFβ receptor (TGF-β1RI), a large petri dish of HEP-G2 cells was affinity labeled with 400 pM of ^{125}I-TGF-β1. The 60-kD TGF-β1 binding protein was released by eluting the culture with 1 M NaCl in binding buffer containing 200 μg/ml β-lactoglobulin as a carrier. The proteins in the eluate were precipitated with 10% TCA, acetone washed and solubilized to 8 M urea, TBS, pH 7.4. To obtain TGF-β1RI after the NaCl extraction, the cells were washed three times with ice cold binding buffer without BSA, scraped off and extracted with solubilization buffer. The NaCl and Triton X-100 extracts were separated on a 4–12% SDS-PAGE gel under reducing conditions. Proteins labeled with ^{125}I-TGF-β1 were electroeluted from gel segments with Centrilutor (Amicon) using Centricon 30-kD cutoff microconcentrators and 50% Laemmli running buffer containing 0.025% SDS. The proteins were precipitated with 10% TCA and V$_8$ protease digestion was done as described (Stone et al., 1990). After digestion the samples were analyzed on a 18% gel under reducing conditions, fixed, dried, and autoradiographed.

Affinity Measurements

Various concentrations of ^{125}I-TGF-β1 alone (from 10 to 300 pM) or a fixed concentration of ^{125}I-TGF-β1 (300 pM) and various concentrations of unlabeled TGF-β1 (total TGF-β1 concentration from 1 nM to 100 nM) was added to HEP-3B cultures on 24-well dishes. After a 3-h incubation, washes, and cross-linking, the cultures were extracted with solubilization buffer supplemented with protease inhibitor cocktail, and the soluble material was analyzed by SDS-PAGE on a 4–20% gel under nonreducing conditions. The autoradiograph of the fixed and dried gel was scanned along each lane. Based on the known quantity of total counts loaded per lane and the relative intensity of individual bands in autoradiogram scans, the amount of bound and free ligand at various TGF-β1 concentrations was determined. Cross-linking efficiency was 20% and the apparent amount of bound ligand was multiplied by a factor of five to take this fact into account. The cross-linking efficiency was assessed by determining the percentage of TGF-β that migrated as a dimer in SDS-PAGE under reducing conditions. The saturation curve and Scatchard analysis were done with the LIGAND-program (National Institutes of Health) using the data points obtained with TGF-β1 concentrations from 300 pM to 100 nM.

Treatment of Cells with Glycosaminoglycan-degrading Enzymes

Hep-3B cells were washed three times with Ham F12/DME (vol/vol 1:1, 37°C) medium containing 0.2% BSA. Heparitinase I and chondroitinase ABC were added to the cultures in the above buffer at a concentration of 0.02 U/ml and incubated for 90 min at 37°C. The cells were washed three times with binding buffer before affinity labeling.
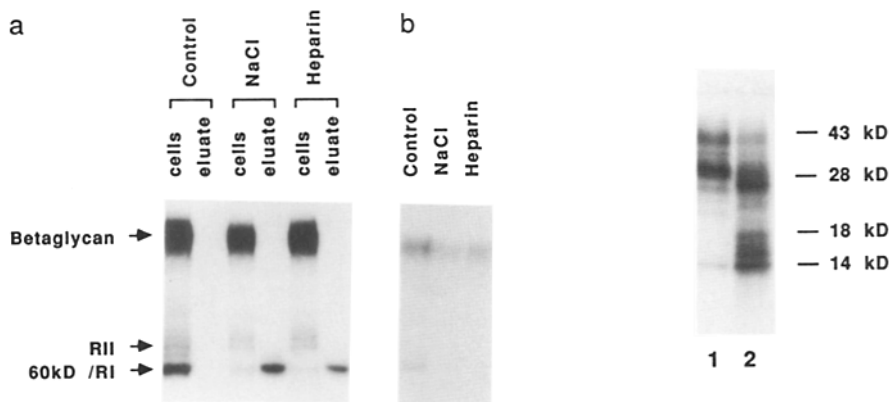
Figure 1. Identification of 60-kD TGF-β1 binding protein. HEP-G2 cells on 6-well dishes were affinity-labeled using 200 pM of ^{125}I-TGF-β1. The cultures were incubated with 0.5 ml buffer (binding buffer with 200 μg/ml β-lactoglobulin as a carrier) with or without 1 M NaCl or 10 μg/ml heparin for 15 min. The proteins in the eluates were TCA-precipitated and washed with acetone. The salt-extracted cultures were then further extracted with buffer containing 1% Triton X-100, and the eluates and the Triton extracts were analyzed by SDS-PAGE followed by autoradiography (a). The Triton X-100-insoluble material containing ECM proteins was solubilized with 8 M urea and 5 × SDS-PAGE sample buffer and analyzed as above (b).

Results

Identification of the 60-kD Binding Protein as a Peripheral Membrane and ECM Protein

Affinity labeling of cultured HEP-G2 cells with ^{125}I-TGF-β revealed three apparent cell surface-binding proteins with relative sizes of 72, 95, and 280 kD in a Triton X-100 extract of the cell layer (Fig 1 a). The sizes of these components correspond to type I and II receptors of TGF-β and betaglycan, respectively. A surprising finding was that much of what appeared to be the TGF-β–labeled 72-kD complex, presumably corresponding to the type I receptor, could be released from the cells and the matrix by treating the cultures with high salt and heparin after the affinity labeling. These results suggested that the 72-kD band may consist of two components: type I receptor (TGF-β1RI), which remains bound to the cell layer upon the heparin and salt treatments, and another TGF-β binding protein that is released by these treatments. Subtracting the molecular weight of the TGF-β subunit from the 72-kD size of the band indicates that the salt-released protein has a molecular mass of 60 kD. The Triton-insoluble material, consisting mainly of ECM, was
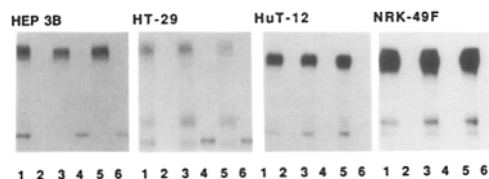

Figure 2. Presence of 60-kD TGF-β1 binding protein in some but not all cell lines. HUT-12, NRK-49F, HEP-3B, and HT-29 cells on 6-well dishes were treated as described above. Buffer control, lanes 1 and 2; 1 M NaCl treatment, lanes 3 and 4; heparin treatment, lanes 5 and 6. Cell extracts, lanes 1, 3, and 5; eluates, lanes 2, 4, and 6.

Figure 3. Peptide profiles of 60-kD TGF-β1 binding protein and TGF-β1RI. HEP-G2 cells were affinity-labeled with 500 pM of ^{125}I-TGF-β1. The 60-kD TGF-β1 binding protein was eluted with 1 M NaCl and the cells were solubilized. The samples were separated on SDS-PAGE under reducing conditions; the 60-kD protein and TGF-β1RI were electroeluted from the gel, alkylated, digested with V_8 protease, and the resulting fragments were separated on SDS-PAGE with an 18% gel. Lane 1, 60-kD protein; lane 2, TGF-β1RI.

solubilized with urea/SDS and also analyzed. The results show that the 72-kD complex and a proteoglycan slightly smaller than betaglycan were present in this material (Fig. 1 b). The proteoglycan component could either be a truncated form of betaglycan or another TGF-β–binding proteoglycan, biglycan, which in SDS-PAGE migrates above 200 kD (Fisher et al., 1987). Analysis of other cell lines showed that the 72-kD complex was also present in heparin and salt extracts of affinity-labeled HEP-3B and HT-29 human colon carcinoma cells, but the HUT-12 human fibroblasts and NRK-49F rat kidney cells, judging from the absence of salt-extractable 72-kD complex, did not have detectable amounts of 60-kD protein (Fig. 2).

TGF-β1RI and 60-kD Binding Protein Are Distinct Proteins

Affinity labeled 60-kD binding protein and TGF-β1RI were analyzed by proteolytic cleavage to examine whether they are structurally related. While two fragments from both proteins appeared to migrate similarly, both proteins also yielded several fragments that were not present in the digest of the other protein (Fig. 3), indicating that the 60-kD protein and TGF-β1RI are two different proteins.

Release of 60-kD TGF-β1 Binding Protein from the Cell Surface at Various NaCl Concentrations

When a stepwise gradient of NaCl was used to elute ^{125}I-TGF-β1/60-kD binding protein complex from HEP-3B cultures after affinity labeling, little dissociation was observed with NaCl concentrations up to 0.20 M (Fig. 4). Half-maximal and maximal elutions were obtained by NaCl concentrations of 0.33 and 0.50 M, respectively. There was an inverse relationship between the amount of ^{125}I-TGF-β1/60-kD protein in the NaCl-eluate and the amount detected in the Triton X-100 extracts of the NaCl-treated cell layer. A 72-kD band was also observed in the Triton X-100 residue solubilized with urea/SDS (not shown). In cultures not treated with NaCl before the Triton extraction, the relative intensity of the 72-kD band present in the urea/SDS fraction representing extracellular matrix proteins was about half of that found in the Triton extract, indicating that a significant amount of the 72-kD complex was bound to the matrix.

Affinity of the 60-kD Binding Protein for ^{125}I-TGF-β1

To determine the affinity of the 60-kD protein for TGF-β1, HEP-3B cells were incubated in the presence of various concentrations of TGF-β1, and the quantity of free TGF-β1 and
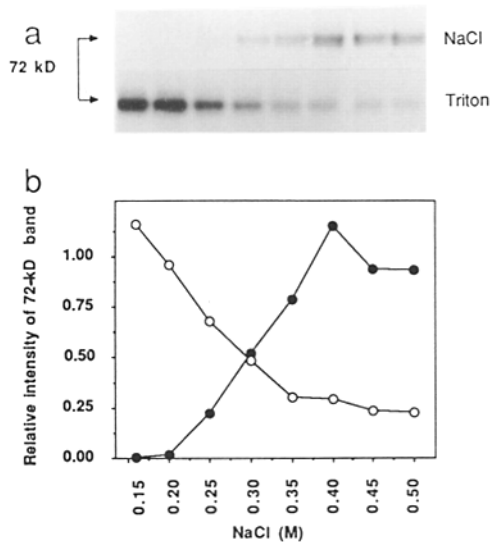
Figure 4. Elution of 60-kD TGF-β1 binding protein from HEP-3B cells by increasing concentrations of NaCl. HEP-3B cells on 6-well dishes were affinity labeled with 500 pM of ¹²⁵I-TGF-β1. The cell layers were eluted with increasing concentrations of NaCl, and the eluates and cells were processed as described in the legend to Fig. 1. Aliquots from NaCl and Triton extracts were separated by 4–12% SDS-PAGE under reducing conditions (*a*). The autoradiograms of the fixed and dried gels were scanned to quantitate the amount of the labeled 60-kD protein (*b*). The exposure time of the autoradiograms was 24 h for the NaCl eluates and 3 d for the Triton X-100 extracts. In the graph, each absorbance from the NaCl eluates was multiplied by three to adjust for the difference in exposure times. (●) NaCl; (○) Triton.

that bound to the 60-kD component was determined. In Scatchard analysis of the data the six highest data points were used to minimize the interference of the binding of TGF-β to the small amount of TGF-β1RI that comigrated with the TGF-β labeled 60-kD protein. As shown in Fig. 5, the binding of TGF-β1 to the 60-kD protein in these cells was saturable, and Scatchard analysis gave a rectilinear plot indicating a single class of binding sites with a K_d of 1.6 nM. The number of binding sites was 30,000 per cell.

The 60-kD Protein Binds to Glycosaminoglycans

The release of the 60-kD protein from the cell layers by high salt treatments suggested that the 60-kD protein may bind to glycosaminoglycan moieties on the cell surface and in the ECM. To study this question we treated HEP-3B cells with heparitinase or chondroitinase to eliminate potential binding sites for the 60-kD protein or with heparin and chondroitin sulfate as competitors for those sites. Subsequent affinity labeling with TGF-β showed that each of these treatments reduced the labeling of the 60-kD component (Fig. 6). Heparitinase was more effective than chondroitinase and heparin was more effective than chondroitin sulfate in reducing the amount of 60-kD protein detected on the cell surface.

Origin of 60-kD TGF-β Binding Protein

To determine whether the 60-kD protein originated from the cells or the serum present in the culture medium, HEP-3B cell cultures were incubated in the presence or absence of cycloheximide and/or 10% FCS. Addition of serum into the
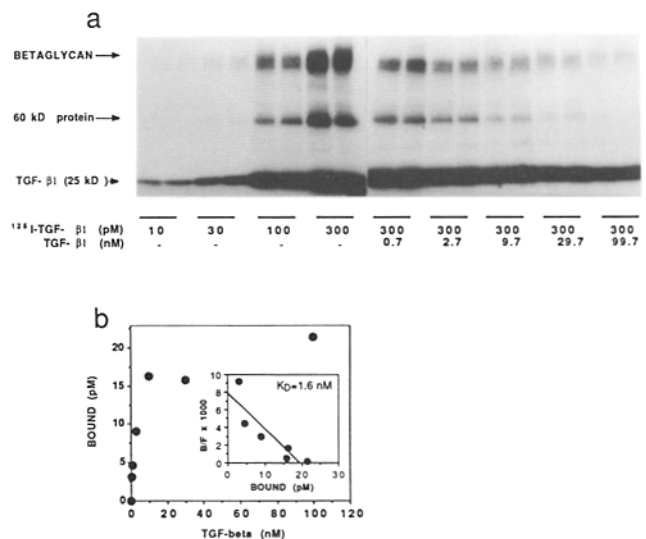

Figure 5. Affinity of 60-kD protein for TGF-β1. HEP-3B cells in duplicate wells of 24-well dishes were incubated for 3 h at 4°C with various concentrations of ¹²⁵I-TGF-β1 (from 10 to 300 pM) or with a fixed concentration of ¹²⁵I-TGF-β1 (300 pM) and various concentrations of unlabeled TGF-β1 (total TGF-β1 from 1 to 100 nM). After cross-linking, the cells were solubilized and extracts were analyzed by 4–20% SDS-PAGE under nonreducing conditions (*a*). The gel bands were quantitated by scanning and the results plotted in *b*. The inset shows a Scatchard plot generated from the results.

cultures decreased the 72-kD labeling to ∼50% relative to the serum-free control (Fig. 7). Cycloheximide caused greatly diminished labeling of the 72-kD band both in serum-free and serum-containing cultures. Taken together these results strongly suggest that the 60-kD binding protein does not originate from the serum but is synthesized by the cells and has a relatively short half-life on the cell surface.

Transfer of the 60-kD Protein from Producer to Nonproducer Cells

We next examined whether it would be possible to extract the 60-kD binding component from 60-kD positive cells (HEP-3B) cells and transfer it to 60-kD negative (HUT-12) cells.
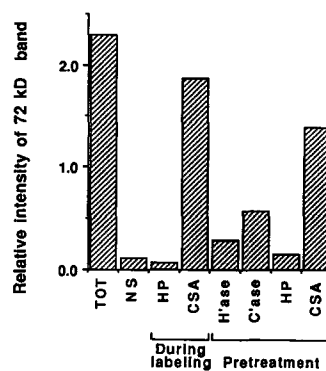

Figure 6. Binding of 60-kD protein to glycosaminoglycans on Hep-3B cells. Hep-3B cells were digested with heparitinase (*H'ase*) or chondroitinase ABC (*C'ase*) as described in Materials and Methods or pretreated with heparin (*HP*; 100 μg/ml) or chondroitin sulfate A (*CSA*; 100 μg/ml) and washed three times with the binding buffer before affinity labeling of the cells with 200 pM of ¹²⁵I-TGF-β1. Affinity labeling was done also in the presence of 100 μg/ml of heparin or chondroitin sulfate A. The relative intensity of ¹²⁵I-TGF-β1-labeled 60-kD protein was quantified by scanning after SDS-PAGE and autoradiography.
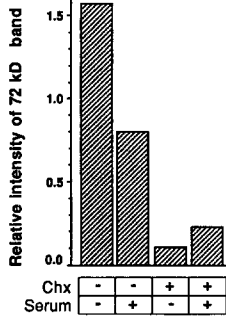
Figure 7. Effect of serum and cycloheximide on 60-kD protein labeling at the cell surface. HEP-3B cells on 6-well dishes were incubated for 18 h in the presence or absence of 10% FCS and/or 20 mM cycloheximide before affinity labeling with 500 pM of ^{125}I-TGF-β-1. The cells were detached, solubilized and analyzed by SDS-PAGE on a 4–20% gel under reducing conditions. The relative intensity of the 72-kD band representing the 60-kD protein-TGF-β complex was quantitated by scanning (these cells have almost no detectable TGF-β1RI; see Fig. 2). The bars represent mean of duplicate experiments.

Treatment of HEP-3B cultures with salt produced an extract that when incubated with HUT-12 cells rendered these cells positive for the 72-kD, heparin-elutable component (Fig. 8). An eluate from the HUT-12 cells themselves, when incubated with HUT-12 cells, did not result in the labeling of a 72-kD heparin-elutable band. An additional cross-linked complex of 30 kD was present both in Hep-3B and HUT-12 eluates; the identity of this component is not known.

Discussion

We show here that one of the cell surface proteins that affinity cross-link to radioactive TGF-β is a 60-kD peripheral membrane protein, and that this protein is bound to cell surfaces and ECMs through an interaction with the glycosaminoglycan component of proteoglycans. Since the 60-kD protein has a high affinity for both TGF-β and glycosaminoglycans, its likely function is to modulate the availability of TGF-β to cells by binding TGF-β to cell surfaces and to the ECM.
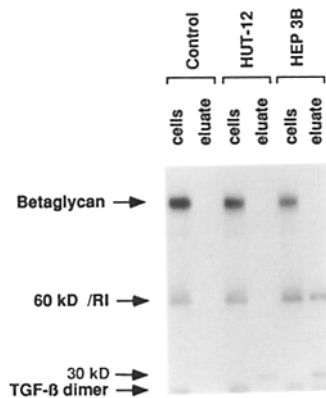

Figure 8. Transfer of 60-kD TGF-β1 binding protein from cells producing it to non-producer cells. High salt extracts were prepared from confluent monolayer cultures of HEP-3B (60-kD protein-positive) and HUT-12 cells (60-kD protein-negative) grown on 140-mm petri dishes by incubating the cell layers with 0.5 M NaCl in DMF containing 1% FCS for 10 min. The extracts were diluted with 25 mM Hepes, pH 7.4, containing 10% FCS to give physiological salt concentration and added, along with control media, to HUT-12 cells on 6-well dishes for 4 h at 37°C. After three washes, the cultures were affinity-labeled using 300 pM of ^{125}I-TGF-β1. The cultures were eluted with heparin (10 μg/ml), and treated and analyzed by SDS-PAGE as described in the legend of Fig. 1. The cells analyzed in the lanes labeled *HUT-12* received the high salt extract from HUT-12 cells, and the lanes labeled *HEP 3B* received the extract from HEP-3B cells. *Control* indicates the cells treated with media only.

Our 60-kD protein is similar to the TGF-β receptors in that it binds TGF-β specifically and with high affinity, but it has the novel property that it can be released from the cells by treatments that reverse protein-glycosaminoglycan interactions. The specificity of the labeling of the 60-kD protein with TGF-β is evident from the fact that the 60-kD protein is one of the few cell surface proteins that become affinity-labeled with TGF-β. Moreover, like the labeling of the known receptors, the labeling of the 60-kD protein with ^{125}I-TGF-β1 was inhibited by unlabeled TGF-β1. The affinity constant of the 60-kD protein for TGF-β1 was 1.6 nM, which is similar to the affinity measured for the type III receptor, betaglycan (1.9 nM; Andres et al., 1989).

The 60-kD protein migrates as a 72-kD protein after affinity labeling with TGF-β. We have not yet been able to identify the protein without association with radiolabeled TGF-β and the designation of 60 kD as its molecular mass is based on the assumption that one 12-kD subunit of TGF-β remains associated with it when the affinity-labeled products are analyzed by gel electrophoresis under reducing conditions. The designation of our protein as a 60-kD protein must, therefore, be considered tentative.

The TGF-β-labeled 60-kD protein essentially comigrates with the type I TGF-β receptor at 72 kD. However, it can be distinguished from the type I receptor, because the receptor behaves as a transmembrane protein (Massagué, 1985), whereas the 60-kD protein can be released from the cell surface by treatments that do not cleave polypeptide chains. Moreover, the peptide maps of the two proteins were different. The comigration of the 60-kD protein with the type I receptor under the affinity-labeling conditions customarily used in the analysis of TGF-β-binding, cell-surface proteins explains why the 60-kD protein has been generally overlooked.

The 60-kD protein is not likely to be one of the other well-characterized TGF-β binding proteins. The proteins known to bind to TGF-β—fibronectin (Fava and McClure, 1987), thrombospondin (Murphy-Ullrich et al., 1992), type IV collagen (Paralkar et al., 1991), decorin (Yamaguchi et al., 1990), betaglycan (Andres et al., 1989), type II receptor (Lin et al., 1992), α_2-macroglobulin (O'Connor-McCourt and Wakefield, 1987), TGF-β latency peptide (Miller et al., 1992), endoglin (Cheifetz et al., 1992), and the amyloid protein (Bodmer et al., 1990)—each have a size different from that of the 60-kD binding protein described here. However, a protein in a rat pituitary cell line (GH$_3$) that binds TGF-β and activin and has a molecular mass of 64 kD (Cheifetz et al., 1988) is a candidate for being the same protein as our 60-kD protein. The possible relationship of this 64-kD protein, also termed type IV receptor, with the 60-kD protein described here remains to be clarified.

Another protein that may have a relationship to our 60-kD protein is follistatin (Vale et al., 1990), a 35-kD activin-binding protein that inhibits the binding of activin to its receptors. Follistatin is clearly not the same protein as the 60-kD protein, because follistatin does not bind TGF-β (Ling, N., personal communication) and the sizes of the two proteins (35 vs. 60 kD) also differ. It is also unlikely that follistatin and the 60-kD protein would be homologues, because follistatin is rich in cysteine and shows marked changes in mobility in SDS-PAGE upon reduction, whereas the 60-kD protein lacks this feature. However, what follistatin and the

60-kD protein do have in common is that they both bind to cell-surface glycosaminoglycans. Follistatin mediates the binding of activin to cell surface proteoglycans (Nakamura et al., 1991), and we show here that the 60-kD protein can do the same for TGF-β. Thus, there is precedent for glycosaminoglycan-binding proteins mediating ligand–cell surface receptor interactions.

Several lines of evidence in this study show that the TGF-β-complexed 60-kD protein is bound to cells through an interaction with the glycosaminoglycan moieties of proteoglycans. First, the binding of the 60-kD protein–TGF-β1 complex to cell layers could be reversed by treating the cells with high salt or with heparin; both of these treatments disrupt protein–glycosaminoglycan interactions. Since TGF-β can interact directly with heparin (McCaffrey et al., 1992), these results leave open the possibility that the complex would be binding to the cellular glycosaminoglycans through its TGF-β component. However, our results indicate that the 60-kD protein interacts directly with cellular glycosaminoglycans and heparin. Thus, treatment of cell layers with heparin or chondroitin sulfate before the TGF-β affinity labeling reduced the subsequent yield of the 60-kD protein–TGF-β complex, indicating that the glycosaminoglycans had released the 60-kD protein from its binding to cell layer glycosaminoglycans. That the 60-kD protein labeling could also be reduced by treating cell layers with glycosaminoglycan-degrading enzymes points to the same conclusion. These results also indicate that the 60-kD protein can bind to both heparan sulfate and chondroitin sulfate proteoglycans. Moreover, the weaker effect of chondroitin sulfate and chondroitinase suggested that, as is usual for protein–glycosaminoglycan interactions (Ruoslahti, 1988), chondroitin sulfate is a weaker binder of the 60-kD protein than heparan sulfate. Since TGF-β itself binds only weakly to heparin, the main force of the binding of the 60-kD protein–TGF-β complex to glycosaminoglycans is likely to be contributed by the 60-kD protein. However, the complex may bind even more strongly than the 60-kD protein alone.

The glycosaminoglycan binding of the 60-kD protein–TGF-β complex may direct the 60-kD protein and the TGF-β bound to it either to cell surfaces or to the ECM. We found that a substantial portion of the 60-kD protein–TGF-β complex was bound to a detergent insoluble ECM fraction in the cell cultures. The distribution of the complex in the detergent soluble (cell membrane) and insoluble (ECM) fractions was markedly different from that of betaglycan (Andres et al., 1989), of which only a minor fraction was in the ECM compartment. The 60-kD protein may, therefore, differ from the membrane protein betaglycan in that the 60-kD protein directs a higher portion of TGF-β bound to it to the ECM. This difference may be even more marked in tissues, which are likely to contain a more abundant ECM than the tumor cell cultures used in this study.

The physiological significance of the 60-kD protein–TGF-β interaction we demonstrate here is unknown. The 60-kD protein could serve as effectors in a negative feedback loop; increased TGF-β effect would lead to increased matrix formation, including decorin (Bassols and Massagué, 1988; Border and Ruoslahti, 1992) and the increased content of decorin along with the matrix-increased binding of the 60-kD protein would then sequester TGF-β to the matrix. This matrix could act as a buffer for TGF-β activity; it would com-

pete with the receptors but would at the same time constitute a potential reservoir for prolonged TGF-β activity and counteract the extremely short plasma half-life (3–5 min) of mature TGF-β. These questions can now be studied with the 60-kD protein described here.

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