Characterization of a 60-kDa Cell Surface-Associated Transforming Growth Factor-β Binding Protein That Can Interfere With Transforming Growth Factor-β Receptor Binding

ESTER PIEK,1* PETRA FRANZEN,2 CARL-HENRIK HELDIN,2 and PETER TEN DIJKE2

1Department of Cell Biology, University of Nijmegen, ED Nijmegen, The Netherlands
2Ludwig Institute for Cancer Research, Biomedical Center, Uppsala, Sweden

We have characterized a 60-kDa transforming growth factor-β (TGF-β) binding protein that was originally identified on LNCaP adenocarcinoma prostate cells by affinity cross-linking of cell surface proteins by using 125I-TGF-β1. Binding of 125I-TGF-β1 to the 60-kDa protein was competed by an excess of unlabeled TGF-β1 but not by TGF-β2, TGF-β3, activin, or osteogenic protein-1 (OP-1), also termed bone morphogenetic protein-7 (BMP-7). In addition, no binding of 125I-TGF-β2 and 125I-TGF-β3 to the 60-kDa binding protein on LNCaP cells could be demonstrated by using affinity labeling techniques. The 60-kDa TGF-β binding protein showed no immunoreactivity with antibodies against the known type I and type II receptors for members of the TGF-β superfamily. Treatment of LNCaP cells with 0.25 M NaCl, 1 μg/ml heparin, or 10% glycerol caused a release of the 60-kDa protein from the cell surface. In addition, we found that the previously described TGF-β type IV receptor on GH3 cells, which does not form a heteromeric complex with TGF-β receptors, could be released from the cell surface by these same treatments. This suggests that the 60-kDa protein and the similarly sized TGF-β type IV receptor are related proteins. The eluted 60-kDa LNCaP protein was shown to interfere with the binding of TGF-β to the TGF-β receptors. Thus, the cell surface-associated 60-kDa TGF-β binding protein may play a role in regulating TGF-β binding to TGF-β receptors.

Transforming growth factor-β (TGF-β) was described originally as a growth stimulator for normal rat kidney cells in soft agar in the presence of TGF-α (Anzano et al., 1983). However, many other biological activities have been ascribed subsequently to this factor, which is the prototype of a large family of structurally related factors (for reviews, see Massagué, 1990; Roberts and Sporn, 1990). Although the three highly similar mammalian TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, mainly exert overlapping biological activities, like inhibition of cell growth, modulation of the immune response, and deposition of extracellular matrix proteins, some isoform-specific activities have been described. TGF-β1, in contrast to TGF-β3, induces scar formation after wounding (Shah et al., 1995). TGF-β1 and TGF-β3 are more potent than TGF-β2 in inhibiting proliferation of hematopoietic progenitor cells and endothelial cells (Cheifetz et al., 1990). Other factors that belong to the TGF-β superfamily are activins and inhibins, bone morphogenetic proteins (BMPs), and the growth and differentiation factors (GDFs).

TGF-β family members mediate their biological activities by binding to cell surface receptors. The TGF-β type I receptor (TβR-I; 53 kDa) and type II receptor (TβR-II; 75 kDa) are transmembrane serine/threonine kinase receptors that are directly involved in signal transduction (for reviews, see ten Dijke et al., 1994b, 1996; Wrana et al., 1994; Attisano et al., 1996). After binding of TGF-β1 to TβR-II, TβR-I is recruited into the complex and becomes phosphorylated by TβR-II on serine and threonine residues in the GS-box. TβR-I is directly responsible for downstream signal transduction that leads to a phosphorylation and heteromerization of particular Smads, which are shuttled subsequently to the nucleus where, in complex with other proteins, they affect the transcription of specific genes (for review, see Massagué, 1996).

Betaglycan (200–400 kDa), also referred to as TGF-β type III receptor (TβR-III; López-Casillas et al., 1991; Wang et al., 1991), and endoglin (180 kDa; Cheifetz et al., 1988) are structurally related transmembrane...
proteins with very short intracellular tails. They probably function in presenting TGF-β to TpR-II rather than in a direct participation in signal transduction. Whereas TpR-III binds all three TGF-β isoforms with equal affinity, endoglin binds TGF-β2 only weakly, which may explain differences in biological potencies of the three TGF-β isoforms in endothelial cell growth inhibition. In rat pituitary GH3 cells, binding of TGF-β to complexes of 70–74 kDa have been detected and referred to as the TGF-β type IV receptor (TpR-IV; Cheifetz et al., 1988; Yamashita et al., 1995). A TpR-V (400 kDa) receptor has been purified from bovine liver (O’Grady et al., 1991).

In addition, there are many other cell surface or extracellular matrix-associated binding proteins for TGF-β that might influence signal transduction indirectly by presenting ligand to the signaling receptors or by inhibiting receptor binding. TGF-β isoform-specific binding proteins have been identified as well (MacKay et al., 1990; MacKay and Danielpour, 1991). Several glycosyl-phosphatidyl-inositol (GPI)-anchored cell surface proteins have been described that bind TGF-βs in an isoform-specific manner (Cheifetz and Massagué, 1991; Dumont et al., 1995). Bützow et al. (1993) reported the characterization of a 60-kDa TGF-β binding protein that associates with heparan sulfate proteoglycans on the cell surface of HepG2 cells and that can be released by 10 μg/ml heparin or 0.25 M NaCl. Human α2-HS glycoprotein and its bovine counterpart, fetuin, a major component of fetal serum, have also been shown to bind several members of the TGF-β superfamily (Demetriou et al., 1996). Other soluble proteins that bind TGF-β are α2-macroglobulin (O’Connor-McCourt and Wakefield, 1987), collagen type IV (Paralkar et al., 1991), fibronectin (Fava and McClure, 1987), decorin and biglycan (Yamaguchi et al., 1990), and thrombospondin (Murphy-Ullrich et al., 1992).

Loss of TGF-β signaling, either by absence of the signaling receptors or by lack of downstream signaling components, has been implicated in several types of cancer (Filmus and Kerbel, 1993; Schutte et al., 1996). The androgen-dependent prostate adenocarcinoma cell line LNCaP lacks expression of TpR-I due to a genetic alteration in the gene for this receptor (Kim et al., 1996a). Under normal in vitro culture conditions, LNCaP cells are insensitive to TGF-β (Wilding et al., 1989). The growth of the cells can be stimulated or inhibited by androgens like dihydrotestosterone (DHT; Schuurmans et al., 1988; Sonnenschein et al., 1989; Lee et al., 1995). Conflicting reports have been published about the responsiveness of LNCaP cells to TGF-β in the presence of proliferation-modulating factors like DHT (Carruba et al., 1994; Kim et al., 1996b,c), epidermal growth factor (EGF) and TGF-α (Schuurmans et al., 1991; Janssen et al., 1995), or retinoic acid (Fong et al., 1993). In a search for novel receptors for TGF-β, we performed affinity cross-linking of cell surface proteins on different cell types. A 60-kDa TGF-β binding protein distinct from the known serine/threonine kinase receptors was identified on LNCaP cells. When it was associated to the extracellular matrix of LNCaP cells, this 60-kDa protein bound only the TGF-β1 isoform. In addition, the protein interfered with binding of TGF-β to TGF-β receptors on mink lung epithelial Mv1Lu cells. Our data suggest that the presently characterized 60-kDa protein may regulate TGF-β binding to its signaling receptors.

MATERIALS AND METHODS
Cell culture

LNCaP and PC3 human prostate cancer cells, GH3 rat pituitary tumor cells, and Mv1Lu cells (CCL64) were obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin. PC3 cells and Mv1Lu cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin. GH3 cells were grown in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin. The cells were grown in 5% CO2 atmosphere at 37°C.

Antibodies

Antisera against activin receptor-like kinase (ALK)-1, activin receptor (ActR) type I, ActR-IB, BMP receptor (BMPR) type IA, BMPR-IB, TpR-I, TpR-II, and endoglin were raised against synthetic peptides corresponding to the intracellular juxtamembrane parts of the type I receptors, the C-terminal tail of TpR-II, and the intracellular part of endoglin, as described previously (Franzén et al., 1993b; ten Dijke et al., 1994b; Yamashita et al., 1994). A monoclonal antibody against TpR-II extracellular domain was obtained from H. Ohashi.
CHARACTERIZATION OF A 60-kDa TGF-β BINDING PROTEIN

A

<table>
<thead>
<tr>
<th>ligand</th>
<th>TGF-β1</th>
<th>TGF-β2</th>
<th>TGF-β3</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>70-74</td>
<td>70-74</td>
<td>70-74</td>
</tr>
</tbody>
</table>

Fig. 2. Analysis of ligand binding specificity of the 60-kDa TGF-β binding protein on LNCaP cells. A: Cross-linking of cell surface proteins on LNCaP cells with different TGF-β isoforms. LNCaP cells (10 × 10^6) were incubated with 200 pM 125I-TGF-β1, 125I-TGF-β2, or 125I-TGF-β3, followed by cross-linking with 0.28 mM DSS. Cell lysates were analyzed by SDS-gel electrophoresis followed by Fuji-X BioImager analysis.

B

<table>
<thead>
<tr>
<th>ligand</th>
<th>125I-TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>competitor</td>
<td>-</td>
</tr>
<tr>
<td>kDa</td>
<td>70-74</td>
</tr>
</tbody>
</table>

Imager analysis. B: Competition of 125I-TGF-β1 binding to LNCaP cell surface proteins by excess cold TGF-β. LNCaP cells (10 x 10^6) were incubated with 100 pM 125I-TGF-β1 in the absence or presence of 400-fold excess cold TGF-β1, TGF-β2, or TGF-β3. After cross-linking with 0.28 mM DSS, cell lysates were analyzed by SDS-gel electrophoresis followed by autoradiography.

at Kirin Brewery Company (Tokyo, Japan). Antiserum to the intracellular part of TβR-III was prepared against the synthetic peptide GETARRQVPTSSPA-SENSS and was obtained from K. Miyazono (Cancer Institute, Tokyo, Japan). Antiserum against the GS-domain of TβR-I was prepared against the synthetic peptide DLIYDMTTGSGSG. The peptides were synthesized with an Applied Biosystems (Foster City, CA) 430A peptide synthesizer by using t-butoxycarbonyl chemistry and were purified by reverse-phase high-performance liquid chromatography. The peptides were coupled to keyhole limpet hemocyanin (Calbiochem-Behring, San Diego, CA) by using glutaraldehyde, mixed with Freund’s adjuvant, and used to immunize rabbits. Antiserum against ActR-II and BMPR-II were raised against synthetic peptides, as described previously (Ichijo et al., 1993; Rosenzweig et al., 1995). Antiserum against bovine fetuin was obtained from K.M. Dziegielewska, and antisera against α2HS-glycoprotein were obtained from K.M. Dziegielewska and W. Jahnen-Dechent.

Affinity cross-linking studies and immunoprecipitations

Human recombinant TGF-β1 was obtained from H. Ohashi at Kirin Brewery Company, TGF-β2 was obtained from B. Pratt at Genzyme Corporation (Framingham, MA), and TGF-β3 was obtained from N. Cerletti at CIBA-GEIGY A.G. (Basel, Switzerland). Activin A was obtained from Y. Eto at Ajinomoto Company (Kawasaki, Japan), and osteogenic protein-1 (OP-1), BMP-2, and murine growth and differentiation factor (GDF)-5 were obtained from T.K. Sampath at Creative Biomolecules, Inc. (Hopkinton, MA). The ligands were iodinated according to the chloramine-T method (Frolik et al., 1984). Cells were incubated for 3 hr on ice in binding buffer [phosphate-buffered saline (PBS) containing 0.9 mM CaCl₂, 0.49 mM MgCl₂, and 0.1% bo-
Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, and 0.3 mM phenylmethylsulfonyl fluoride (PMSF) or with PBS buffer containing 10 mM Tris-HCl, pH 7.5, and 0.3 mM PMSF. The cells were scraped off the plates in detachment buffer, and they were lysed after centrifugation in solubilization buffer (125 mM NaCl, 10 mM Tris-HCl, pH 8.8, 0.01% bromphenol blue, 24% glycerol, 2% SDS, and 1% Triton X-100, 0.1% SDS, and 1% 2-mercapto ethanol at 37°C for 8 min and were then washed with PBS buffer containing 10 mM Tris-HCl, pH 7.5, and 1% Triton X-100, 0.1% SDS, and 1% 2-mercapto ethanol at 37°C for 40 min on ice. In case of subsequent immunoprecipitations with anti-TGF-β receptor antibodies, the immunocomplexes were collected on protein-A sepharose beads (Immunosorb; EC Diagnostics AB, Uppsala, Sweden). The beads were centrifuged and washed three times with solubilization buffer followed by one wash in distilled water. Immuno-complexes and solubilized cell extracts were mixed with sodium dodecyl sulfate (SDS) sample buffer (80 mM Tris-HCl, pH 8.8, 0.1% bromphenol blue, 24% glycerol, 4% SDS, and 10 mM dithiothreitol), boiled for 3 min, and separated by SDS-gel electrophoresis using a 5.0–12.5% gradient polyacrylamide gel. For autoradiographic analysis, gels were exposed to Hyperfilm MP (Amersham, Buckinghamshire, United Kingdom).

**Dithiothreitol treatment**

LNCaP cells and PC3 cells were treated with or without 1 mM dithiothreitol (DTT) in binding buffer without bovine serum albumin at 37°C for 8 min and were then affinity labeled with 200 pM 125I-TGF-β followed by cross-linking with 0.28 mM DSS. The cross-linked complexes were analyzed by SDS-gel electrophoresis and were visualized by using a Fuji-X BioImager (Tokyo, Japan).

**Endoglycosidase F treatment**

LNCaP cells and PC3 cells were incubated with 200 pM 125I-TGF-β and cross-linked with 0.28 mM DSS. Cross-linked complexes were incubated with 0.5 unit of endoglycosidase F (Endo F) for 200 pM 125I-TGF-β followed by cross-linking with 0.28 mM DSS. Aliquots of cell lysates were analyzed directly by SDS-gel electrophoresis followed by Fuji-X BioImager analysis or were first subjected to immunoprecipitation (IP) by using antisera against TGF-β type I (TβRI), TβRII, TβR-III, and endoglin. B: Endoglycosidase F treatment of 125I-TGF-β affinity cross-linked protein complexes from LNCaP cells with TGF-β receptor antisera. LNCaP cells (40 × 10^6) were affinity labeled with 200 pM 125I-TGF-β followed by cross-linking with 0.28 mM DSS. Aliquots of cell lysates were analyzed directly by SDS-gel electrophoresis followed by Fuji-X BioImager analysis or were first subjected to immunoprecipitation (IP) by using antisera against TGF-β type I (TβRI), TβRII, TβR-III, and endoglin.

**Northern blot analysis**

Isolation of total RNA and Northern blotting were performed as described previously (Franzén et al., 1993a). The filters were hybridized and washed with a TβR-I-specific cDNA probe, also as described previously (Franzén et al., 1993b).

**Mitogenic assays**

For growth inhibition assays, LNCaP cells were seeded in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics at a density of $5.0 \times 10^4$ cells per 24-well plate. The medium was changed every 2 days. The plates were incubated at 37°C with 5% CO₂. At the end of the experiment, 0.2% Triton X-100 was added to the medium, and the absorbance at 540 nm was measured using a microplate reader.
CHARACTERIZATION OF A 60-kDa TGF-β BINDING PROTEIN

<table>
<thead>
<tr>
<th>Endo F</th>
<th>LNCaP</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>PC-3</th>
<th>DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 3. (Continued.)
cells per well in 24-well tissue culture plates. The next
day, growth factors were added at various concentra-
tions. After treatment for 20 hr, cells were incubated
with 0.1 μCi/ml [3H]thymidine (Amersham) for an addi-
tional 2 hr. Cells were fixed in ice-cold 5% trichloroacetic
acid for 20 min, washed with water, and solubilized
in 200 μl 0.1 M NaOH at room temperature. [3H]thymi-
dine incorporation was measured in a liquid-scintilla-
tion β counter by using Ecoscint (National Diagnostics,
Manville, NJ).

The effect of TGF-β on DHT-mediated cellular prolifera-
tion of LNCaP cells was measured by [3H]thymidine
incorporation assays. Cells were seeded at a density of
2.5 × 10⁴ cells per well in 24-well tissue culture plates
in phenol red-free RPMI 1640 medium containing 10% charcoaled fetal bovine serum and antibiotics. After 2
days, culture medium was refreshed, and cells were
stimulated with various concentrations of DHT in the
presence or absence of 400 pM TGF-β1. After 2
days, [3H]thymidine was added for 2 hr, and [3H]thymi-
dine incorporation was measured, as described above.

**Extraction of the 60-kDa TGF-β binding protein from the cell surface**

LNCaP cells and GH3 cells that were grown to conflu-
ency in 25-cm² flasks were affinity labeled with 200 pM
[125I]-TGF-β1 followed by DSS cross-linking. Extraction of
TGF-β binding proteins from the cell surface was per-
fomed by incubations twice for 15 min on ice in PBS
buffer containing different concentrations of NaCl, hepa-in, or glycercer. Cells were solubilized and prepared for
SDS-gel electrophoresis, as described above. The two
washes were pooled, and proteins were precipitated in
10% trichloroacetic acid for 30 min on ice followed by 15
min centrifugation and an acetone wash. The samples
were boiled in sample buffer and separated by SDS-gel
electrophoresis followed by autoradiography.

**Competition binding assay with the 60-kDa
TGF-β binding protein**

Confluent LNCaP cells (25 × 10⁶ cells) in 175-cm²
flasks were washed twice in binding buffer in the ab-
sence of bovine serum albumin. Cells were scraped off
the flasks in 10 ml of bovine serum albumin-free bind-
ing buffer, spun down for 5 min at 4°C, and resus-
pended in 1 ml of PBS containing 10% glycerol. Extrak-
tion of the 60-kDa TGF-β binding protein was done for
30 min at 4°C. Thereafter, cells were spun down, and
the supernatant was centrifuged at 13,000 rpm for 30
min at 4°C. An affinity cross-linking assay was per-
fomed on 2.5 × 10⁹ MvLu cells in six-well culture
plate dishes using 20 pM [125I]-TGF-β in 1 ml of PBS
containing 10% glycerol and 0.1% bovine serum albumin
in the presence or absence of 60-kDa TGF-β binding
protein eluted from 25 × 10⁶ LNCaP cells. Samples
were analyzed by SDS-gel electrophoresis and autora-
diography.

**RESULTS**

**Identification of a 60-kDa TGF-β1 cell surface
binding protein in LNCaP cells distinct from TGF-β receptors**

Binding of [125I]-TGF-β1 to LNCaP cells followed by
cross-linking with DSS revealed complexes of approxi-
mately 70–74 kDa (Fig. 1). Subtracting the molecular
mass of the cross-linked monomeric TGF-β1 results in
the estimated size of approximately 60 kDa. Under
nonreducing conditions, only one TGF-β1 cross-link-

![Fig. 4. Analysis of responsiveness of LNCaP cells to members of the TGF-β superfamily and identification of ligand binding to receptors. A: Cellular proliferation of LNCaP cells in the presence of different members of the TGF-β superfamily. LNCaP cells were seeded at a density of 50,000 cells/well in 24-well plates and were stimulated for 20 hr with 800 pM activin A, 6.25 nM BMP-2, 6.25 nM growth and differentiation factor 5 (GDF-5), 6.25 nM osteogenic protein-1 (OP-1), and 800 pM TGF-β1. Inhibition of cellular proliferation was measured by [3H]-thymidine incorporation, and results are expressed in percentage of unstimulated control. Each column represents the mean of triplicate observations. The vertical bars denote standard deviations. B: Cellular proliferation of LNCaP cells in the presence of different concentrations of dihydrotestosterone (DHT) and/or TGF-β1. LNCaP cells were seeded at a density of 25,000 cells/well in 24-well plates. Cells were grown in the presence of different concentrations of DHT and/or 400 pM TGF-β1 for 4 days. [3H]-thymidine incorporation was measured, and results are expressed in percentage of unstimulated control. Each column represents the mean of triplicate observations. The vertical bars denote standard deviations. C: Analysis of activin receptor expression in LNCaP cells. LNCaP cells (30 × 10⁶) were incubated with 400 pM [125I]-activin A followed by cross-linking with 0.28 mM DSS. Aliquots of cell lysates were analyzed directly by SDS-gel electrophoresis followed by autoradiography or were first subjected to immunoprecipitation (IP) by using antisera against activin receptors. D: Analysis of OP-1 receptor expression in LNCaP cells. LNCaP cells (60 × 10⁶) were incubated with 250 pM [125I]-OP-1 followed by cross-linking with 0.28 mM DSS. Aliquots of cell lysates were analyzed directly by SDS-gel electrophoresis followed by Fuji-X Bioimager analy-
as or were first subjected to immunoprecipitation (IP) by using antisera against OP-1 receptors followed by autoradiography.
studies using $^{125}$I-TGF-β$^2$ and $^{125}$I-TGF-β$^3$ gave weaker bands, suggesting that the affinity of the 60-kDa protein for these TGF-β isoforms is much lower than the affinity for TGF-β$^1$ (Fig. 2A). The binding of $^{125}$I-TGF-β$^1$ to the 60-kDa protein as well as to the TβR-III (for its identification, see Fig. 3A) was fully competed with 400-fold excess of unlabeled TGF-β$^1$ (Fig. 2B), indicating specificity in binding. Compared with TGF-β$^1$, a 400-fold excess of TGF-β$^2$ or TGF-β$^3$ competed to a lesser extent with $^{125}$I-TGF-β$^1$ for binding to the 60-kDa protein, whereas the binding of $^{125}$I-TGF-β$^1$ to TβR-III was clearly displaced. Activin and OP-1 also failed to compete for binding of $^{125}$I-TGF-β$^1$ to the TGF-β binding proteins on LNCaP cells (data not shown). These results indicate that the 60-kDa protein on LNCaP cells displays TGF-β isoform-specific binding.

Immunoprecipitations with antisera raised against the intracellular domain of TβR-I, TβR-II, TβR-III, and endoglin failed to bring down the 60-kDa $^{125}$I-TGF-β$^1$ cross-linked protein complexes (Fig. 3A). TβR-III appeared to be expressed by LNCaP cells, because its presence was confirmed by immunoprecipitation of the $^{125}$I-TGF-β$^1$ cross-linked, 200-kDa protein complexes using the TβR-III antiserum. To further explore the possibility that the 60-kDa TGF-β binding protein on LNCaP cells is related to the similarly sized TβR-I, the following experiments were performed. TβR-I is known to contain N-linked glycosylation, which can be removed by treatment with endoglycosidase F. However, endoglycosidase F treatment of LNCaP cells did not alter the size of the TGF-β$^1$ binding complexes (Fig. 3B), indicating that the proteins do not contain N-linked carbohydrate chains. Binding of TGF-β to TβR-I can be abolished by DTT. However, when LNCaP cells were treated with DTT preceding affinity cross-linking studies, the 70-74 kDa protein complexes could still be detected (Fig. 3C).

In PC-3 cells, TβR-I was clearly affected by endoglycosidase F or DTT treatment. In addition, these experiments revealed that PC-3 cells also contain the 70–74 kDa protein complexes. Moreover, Northern blot analysis revealed that LNCaP cells lack expression of TβR-I in contrast to other cell lines examined (Fig. 3D). Taken together, these observations indicate that the 60-kDa LNCaP protein is distinct from TβR-I.

To examine whether the TGF-β binding components are related to other known serine/threonine kinase type I receptors, we subjected the $^{125}$I-TGF-β$^1$ affinity-labeled, cross-linked complexes from LNCaP cells to immunoprecipitation with specific antisera raised against the known type I receptors [activin receptor-like kinases (ALK) 1–6; ten Dijke et al., 1994a] and with an antibody raised against the GS domain. These antisera as well as antisera against the extracellular domain of TβR-II did not immunoprecipitate any of the TGF-β$^1$ protein complexes in LNCaP cells (Fig. 3E and data not shown).

**LNCaP cells are TGF-β resistant but respond to other TGF-β superfamily members**

The growth-modulating effects of different members of the TGF-β superfamily on LNCaP cells were tested by $[^3H]$-thymidine incorporation assays. TGF-β$^1$ had no significant antimitogenic effect on LNCaP cells, whereas activin and GDF-5 clearly inhibited the growth of LNCaP cells to less than 50% of control cells (Fig. 4A). The inhibition was even more pronounced with the addition of BMP-2 or OP-1 (Fig. 4A). Kim et al. (1996c) have reported the abolition of DHT-mediated cellular proliferation by TGF-β. Therefore, the potential effect of TGF-β on DHT-stimulated LNCaP cells was investigated (Fig. 4B). Although stimulation of LNCaP cells with increasing concentrations of DHT resulted in a typi-
PIEK ET AL.

A cell lysate release in medium

0 0.15 0.20 0.25 0.30 0.40 0.50 0 0.15 0.20 0.25 0.30 0.40 0.50

NaCl [M]

70-74 kDa

B

70-74 kDa

C

10% glycerol

100 µg/ml heparin

70-74 kDa

Fig. 5. Determination of the elution characteristics of the 60-kDa TGF-β binding protein. LNCaP cells were grown to confluency in 25-cm² flasks. Cell surface proteins on LNCaP cells were affinity cross-linked with 200 nM ¹²⁵I-TGF-β1 followed by two washes with different concentrations of NaCl (A) and heparin (B) or glycerol in combination with heparin (C). The two washes from each flask were pooled and trichloroacetic acid precipitated. Cells were lysed, and all samples were analyzed by SDS-gel electrophoresis followed by autoradiography.

cal bell-shaped growth curve (Schuurmans et al., 1988; Sonnensohn et al., 1989; Lee et al., 1995), showing maximum stimulation of cell growth at 10⁻¹⁰ M DHT, the addition of TGF-β1 did not affect this DHT-induced proliferation. Thus, although LNCaP cells do have the potency to react on several members of the TGF-β superfamily, they are nonresponsive to TGF-β itself.

Cross-linking studies with radiolabeled ligands showed that, in LNCaP cells, activin A bound to ActR-IB and ActR-II (Fig. 4C), whereas OP-1 bound to BMPR-I and BMPR-II and weakly to ActR-II (Fig. 4D). Apparently, activin A and OP-1 did not bind to the 60-kDa TGF-β binding protein.

TGF-β 60-kDa binding protein is a cell surface-associated protein

To explore the basis for the cell membrane association of the 60-kDa TGF-β binding protein, we investigated whether the ¹²⁵I-TGF-β1 cross-linked protein complexes could be released by treatment of the cell cultures with NaCl (Fig. 5A). Elution of the protein complexes from the cell surface into the medium could be detected clearly after incubation in buffers containing 0.25 M or more of NaCl. Incubation of LNCaP cells in 1 µg/ml or more of heparin in PBS after affinity cross-linking also resulted in a release of the TGF-β-bound complexes (Fig. 5B). Heparin is known to dissociate proteins that are attached to glycosaminoglycans. In addition, release of the TGF-β-bound protein complexes could be achieved after incubation in 10% glycerol in PBS (Fig. 5C).

TGF-β 58–62 kDa binding proteins on GH3 cells have properties similar to the 60-kDa TGF-β1 binding protein from LNCaP cells

GH3 cells contain TGF-β binding proteins similar in size to the 60-kDa protein on LNCaP cells and that have been designated TGF-β type IV receptors. Therefore, we investigated whether the TGF-β binding protein on GH3 cells showed the same isoform specificity and cell surface-associated characteristics as the LNCaP TGF-β binding protein. Regarding the isoform specificity of the proteins on GH3 cells, the 69-kDa, cross-linked TGF-β type I receptor, which had high affinity for all three TGF-β isoforms, partly overlapped with the complex containing the 60-kDa component (Fig. 6A), making firm conclusions difficult. The upper protein band in the 70–74 kDa region appeared to be somewhat weaker when ¹²⁵I-TGF-β2 or ¹²⁵I-TGF-β3 were used in the cross-linking studies, which is in line with our observations for the 60-kDa TGF-β binding protein from LNCaP cells. Similar to our findings for the TGF-β1 binding component in LNCaP cells, the 70-74 kDa complexes on GH3 cells were released upon treatment with 0.5 M NaCl, 10 µg/ml heparin (Fig. 6B).
**A**

<table>
<thead>
<tr>
<th>IP</th>
<th>70-74 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>70-74 kDa</td>
</tr>
<tr>
<td>H</td>
<td>70-74 kDa</td>
</tr>
<tr>
<td>N</td>
<td>70-74 kDa</td>
</tr>
</tbody>
</table>

**B**

**DISCUSSION**

This study describes the characterization of a 60-kDa TGF-β binding protein that was originally identified on LNCaP cells by affinity cross-linking of cell surface proteins with 125I-TGF-β1. Our results show that the 60-kDa TGF-β binding protein is distinct from TpR-I, TpR-II, and other known type I receptors for members of the TGF-β superfamily. Rather than being a transmembrane protein, the 60-kDa TGF-β binding protein is associated to the cell surface of LNCaP cells. It appears to be present on multiple cell types, including PC-3 cells and GH3 cells, and can regulate the access of TGF-β to its receptors.

The cell surface-associated TGF-β binding protein on LNCaP cells exists in multiple forms, observed as 70–74 kDa. TGF-β bound protein complexes upon analysis by SDS-gel electrophoresis (Fig. 1). Because the 70–74 kDa protein complexes include 12-kDa subunits of monomeric cross-linked TGF-β, the actual size of the binding protein is estimated to approximately 60 kDa. Under nonreducing conditions, the multiple TGF-β bound protein complexes run slightly faster, as a 67-kDa component (Fig. 1), suggesting that the 60-kDa TGF-β binding protein contains cysteine bridges. Although LNCaP cells do express TpR-III (Fig. 3A), they lack expression of TpR-I (Fig. 3A–E) due to a genetic alteration in the gene encoding this receptor (Kim et al., 1996a), and the expression level of TpR-II is very low (Kim et al., 1996c). Transfection of TpR-I into LNCaP cells results in recovery of TGF-β responsiveness, showing that the TGF-β signaling pathway downstream of TpR-I is intact in LNCaP cells (Kim et al., 1996a). There are contradictory reports, however, on the responsiveness of LNCaP cells to TGF-β in the presence of proliferation-inducing factors, like dihydrotestosterone (Schuurmans et al., 1988; Wilding et al., 1989; Kim et al., 1996b,c). Although TGF-β has been described to suppress the mitogenic activity of 10^{-10} M DHT in LNCaP cells (Kim et al., 1996c), we observed no inhibition of the growth-stimulatory effect of 10^{-10} M DHT on LNCaP cells by the addition of TGF-β. Cross-linking studies on 10^{-10} M DHT-stimulated LNCaP cells did not indicate an up-regulation of expression of TGF-β receptors or TGF-β binding proteins compared with untreated cells (data not shown). Apparently, the TGF-β 60-kDa protein interferes with TGF-β receptor binding

The 60-kDa protein extracted from LNCaP cells by a wash with 10% glycerol in PBS was tested for its effect on 125I-TGF-β binding to the TGF-β receptors on MV1Lu cells (Fig. 7). MV1Lu cells do not contain the 60-kDa TGF-β binding protein, yet they express high levels of TpR-I, TpR-II, and TpR-III. Therefore, it seems to be a suitable model system to investigate the possible function of the 60-kDa TGF-β binding protein from LNCaP cells. In the presence of 60-kDa LNCaP-derived protein, the binding of all three TGF-β isoforms to the TGF-β receptors was inhibited. After incubation of 125I-TGF-β1 with the glycerol extract followed by cross-linking, only the 70–74 kDa protein complexes were detected (data not shown), indicating that the decrease in TGF-β binding to its receptors was mediated by the 60-kDa protein and not by other proteins present in the wash. These results suggest that, in a soluble form, the 60-kDa protein has affinity for all three TGF-β isoforms. Thus, the 60-kDa TGF-β binding protein can modulate the binding of TGF-β to its receptors, implying that it might regulate TGF-β receptor-mediated signaling.

**or 10% glycerol (data not shown), whereas TpR-I was not eluted from the cells under these conditions.**

**TGF-β 60-kDa protein interferes with TGF-β receptor binding**

**The 60-kDa protein extracted from LNCaP cells by a wash with 10% glycerol in PBS was tested for its effect on 125I-TGF-β binding to the TGF-β receptors on**
Fig. 7. Functional characterization of the 60-kDa TGF-β binding protein. Affinity cross-linking of $^{125}$I-TGF-β to TGF-β receptors on mink lung epithelial cells (Mv1Lu) in the presence of LNCaP-eluted 60-kDa TGF-β binding protein. The 60-kDa TGF-β binding proteins were eluted from LNCaP cells with 10% glycerol in PBS. Mv1Lu cells (2.5 x 10^6) were incubated with 20 pM $^{125}$I-TGF-β1, $^{125}$I-TGF-β2, or $^{125}$I-TGF-β3 in binding buffer without glycerol or LNCaP-eluted proteins, or in binding buffer with 10% glycerol but in the absence of LNCaP-eluted proteins, or in binding buffer containing 10% glycerol and cell surface-eluted proteins from 25 x 10^6 LNCaP cells, followed by cross-linking with 0.28 mM DSS. Cell lysates were analyzed directly by SDS-gel electrophoresis followed by autoradiography.

The 60-kDa TGF-β binding protein on LNCaP cells is not able to directly transduce growth-modulating TGF-β activities.

The 60-kDa protein identified on LNCaP cells appeared to bind TGF-β1 with higher affinity than TGF-β2 and TGF-β3. The affinity constant of the 60-kDa protein for TGF-β1 remains to be determined. Activin A and OP-1 did not bind to the 60-kDa protein. Isoform-specific TGF-β binding proteins have been described that are attached to the cell membrane via a GPI anchor (Cheifetz and Massagué, 1991; Dumont et al., 1995). The 60-kDa TGF-β binding protein from LNCaP cells is not GPI anchored, because its release could not be induced by phosphatidylinositol-phospholipase C (data not shown). To our knowledge, this is the first demonstration of a protein that binds TGF-β1 and TGF-β3 with different affinity. TGF-β1 and TGF-β3 have very similar in vitro biological activities (Cheifetz et al., 1990). However, they differ in their activity with respect to scar formation (Shah et al., 1995).

Dissociation of the 70–74 kDa TGF-β cross-linked protein complexes from LNCaP cells by sodium chloride or heparin indicated that the 60-kDa TGF-β binding protein is associated to structures at the cell surface, perhaps to heparan sulfate proteoglycans. Heparin can compete for binding of proteins that are attached to heparan sulfate proteoglycans. The ability of the 60-kDa TGF-β binding protein from LNCaP cells to be eluted by salt or heparin is reminiscent of the characteristics of a previously described 60-kDa TGF-β binding protein localized in the extracellular matrix of HepG2 cells (Bützow et al., 1993). The affinity constant

---

<table>
<thead>
<tr>
<th>TGF-β1</th>
<th>TGF-β2</th>
<th>TGF-β3</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>glycerol</td>
<td>LNCaP</td>
</tr>
<tr>
<td>control</td>
<td>glycerol</td>
<td>LNCaP</td>
</tr>
<tr>
<td>control</td>
<td>glycerol</td>
<td>LNCaP</td>
</tr>
</tbody>
</table>
The identity of the 60-kDa TGF-β binding protein is unknown. Certain components known to bind TGF-β, like α2-macroglobulin, fibronectin, and decorin (Fava and McClure, 1987; O’Connor-McCourt and Wakefield, 1987; Yamaguchi et al., 1990), can be excluded as candidates in view of their differences in size. Recently, bovine fetuin and its human counterpart, α2-HS glycoprotein, with molecular sizes of 60 kDa, have been described to bind several members of the TGF-β superfamily (Demetriou et al., 1996). However, the characteristics of the 60-kDa protein from LNCaP cells make it unlikely that it is fetuin or α2-HS glycoprotein. Whereas fetuin and α2-HS glycoprotein have highest affinity for BMPs and bind with weaker affinity to TGF-β, the 60-kDa protein from LNCaP cells has highest affinity for TGF-β and no appreciable affinity for activin A or BMPs. Fetuin and α2-HS glycoprotein contain a number of N- and O-glycosylated chains (Dziegielewska et al., 1990). However, endoglycosidase F treatment of the 60-kDa protein from LNCaP cells cross-linked with 125I-TGF-β did not result in faster migration on SDS-gel electrophoresis. Cross-linking of radiolabeled TGF-β to calf serum, in which fetuin is abundantly present (for review, see Brown et al., 1992), or to commercial α2-HS glycoprotein revealed TGF-β bound protein complexes distinct from the 70–74 kDa TGF-β cross-linked complexes on LNCaP cells (data not shown). In addition, we were unable to immunoprecipitate cross-linked 125I-TGF-β1 complexes from LNCaP cells by using antisera against α2-HS glycoprotein; moreover, these antisera did not recognize the eluted 60-kDa TGF-β binding protein by using immunoblotting (data not shown). The 60-kDa TGF-β binding protein identified from HepG2 cells by Bützow et al. (1993) was shown to be produced by these cells, thereby excluding serum delivery.

The 60-kDa TGF-β binding protein is possibly a follistatin-like protein. Follistatin is an activin binding protein that inhibits activin receptor binding. Like follistatin (Ueno et al., 1987; Inouye et al., 1992; Sugino et al., 1993), the 60-kDa TGF-β binding protein inhibits signaling of TGF-β superfamily members, and it may associate to heparan sulfate proteoglycans on the cell surface.

The 70–74 kDa TGF-β binding protein complexes have been observed in several cell lines, like HEP-G2 cells, HEP-G3 cells, HT-12 cells (Bützow et al., 1993), and PC-3 cells (Fig. 3B,C). In rat pituitary GH3 cells, TGF-β binding protein complexes with sizes similar to the 60-kDa binding protein on LNCaP cells have been identified (Cheifetz et al., 1988; Yamashita et al., 1995) and are referred to as TGF-β type IV receptor. We showed that TβR-IV can be eluted from the cell surface of GH3 cells by salt, heparin, and glycerol in a manner similar to what we observed for the 60-kDa protein on LNCaP cells. In addition, Yamashita et al. (1995) previously showed that a treatment of GH3 cells with endoglycosidase F did not result in a shift in molecular weight of the 70–74 kDa TGF-β bound protein complexes in SDS-gel electrophoresis and that transient DTT treatment did not abolish the binding of TGF-β, similar to the 60-kDa protein from LNCaP cells. Therefore, we conclude that TβR-IV is a cell-associated TGF-β binding protein that may be related to the 60-kDa protein from LNCaP cells. We are currently investigating whether the TβR-IV on GH3 cells and the 60-kDa TGF-β binding protein on LNCaP cells exert the same biological activities.

In soluble form, the 60-kDa LNCaP protein was shown to impede the binding of TGF-β to the TβRs, suggesting that it may inhibit TGF-β signaling. The effects of the 60-kDa component on TGF-β-induced growth inhibition could not be tested directly due to the presence of growth-promoting contaminants in the partially purified preparation of the 60-kDa component. Although affinity cross-linking of iodinated TGF-β to the glycerol-eluted LNCaP extract revealed that the 60-kDa protein is the only TGF-β binding protein, the 60-kDa TGF-β binding protein is present in very low abundance (data not shown). Therefore, it seems to be inevitable that, in order to clarify the role of the 60-kDa TGF-β binding protein on modulation of TGF-β action, the 60-kDa TGF-β binding protein has to be purified. Interestingly, soluble 60-kDa TGF-β binding protein, in contrast to its cell surface-associated configuration, does have affinity for all three TGF-β isoforms. Discrepancies in TGF-β binding affinities of soluble and cell surface-associated 60-kDa protein are subject for further studies. When it is bound to the cell surface, the 60-kDa TGF-β binding protein may also regulate binding to TβRs. The 60-kDa protein was not immunoprecipitated with TβR-I in GH3 cells (Fig. 6A; Yamashita et al., 1995), suggesting that it may not be involved in ligand presentation to the receptors. Thus, the 60-kDa TGF-β binding protein most likely sequesters ligand and neutralizes TGF-β bioactivity. Alternatively, it may have a storage function for TGF-β at the cell surface and extracellular matrix from which it can be released in active form, e.g., during wound repair. Thus, the 60-kDa cell surface-associated TGF-β binding protein identified on LNCaP cells might play an important role in modulating isoform-specific TGF-β activities.

ACKNOWLEDGMENTS

We thank E.J.J. van Zoelen, K. Miyazono, and R. Bützow for valuable discussions. We also thank H. Ohashi for TGF-β1; B. Pratt for TGF-β2; N. Cerletti for TGF-β3; Y. Eto for activin A; T.K. Sampath for OP-1, BMP-2, and GDF-5; Kirin Brewery Company for TβR-II antiserum; K. Miyazono for TβR-III antiserum; W. Jahn-Dechent for antiserum against human α2HS glycoprotein; and K.M. Dziegielewska for antiserum against bovine fetuin and human α2HS glycoprotein. E.P. was supported by a fellowship from the Netherlands Organization for Scientific Research (NWO).

LITERATURE CITED


