Betaglycan Can Act as a Dual Modulator of TGF-β Access to Signaling Receptors: Mapping of Ligand Binding and GAG Attachment Sites

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Abstract. Betaglycan, also known as the TGF-β type III receptor, is a membrane-anchored proteoglycan that presents TGF-β to the type II signaling receptor, a transmembrane serine/threonine kinase. The betaglycan extracellular region, which can be shed by cells into the medium, contains a NH_2-terminal domain related to endoglin and a COOH-terminal domain related to uromodulin, sperm receptors Zp2 and 3, and pancreatic secretory granule GP-2 protein. We identified residues Ser^{535} and Ser^{546} in the uromodulin-related region as the glycosaminoglycan (GAG) attachment sites. Their mutation to alanine prevents GAG attachment but does not interfere with betaglycan stability or ability to bind and present TGF-β to receptor II. Using a panel of deletion mutants, we found that TGF-β binds to the NH_2-terminal endoglin-related region of betaglycan. The remainder of the extracellular domain and the cytoplasmic domain are not required for presentation of TGF-β to receptor II; however, membrane anchorage is required. Soluble betaglycan can bind TGF-β but does not enhance binding to membrane receptors. In fact, recombinant soluble betaglycan acts as potent inhibitor of TGF-β binding to membrane receptors and blocks TGF-β action, this effect being particularly pronounced with the TGF-β2 isoform. The results suggest that release of betaglycan into the medium converts this enhancer of TGF-β action into a TGF-β antagonist.

The binding of some cytokines to their signaling receptors is modulated by accessory receptors. A case in point is the binding of TGF-β which is assisted by betaglycan, a membrane-anchored proteoglycan also known as the TGF-β type III receptor (López-Casillas et al., 1991, 1993; Wang et al., 1991). TGF-β and related cytokines signal through complexes of two distantly related transmembrane protein serine/threonine kinases known as receptors I and II (Attisano et al., 1992, 1993; Ebner et al., 1993; Estevez et al., 1993; Franzén et al., 1993; Lin and Lodish, 1993; Lin et al., 1992; Massagué, 1992; Mathews and Vale, 1991). When expressed alone, the type I receptors cannot bind ligand, and the TGF-β type II receptor has low affinity for TGF-β1 and no affinity for TGF-β2. When expressed together, receptors I and II bind TGF-β1 with high affinity ($K_D \sim 10^{-11}$ M), forming a signaling receptor complex, but still bind TGF-β2 with low affinity ($K_D > 10^{-9}$ M) (Attisano et al., 1993; Franzén et al., 1993; Wrana et al., 1992).

These limitations in ligand-binding ability can be overcome by the action of betaglycan. When expressed alone or purified from tissue, betaglycan binds all TGF-β isoforms with relatively high affinity ($K_D \sim 10^{-10}$ M) (Andres et al., 1991; Massagué et al., 1990). In the presence of TGF-β, beta glycan on the cell surface associates with the signaling receptors (López-Casillas et al., 1993; Moustakas et al., 1993). Receptor II binds TGF-β tethered to membrane betaglycan better than it binds TGF-β free in the medium, and forms a stable ternary complex with TGF-β and betaglycan (López-Casillas et al., 1993). Betaglycan is thought to be displaced from this complex by receptor I as it becomes available on the cell surface. As a result, betaglycan action increases binding of TGF-β to its signaling receptors. This positive action is particularly evident with TGF-β2, an isoform that has low affinity for receptors I and II in the absence of betaglycan. Various cell lines that do not express betaglycan are less sensitive to TGF-β2 than they are to TGF-β1 (Cheifetz et al., 1990; Ohta et al., 1987), and gain sensitivity to TGF-β2 when transfected with betaglycan (López-Casillas et al., 1993). Membrane betaglycan is therefore considered a positive regulator of TGF-β access to signaling receptors.

The deduced primary structure of betaglycan reveals a 853-amino acid precursor protein with a large extracellular domain (López-Casillas et al., 1991; Moren et al., 1992; Wang et al., 1991). This domain bears no resemblance to those of receptors I and II, which are short and contain characteristic cysteine arrangements (Attisano et al., 1993; Childs et al., 1993; Ebner et al., 1993; Franzén et al., 1993; Lin et al., 1992). The extracellular domain of betaglycan contains heparan sulfate and chondroitin sulfate glycosaminoglycan (GAG)[1] chains. Betaglycan binds TGF-β through

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1. *Abbreviation used in this paper*: GAG, glycosaminoglycan.

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the core protein (Cheifetz et al., 1988a; Segarini and Seyedin, 1988), and can bind basic fibroblast growth factor (bFGF) through the heparan sulfate chains (Andres et al., 1992). The extracellular domain is followed by a single transmembrane segment and a short cytoplasmic region with no discernable signaling structure.

Betaglycan contains regions of similarity to other proteins (see Fig. 1). In the NH_2-terminal region, as well as in the transmembrane and cytoplasmic regions, it shows amino acid sequence similarity to endoglin (López-Casillas et al., 1991; Wang et al., 1991). Endoglin is a disulfide-linked homodimeric membrane glycoprotein from endothelial cells (Gougos and Letarte, 1990) that binds TGF-β1 and β3 but not TGF-β2 (Cheifetz et al., 1992). The second half of the betaglycan extracellular domain resembles various proteins of diverse function (Bork and Sander, 1992). These proteins include the kidney-derived urinary protein, uromodulin, the sperm receptors Zp2 and Zp3, and the major pancreatic secretory granule membrane protein GP-2. In betaglycan, this domain contains the two serines that most closely fit the consensus for GAG chain attachment.

In addition to existing as a membrane-anchored form, the ectodomain of betaglycan is shed by cells as a soluble form into the medium (Andres et al., 1989; López-Casillas et al., 1991). Although one function of membrane-anchored betaglycan is to present TGF-β to signaling receptors, the function of the soluble form and the participation of the various structural domains of betaglycan in these functions have been unknown. The present studies address these questions with the use of mutant versions of betaglycan that lack specific extracellular segments, GAG attachment sites, a membrane anchor or a cytoplasmic domain. Analysis of these betaglycan mutants has allowed identification of the TGF-β-binding region and GAG attachment sites, and indicates that the uromodulin-related region is dispensable for ligand binding and presentation to signaling receptors. Furthermore, the results show that betaglycan devoid of its membrane anchor inhibits TGF-β binding to cell surface receptors and prevents TGF-β action. Based on these results, betaglycan is identified as a dual regulator of TGF-β access to signaling receptors.

Materials and Methods

Construction and Expression of Mutant Betaglycan cDNAs

The rat betaglycan cDNA containing a human c-myc epitope sequence (López-Casillas et al., 1993) was used as the template for all mutant constructs. The nucleotide base pair and amino acid sequence numbering is as in the wild-type betaglycan sequences (López-Casillas et al., 1991) and disregards the bases/residues added by the c-myc epitope. To render the AflIII site at bp 929 unique in the parental plasmid, the AflIII site located in the pGEM-4Z vector backbone was eliminated by AflIII digestion, mung bean nuclease trimming and self ligation. DNA manipulations were done according to standard procedures (Sambrook et al., 1989) and reagents: endonucleases (Boehringer-Mannheim Corp., Indianapolis, IN or Bethesda Research Laboratories, Gaithersburg, MD), DNA polymerase Klenow fragment (Boehringer-Mannheim), mung bean nuclease (Pharmacia LKB Biotechnology, Piscataway, NJ), T4 DNA ligase (Boehringer-Mannheim), and competent *E. coli* HB101 cells (Bethesda Research Laboratories). All constructs were confirmed by sequencing (USB Sequenase Kit).

The specific constructs were obtained as follows. Δ1: self ligation of the large DNA fragment resulting from digestion with StuI (at bp 462) and AflIII (at bp 929) repaired with Klenow fragment. Δ2: self ligation of the large DNA fragment resulting from digestion with AflIII (at bp 929) repaired with Klenow fragment, followed by digestion with XhoI (at bp 1829) trimmed with mung bean nuclease; in addition, this manipulation introduces a Trp at position 200. Δ3: self ligation of the large DNA fragment resulting from digestion with XhoI (at bp 1829) and NcoI (at bp 2677) and trimming with mung bean nuclease; in addition, this manipulation introduces an Arg at position 499. Δ5: self ligation of the large DNA fragment resulting from digestion with AflIII (at bp 929) and BclI (at bp 1188) and repair with Klenow fragment; in addition, this manipulation introduces a Trp at position 200. Δ6: as previously described (López-Casillas et al., 1993). Δ8: annealed 5'CCGGCAACTGGGT3' and 5'GATCACCCAGTTGCCGG3' oligonucleotides were inserted into the large DNA fragment that results from digestion with StuI (at bp 462) and BclI (at bp 1188); in addition, this manipulation introduces a Gly at position 45. Δ9: self ligation of the large DNA fragment resulting from digestion with EcoRV (at bp 1559) and BclI (at bp 1188) repaired with Klenow fragment. Δ 10: self ligation of the large DNA fragment resulting from digestion with EcoRV (at bp 1559) and NcoI (at bp 2677) repaired with Klenow fragment. Δtail: a synthetic double-stranded 100-bp long oligomer encoding the last 4 residues of the ectodomain and the complete transmembrane region followed by Asp-Lys-Lys-Stop, contained NcoI and AvrII sites which were used for insertion at the same sites in betaglycan cDNA, replacing the original sequences comprised between them. In addition to deleting the cytoplasmic tail, these changes created unique HpaI and ClaI sites flanking the transmembrane region. S535A, S546A, and gag^- were created by site directed mutagenesis using a PCR strategy, as previously described (Wrana et al., 1992). LS, $LSgag^-$, and SS were created by the insertion of an annealed pair of oligonucleotides encoding a stop codon at the NcoI site (bp 2677) for the long forms, or at the SalI site (bp 1870) for the short form. The gag^- mutant plasmid was used as the template for the $LSgag^-$ construct. In addition, a hexa-histidine sequence was engineered before the stop codon in the long soluble forms in order to facilitate the purification of their products.

For expression in COS-1 cells, betaglycan cDNA inserts were transferred from pGEM-4Z to the pCMV5 mammalian expression vector (Andersson et al., 1989) using the flanking EcoRI and HindIII sites. Transient transfections were done by the diethylaminoethyl-dextran method (Seed and Aruffo, 1987) and assayed 48–72 h later.

Cell Surface Biotinylation

Cell monolayers were labeled with sulfosuccinimidyl 6-(biotinoamido) hexanoate (NHS-LC-biotin, a membrane-impermeable biotinylation reagent, Pierce Chem. Co., Rockford, IL) as previously described (López-Casillas et al., 1993). Labeled products were displayed by immunoprecipitation from cell lysates with the anti-myc epitope monoclonal antibody 9E10 (Evan et al., 1985), followed by SDS-PAGE, electroblotting, and probing with peroxidase-conjugated streptavidin as previously described (López-Casillas et al., 1993).

TGF-β Receptor Assays

TGF-β1 and -β2 from porcine platelets (R & D Systems, Inc., Minneapolis, MN) were labeled with ^{125}I as previously described (Cheifetz et al., 1990). Cell surface TGF-β receptor binding assays and affinity-labeling assays were done as previously described (Massagué, 1987). Where indicated, labeled receptors were immunoprecipitated from cell lysates with the anti-myc antibody 9E10 (Evan et al., 1985), or with the anti-HA1 epitope monoclonal antibody 12CA5 (Meloche et al., 1992) as previously described (López-Casillas et al., 1993). For affinity-labeling of soluble receptors, conditioned media received Triton-X 100 to a final concentration of 0.05%, and was incubated with the indicated concentration ^{125}I-TGF-β for 2–3 h at 4°C. Disuccinimidyl suberate (Pierce Chem. Co.) dissolved at 10 mg/ml in DMSO was added to a final concentration of 0.1 mg/ml, and incubation continued for another 15 min. The cross-linker was then quenched by adding glycine (pH 7.5) to a final concentration of 10 mM, and the reaction mixture was immediately mixed with SDS-PAGE sample buffer, boiled, and separated in a 7% polyacrylamide gel. The region of the gel containing the free radiolabeled ligand was removed before autoradiography.

For treatment of betaglycan with chondroitinase or heparitinase, lysates from affinity-labeled cells were immunoprecipitated with antibody 9E10 and the immune complexes were collected with protein-G Sepharose beads. After the regular detergent washings, the beads were washed once with digestion buffer (10 mM Tris-Cl pH 7.5; 50 mM sodium acetate pH 7.5; 2.5 mM calcium chloride; 0.1% Triton X-100; 0.01% BSA; 10 μg/ml leupeptin; 10 μg/ml antipain; 100 μg/ml benzamidine hydrochloride; 50 μg/ml

aprotinin; 100 μg/ml soybean trypsin inhibitor; 10 μg/ml pepstatin; and 1 mM PMSF and then resuspended in 0.1 ml of digestion buffer containing either no enzymes or 100 mU of chondroitinase ABC (ICN Biochemicals, Costa Mesa, CA) alone or together with 20 mU of heparitinase (ICN Biochemicals). These samples were incubated for 12 h at 37°C. Incubations were stopped by the addition of SDS-PAGE sample buffer and boiling. The products resulting from these treatments were separated in a 6% polyacrylamide gel and revealed by autoradiography.

Growth Inhibition Assay

For growth inhibition assays, MvlLu cells (CCL64, ATCC) were seeded in minimal essential medium supplemented with non-essential amino acids and 0.2% FBS, at 30,000 cells/cm^2 in 24-well clusters. ^{125}I-iododeoxyuridine incorporation into DNA was measured as previously described (Cheifetz et al., 1990).

Baculoviral Betaglycan

The insert in the pCMV5 subclone of the LS construct was transferred to the pVL1392 plasmid using the NotI and XbaI sites. Cotransfection of Sf21 insect cells with the pVL1392 subclone of the LS construct and the BaculoGold Viral DNA, as well as the single plaque purification of the resulting recombinant clones, were carried out as described in the BaculoGold Transfection Kit (Pharmigen). Additional amplification and expression of the baculoviral strain expressing LS was carried out with Sf9 cells.

Media conditioned by Sf9 cells was collected 2–3 d after infection with either the AcNPV wild-type baculovirus or the recombinant strain expressing the betaglycan LS construct. Before being used in TGF-β-binding assays and growth inhibition assays, the media were dialyzed (Spectra/Por 6 membrane; 50,000 dalton cut-off) at 4°C against two changes of minimal essential medium supplemented with non-essential amino acids. Samples used for growth inhibition were supplemented with 0.2% FBS and those used in binding assays received a 1/10 vol of 10 × binding buffer (Massagué, 1987). The amount of soluble betaglycan in the samples was quantitated by Western blot analysis using antiserum raised against bacterially expressed betaglycan ectodomain (López-Casillas et al., 1993), and using known amounts of this protein as a standard. The various dilutions of media containing baculoviral betaglycan were made in media conditioned by Sf9 cells infected with wild-type baculovirus, which served as a negative control.

Results

To determine the functional properties of various regions in betaglycan, we modified the rat cDNA (López-Casillas et al., 1991) to generate a panel of betaglycan mutants with deletions of the extracellular endoglin-related domain, the uromodulin-related domain, the membrane anchor or the cytoplasmic domain. We constructed additional mutants in which one or both putative GAG attachment sites were altered by single codon substitutions. To facilitate the isolation and visualization of all betaglycan forms with a common antibody, all constructs included a sequence encoding the human c-myc epitope that is recognized by monoclonal antibody 9E10 (Evans et al., 1985). This epitope tag was engineered six amino acids downstream of the putative signal peptide cleavage site. The ligand-binding properties of myc-tagged betaglycan are indistinguishable from those of wild-type betaglycan (López-Casillas et al., 1993). The structure of the most relevant betaglycan mutants studied are shown schematically in Fig. 1.

Identification of Glycosaminoglycan Attachment Sites

Betaglycan (López-Casillas et al., 1991; Wang et al., 1991) contains 6 serine-glycine sequences of which only Ser^{535}-Gly and Ser^{546}-Gly are surrounded by acidic residues, making them the most likely sites for GAG chain attachment (Bourdon et al., 1987). We mutated either one (in S535A or

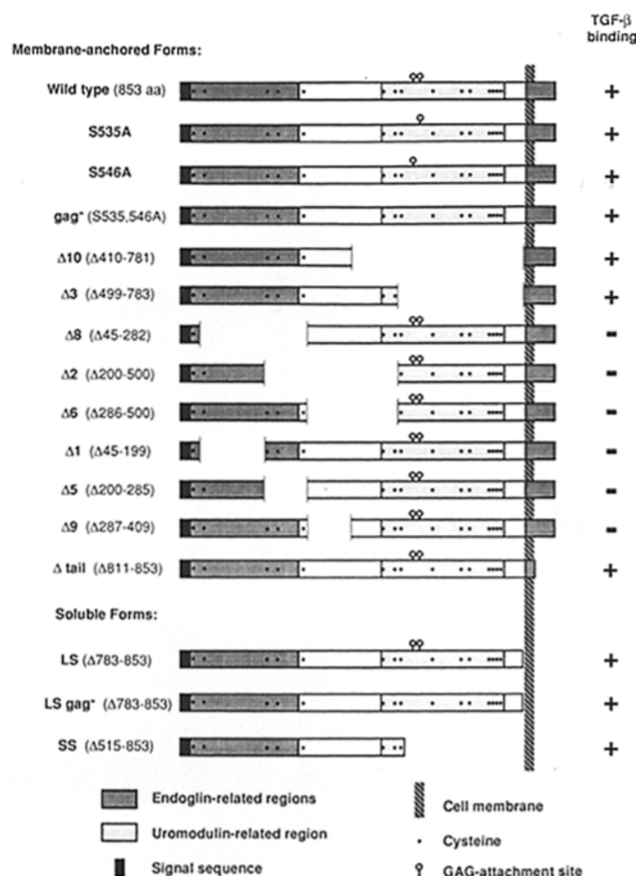

Figure 1. Betaglycan mutants. The various betaglycan constructs analyzed in this study are presented schematically, along with their TGF-β-binding activity. The amino acid positions deleted or modified are indicated in parentheses. Flanking the uromodulin-related domain there are regions (*non-shadowed boxes*) without similarity to known proteins. The first three cysteines within the uromodulin-related domain delimit a short central region of similarity to endoglin. The GAG chain attachment sites, at serines 535 and 546, are indicated.

S546A) or both [in BG(gag^-)] of these serines to alanine, and transiently expressed these constructs in COS-1 cells. Cells were affinity-labeled by incubation with ^{125}I-TGF-β1 and cross-linking with disuccinimidyl suberate. Detergent lysates from these cells were separated by gel electrophoresis and the labeled receptors revealed by autoradiography.

The proteoglycan form of betaglycan typically migrates as a broad species above 200 kD, and its core lacking GAG chains migrates as a doublet at ~130 kD (Fig. 2 *A*; Andres et al., 1991; López-Casillas et al., 1991). Mutation of either serine yielded proteoglycan forms of faster electrophoretic migration (Fig. 2) and increased the proportion of core devoid of GAG chains, the latter effect being more pronounced in the S535A mutant (Fig. 2 *A*). Mutation of both serines completely abolished the addition of GAG chains (lane gag^-, Fig. 2 *A*). The same effect was observed when the double mutant BG(gag^-) was stably expressed in L_6E_9 skeletal rat myoblasts (not shown). These results identified serines 535 and 546 as the sites for GAG attachment, and suggested that betaglycan is heterogeneously glycosylated with some molecules containing only one GAG chain.

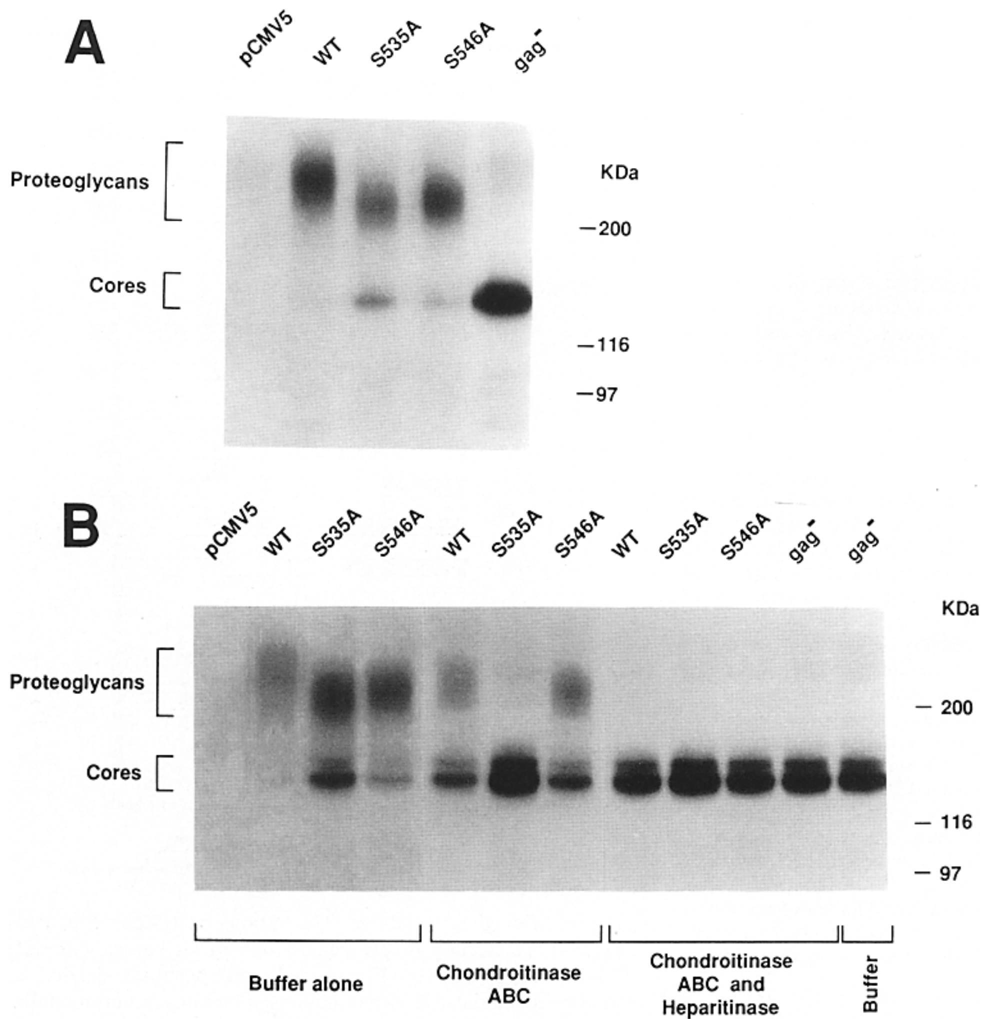

Figure 2. Serines 535 and 546 are the GAG attachment sites. COS-1 cells were transiently transfected with the indicated mutant betaglycan vectors or the empty vector (*pCMV5*), and then affinity labeled with 100 pM of ^{125}I-TGF-β1. Labeled products were subjected directly to SDS-PAGE and autoradiography in *A*. In *B*, they were immunoprecipitated with the antibody 9E10 and then incubated, as indicated, with buffer alone, chondroitinase ABC, or chondroitinase plus heparitinase. Incubations, carried out as described in Materials and Methods, were terminated by heating the samples in SDS-PAGE buffer. The resulting digestion products were subjected directly to SDS-PAGE and autoradiography. Fully glycosylated betaglycans (*Proteoglycans*) and their GAG-less cores (*Cores*) are identified by their characteristic mobility. The size and mobility of protein standards are indicated in kilodaltons (*kDa*).

To determine the type of GAG chains that were attached to these two sites, affinity labeled wild-type and mutant betaglycan forms were immunoprecipitated and subjected to digestion with chondroitinase and heparitinase. Complete removal of GAG chains from wild-type betaglycan required digestion with both enzymes (Fig. 2 *B*), confirming the presence of both heparan sulfate and chondroitin sulfate chains (Segarini and Seyedin, 1988; Cheifetz et al., 1988*a*; López-Casillas et al., 1991). Chondroitinase alone was sufficient to remove all GAG chains from BG(S535A) but had little effect on BG(S546A) (Fig. 2 *B*). Heparitinase was required for full removal of GAG chains from BG(S546A) (Fig. 2 *B*). These results indicated that betaglycan expressed in COS cells carries heparan sulfate attached primarily to Ser^{535} and chondroitin sulfate attached to Ser^{546}.

The concentration of ^{125}I-TGF-β1 (100 pM) used in these binding assays is close to the K_D value of betaglycan (1-4 $\times$ 10^{-10} M; Massagué et al., 1990). Therefore, these assays should reveal any gross alteration of the binding affinity of betaglycan caused by the mutations. The overall level of ligand binding (not shown) and affinity-labeling intensity (Fig. 2) of betaglycan lacking GAG chains were similar to those of the proteoglycan form, indicating that the GAG chains do not have a major effect on TGF-β1 binding to membrane-anchored betaglycan. This result agrees with the observation that cells deficient in GAG biosynthetic pathways express betaglycan as a core devoid of GAG chains but capable of binding TGF-β (Cheifetz and Massagué, 1989).

TGF-β Binds to the Endoglin-related, NH_2-Terminal Domain of Betaglycan

To identify the TGF-β-binding region, betaglycan cDNA constructs harboring deletions that removed various portions of the extracellular domain were transiently transfected in COS-1 cells. Expression of these products on the cell surface was confirmed by cell surface biotinylation using sulfo-NHS-biotin (Staros, 1982), followed by immunoprecipitation of cell lysates with 9E10 antibody and visualization of electrophoretically separated proteins with streptavidin-coupled chemiluminescence (Fig. 3 *A*).

The TGF-β-binding activity of these mutants was determined in binding assays and in affinity-labeled assays, and is summarized in Fig. 1 with representative examples shown in Fig. 3 *B*. Deletion of the extracellular COOH-terminal half downstream of Ile^{410}, such as in BGΔ3 and BGΔ10 (Fig. 3 *B*), did not cause any gross alteration in the ability to bind ^{125}I-TGF-β1 or β2. These two mutant betaglycan forms yielded affinity-labeled products of the expected size, and minor products of approximately twice this size (Fig. 3 *B*). These minor products might represent internally cross-linked betaglycan dimers (Massagué, 1985).

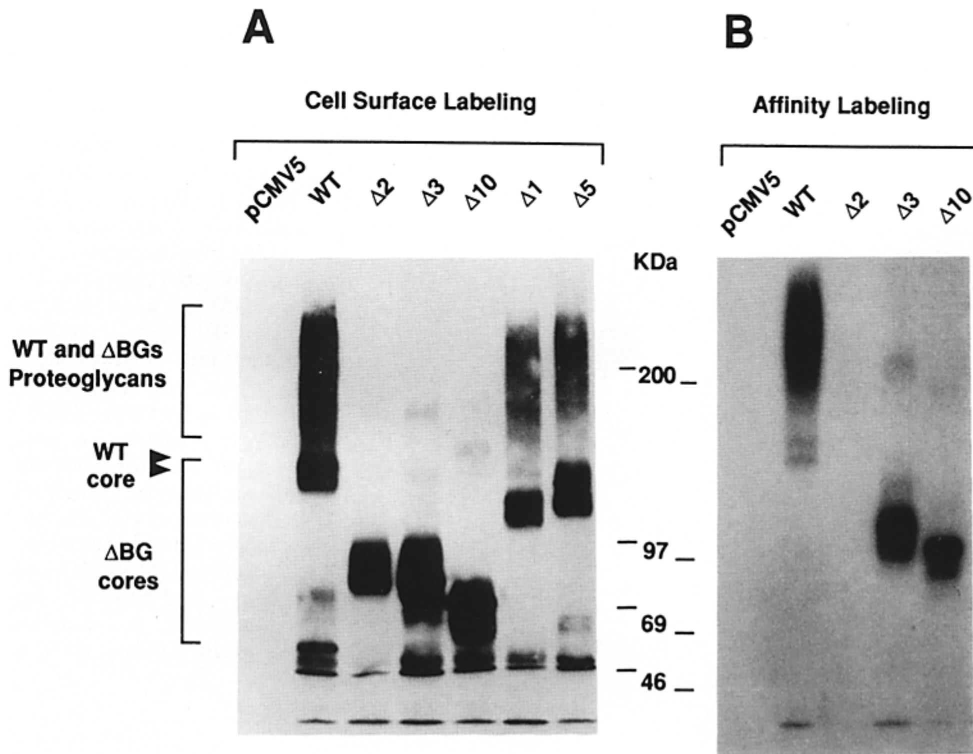

Figure 3. Expression and TGF-β binding activity of betaglycan mutants. COS-1 cells were transiently transfected with the indicated betaglycan constructs or the empty vector (*pCMV5*). Parallel cultures of transfectants were surface-labeled by biotinylation (*A*) or affinity labeled with 100 pM ^{125}I-TGF-β1 (*B*). Cell lysates were immunoprecipitated with antibody 9E10, and the immunoprecipitates analyzed by SDS-PAGE and visualized by streptavidin-coupled chemiluminescence (*A*) or autoradiography (*B*).

Deletion of segments upstream to Ile^{410} yielded products unable to bind ^{125}I-TGF-β1 or β2 (Fig. 3 *B*). The segments removed by these mutations (see Fig. 1) included the NH_2-terminal endoglin-related region (deleted in Δ8) and an adjacent region with no similarity to any known protein (deleted in Δ2). Each of a series of smaller subdomain deletions (Δ1, Δ5, Δ6, and Δ9) affecting these regions also eliminated TGF-β binding. These results indicated that the NH_2-terminal half of the betaglycan extracellular domain including the endoglin-related domain contains the TGF-β-binding site.

Cells expressing wild-type betaglycan produced equivalent proportions of proteoglycan form and GAG-less core (Fig. 3 *A*; Andres et al., 1991; López-Casillas et al., 1991). The GAG-less core from this source labeled poorly with ^{125}I-TGF-β1 (compare the proteoglycan forms with the core forms in the WT lanes of Fig. 3, *A* and *B*). However, the results with the BG(gag$^-$) mutants (see below) and endogenous betaglycan in CHO cells deficient in GAG synthesis (Cheifetz and Massagué, 1989) argue that the binding activity of GAG-less betaglycan is similar to that of the proteoglycan form. Limitations in the ability of COS cells to completely fold and glycosylate all the overexpressed betaglycan may explain the high levels of GAG-less core present in cells transfected with wild-type betaglycan, and the low-binding activity of this core form.

Deletion mutants BG(Δ3) and (Δ10) which lack the COOH-terminal half of the extracellular domain including the two GAG attachment sites (Fig. 1), yielded GAG-less products of 75 and 60 kD, respectively (Fig. 3 *A*), as expected. Unexpectedly, various deletions in the NH_2-terminal half that spared the GAG attachment sites also yielded predominantly GAG-less forms [BG(Δ1), (Δ2) and (Δ5), Fig. 3 *A*; BG(Δ6), (Δ8) and (Δ9), not shown]. This suggested that deletions in the NH_2-terminal region interfere with the use of betaglycan as a substrate for GAG chain attachment, owing perhaps to misfolding of these mutant forms.

The TGF-β Binding Domain Is Sufficient to Support Association of Membrane Betaglycan with the Type II Receptor

In the presence of TGF-β, betaglycan associates with the type II receptor, TβR-II, forming a ternary complex, and we have previously shown that the binding-defective mutant BG(Δ6) does not associate with TβR-II (López-Casillas et al., 1993). To determine whether regions in membrane betaglycan other than the TGF-β binding domain were required for its association with TβR-II, COS-1 cells were cotransfected with betaglycan constructs of interest together with TβR-II tagged with the influenza virus hemagglutinin HA1 epitope (López-Casillas et al., 1993). The cotransfected cells were affinity labeled with ^{125}I-TGF-β1 or β2 and immunoprecipitated with anti-HA1 antibody. This antibody does not precipitate betaglycan expressed alone in COS-1 cells (López-Casillas et al., 1993). Betaglycan mutants lacking all extracellular regions outside the TGF-β-binding domain, or lacking the cytoplasmic region were able to associate with TβR-II, as determined by their ability to coprecipitate with this receptor (Fig. 4). The mutants also retained the ability to enhance the binding of ^{125}I-TGF-β2 to TβR-II (Fig. 4), a previously described property of wild-type betaglycan (López-Casillas et al., 1993). These results indicated that the TGF-β-binding domain of membrane-anchored betaglycan is sufficient for formation of a ternary complex with TGF-β and TβR-II and for enhancement of TGF-β binding to this receptor.

In these experiments, the amount of label associated with TβR-II was lower than the amount of label associated with the coprecipitating betaglycan. It is not possible to draw any

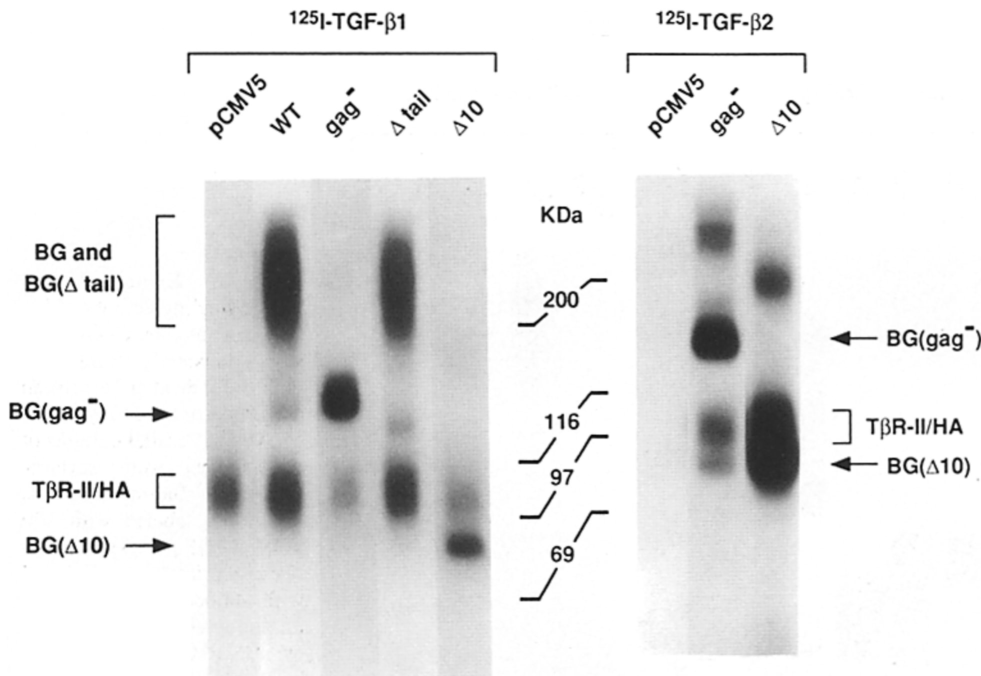

Figure 4. Complex formation between betaglycan and the TGF-β type II receptor. COS-1 cells were cotransfected with the indicated betaglycan mutants or the empty vector (*pCMV5*) and a HA1 epitope tagged TGF-β type II receptor vector. Lysates from cells affinity-labeled with 100 pM ^{125}I-TGF-β1 or 100 pM ^{125}I-TGF-β2 were immunoprecipitated with the anti-HA1 antibody and analyzed by SDS-PAGE and autoradiography. The immunoprecipitated TGF-β type II receptor (*TβR-II/HA*) and coprecipitated molecules are indicated. Migration of molecular weight standards (*kDa*) is shown.

conclusions from these data about the stoichiometry of the complex because the relative efficiency of the ^{125}I-TGF-β cross-linking reactions with betaglycan and TβR-II is not known.

Binding Activity of Soluble Betaglycan

Secreted versions of betaglycan were created by engineering a stop codon at appropriate sites in the cDNA. A long soluble betaglycan form, BG(LS), was generated by placing a stop codon just upstream of the transmembrane region (Fig. 1). A GAG-less version of this form, BG(LS gag$^-$) was similarly constructed using the BG(gag$^-$) cDNA. When transfected into COS-1 cells, these constructs yielded secreted products of the predicted size, as determined by immunoprecipitation or Western blotting of samples from the conditioned media (not shown). ^{125}I-TGF-β1 binding and cross-linking to these products in solution, followed by gel electrophoresis and autoradiography, revealed strong TGF-β-binding activity (Fig. 5). Similar results were obtained if the secreted proteins were isolated with 9E10 antibody bound to protein-G Sepharose beads and then tested for ligand-binding activity (not shown).

A shorter form of soluble betaglycan, BG(SS), containing the TGF-β-binding domain and lacking most of the uromodulin-related domain was generated by inserting a stop codon at position 515 (Fig. 1). When assayed immediately after collection of the conditioned media, the binding activity of this form expressed in COS-1 cells was comparable to that of the larger soluble forms (Fig. 5). However, the stability of these proteins differed greatly. When stored at 4°C, BG(SS) lost all TGF-β-binding activity (as determined by binding and cross-linking assays) within the first 2 d of storage whereas BG(LS) and (LS gag$^-$) showed no significant loss of activity after 2 months of storage at 4°C under sterile conditions (data not shown). The preparations of BG(SS) progressively aggregated forming disulfide-linked complexes, as deter-

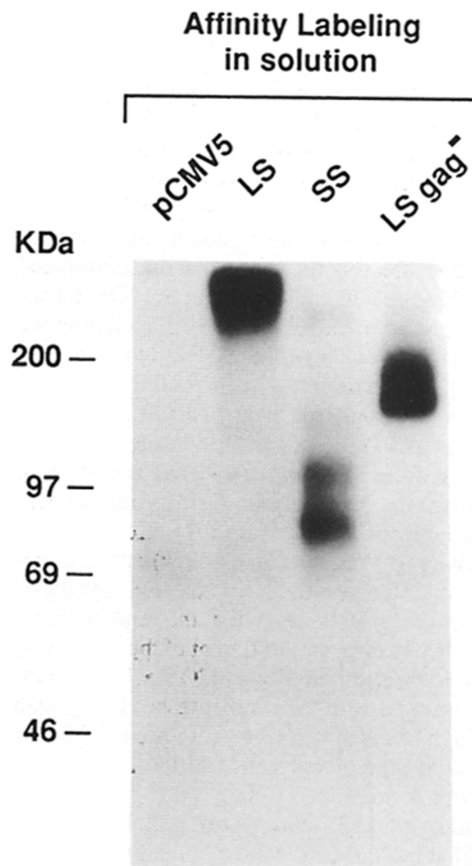

Figure 5. Soluble betaglycan forms bind TGF-β. Samples of freshly collected media from COS-1 cells expressing the indicated betaglycan constructs or the empty vector (*pCMV5*) were affinity-labeled with 370 pM ^{125}I-TGF-β1 and analyzed by SDS-PAGE and autoradiography.

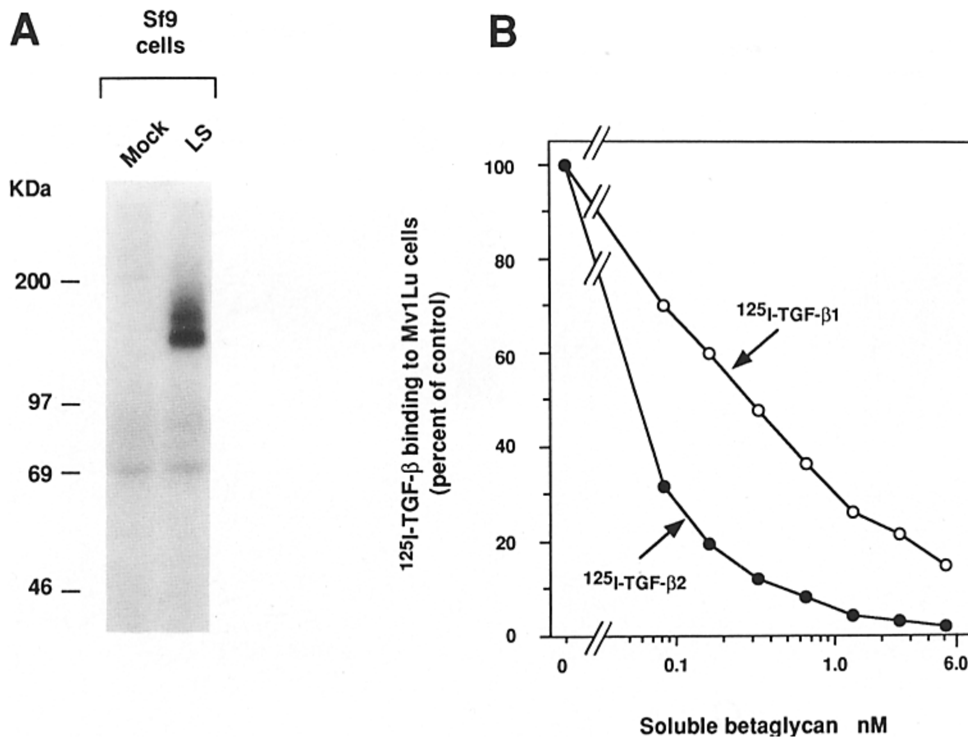

Figure 6. Inhibition of TGF-β binding to MvlLu cells by soluble betaglycan. (*A*) Media conditioned by Sf9 insect cells, infected with wild-type baculovirus (*mock*) or a recombinant baculovirus containing the BG(LS) construct, were analyzed by affinity labeling. (*B*) MvlLu cells were incubated with 25 pM ^{125}I-TGF-β1 (*open symbols*) or 25 pM ^{125}I-TGF-β2 (*closed symbols*) in the presence of Sf9-conditioned medium containing the indicated concentrations of soluble betaglycan. The betaglycan concentration was determined as described under Materials and Methods. The various dilutions of media containing baculoviral betaglycan were made in media conditioned by Sf9 cells infected with wild-type baculovirus, which therefore served as a negative control. At the end of the incubation, cell monolayers were washed with binding buffer, and the associated radioactivity was determined. The amount of bound TGF-β is plotted relative to the amount bound in cell monolayers that received no exogenous betaglycan.

mined by Western blotting of samples electrophoresed in the absence of reductant (data not shown). A shorter form created by introduction of a stop codon at position 410 was more stable than BG(SS) but in contrast to its membrane-anchored counterpart, this form showed markedly decreased TGF-β-binding activity (data not shown). These results confirmed that the NH_2-terminal half of betaglycan is sufficient for TGF-β binding, but removal of segments outside this region may severely decrease the stability or binding activity of soluble betaglycan.

Inhibition of TGF-β Binding to Membrane Receptors by Soluble Betaglycan

Cells release soluble betaglycan by proteolytic cleavage at a site near the transmembrane region (Andres et al., 1989; López-Casillas et al., 1991). Since this form is similar in size to BG(LS), we used this recombinant protein to study the ability of soluble betaglycan to affect TGF-β binding to cell surface receptors. The BG(LS) cDNA, subcloned into a baculovirus vector and expressed in insect Sf9 cells, yielded high levels of secreted protein capable of binding TGF-β (Fig. 6 *A*). The baculoviral BG(LS) was devoid of GAG chains (Fig. 6 *A*). When added to MvlLu cell cultures, medium conditioned by Sf9 cells infected with wild-type baculovirus, induced a loss of GAG chains from cell surface betaglycan (data not shown, but see Fig. 7 *A*). This result suggested that a glycosaminidase present in these preparations was responsible for the removal of GAG chains from baculoviral betaglycan. Nevertheless, the stability and TGF-β-binding activity of the GAG-less BG(LS) produced in Sf9 cells was similar to that of BG(LS) produced in COS-1 cells.

The ability of soluble betaglycan to affect the TGF-β interaction with cell surface receptors was tested using MvlLu mink lung epithelial cells that are very sensitive to growth inhibition by TGF-β and have well characterized TGF-β receptors (Boyd and Massagué, 1989; Laiho et al., 1990*a*,*b*; Wrana et al., 1992). Addition of soluble betaglycan from insect cells during incubation of MvlLu cells with 25 pM ^{125}I-TGF-β1 or β2 strongly inhibited binding of these ligands to cell surface receptors (Fig. 6 *B*). The various dilutions of media containing baculoviral betaglycan were made in media conditioned by Sf9 cells infected with wild-type baculovirus, which served as a negative control. The betaglycan concentration required for a 50% of inhibition of binding was 0.3 nM for ^{125}I-TGF-β1 and ~50 pM for ^{125}I-TGF-β2. At the highest concentrations tested (5.2 nM), betaglycan reduced ^{125}I-TGF-β1 binding by 80% and ^{125}I-TGF-β2 binding by >98% (Fig. 6 *B*). This difference between isoforms is consistent with the higher affinity of betaglycan for TGF-β2 (Andres et al., 1991; Mitchell et al., 1992; Segarini et al., 1987).

A similar inhibitory effect of soluble betaglycan was observed when TGF-β interaction with cell surface receptors was determined by receptor affinity-labeling. Soluble betaglycan inhibited the labeling of type I and II receptors with an ID_{50} of 0.3 nM when 25 pM ^{125}I-TGF-β1 was used as the ligand and ~80 pM with 25 pM ^{125}I-TGF-β2 as the ligand (Fig. 7). Complete inhibition of the ^{125}I-TGF-β2 binding was observed with 1 nM soluble betaglycan, and nearly complete inhibition of ^{125}I-TGF-β1 binding with 5 nM soluble betaglycan.

Soluble Betaglycan Acts as a TGF-β Antagonist

To test whether addition of soluble betaglycan would inhibit

Figure 7. Inhibition of TGF-β binding to signaling receptors by soluble betaglycan. MvlLu cells were incubated with 25 pM ^{125}I-TGF-β1 or 25 pM ^{125}I-TGF-β2 in the presence of the indicated concentrations of baculoviral soluble betaglycan, as described in Fig. 6. After washing unbound ligand, cell monolayers were incubated with the bifunctional crosslinker (disuccinimidyl suberate), and the resulting affinity-labeled products were subjected to SDS-PAGE and revealed by autoradiography (*A*). The characteristic mobilities of the MvlLu TGF-β receptors (*BG, BG core, TβR-II, and TβR-I*) are indicated. The relatively high proportion of GAG-less BG core seen in this experiment is due to glycosaminidase activity present in the Sf9 conditioned media (see text). This activity does not affect the labeling of the other receptors. The relative labeling intensity of the TGF-β type I receptor band (*B*) and type II receptor band (*C*) were quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Certain betaglycan concentrations caused an apparently complete inhibition of receptor labeling with ^{125}I-TGF-β2 as detected by autoradiography. However, the higher sensitivity of the Phosphorimager revealed that the level of inhibition in some of these conditions was still partial (compare *A* with *B* and *C*). (*open symbols*) ^{125}I-TGF-β1; (*closed symbols*) ^{125}I-TGF-β2.

the biological activity of TGF-β, we determined its effect on the growth inhibitory response of MvlLu cells to TGF-β as measured by ^{125}I-deoxyuridine incorporation into DNA (Laiho et al., 1990*a*). Addition of 5.8 nM soluble baculoviral betaglycan decreased the growth inhibitory potency of TGF-β2 approximately 20-fold, from ED_{50} = 0.5 pM to ED_{50} = 10 pM (Fig. 8 *A*). Soluble betaglycan reduced the effect of TGF-β2 in a concentration-dependent manner (Fig. 8 *B*), correlating closely with the inhibition of TGF-β2 binding to receptors. For example, a reduction of the effective TGF-β2 concentration from 5 pM to 0.5 pM, or 90%, would decrease the growth inhibitory effect by 50% (Fig. 8 *A*). Indeed, addition of 0.6 nM soluble betaglycan, which reduced TGF-β2 binding to cell surface receptors by ~90% (see Figs. 6 and 7), decreased the growth inhibitory effect of 5 pM TGF-β2 by 50% (Fig. 8 *B*). As expected from its lower potency as

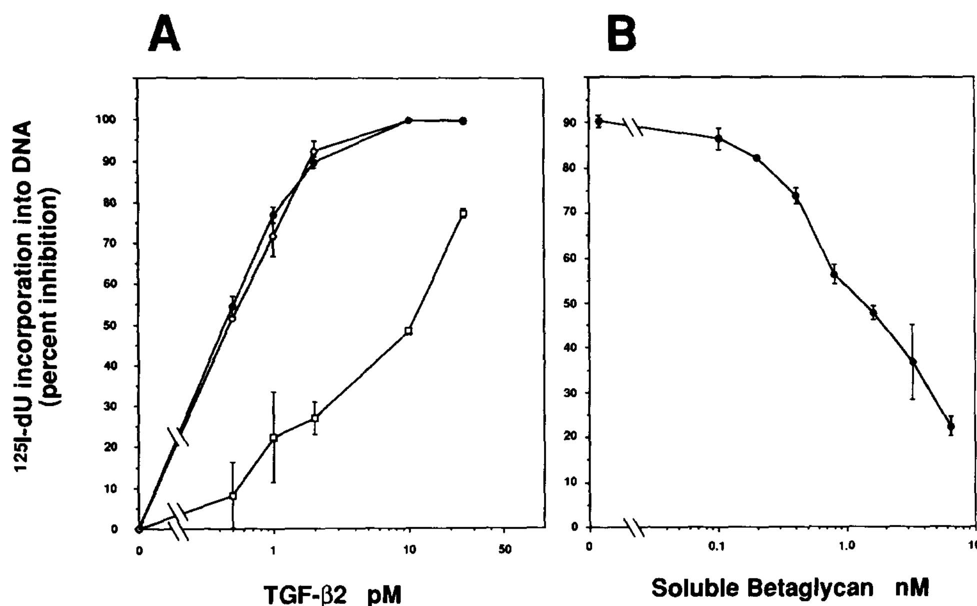

Figure 8. Inhibition of TGF-β action by soluble betaglycan. MvlLu cells were incubated for 24 h with the indicated concentrations of TGF-β2 and Sf9 conditioned media, and allowed to incorporate ^{125}I-deoxyuridine into DNA during the last 4 h of this incubation. Before their use in these assays, conditioned media from Sf9 cells were dialyzed against standard growth inhibition assay media. In *A*, incubations were done in standard media (*open circles*), dialyzed conditioned media from Sf9 cells infected with wild-type baculovirus (*closed circles*), or dialyzed conditioned media from Sf9 cells containing 5.8 nM soluble baculoviral betaglycan (*open squares*). In *B*, cells were incubated with 5 pM TGF-β2 added in dialyzed conditioned media from betaglycan-secreting Sf9 cells mixed with control conditioned media (Sf9 cells infected with wild-type baculovirus) to achieve the indicated final concentrations of soluble baculoviral betaglycan. Data are average from triplicate assays, ±standard errors.

an inhibitor of TGF-β1 binding, soluble betaglycan added at 5.8 nM decreased the growth inhibitory potency of TGF-β1 only ~50% (data not shown).

Discussion

Betaglycan is a structurally complex membrane proteoglycan, and the present studies investigate the functional role of its various domains. One of the most significant outcomes of this work is the finding that whereas membrane-anchored betaglycan acts as an enhancer of TGF-β action, the released form of betaglycan acts as a TGF-β antagonist by preventing its access to cell surface receptors.

Glycosaminoglycan Attachment Sites in Betaglycan

The present studies confirm the earlier prediction (López-Casillas et al., 1991) that Ser[535] and Ser[546] are the GAG chain attachment sites in betaglycan. These residues are located in Ser-Gly sequences surrounded by acidic residues, which is consensual for GAG chain attachment (Bourdon et al., 1987). Mutation of either serine to alanine decreases the extent of betaglycan glycosylation, and mutation of both serines prevents GAG chain attachment completely.

In most cell lines examined including COS cells, betaglycan contains both heparan sulfate and chondroitin sulfate chains as determined by digestion with heparitinase and chondroitinase (Segarini and Seyedin, 1988; Cheifetz et al., 1988*a*; López-Casillas et al., 1991). Mutant betaglycan with Ser[546] as the sole GAG attachment site was completely converted to GAG-less core by chondroitinase alone, indicating that Ser[546] carries only chondroitin sulfate chains. Betaglycan with Ser[535] as the sole GAG attachment site showed some susceptibility to digestion by chondroitinase and more to heparitinase, indicating that Ser[535] carries predominantly heparan sulfate chains.

The GAG-less betaglycan cores obtained by enzymatic removal of the GAG chains or by mutational elimination of the GAG attachment sites are heterogeneous in size. The heterogeneity is not eliminated by enzymatic or chemical treatments that remove other carbohydrate chains from the GAG-less core (Cheifetz et al., 1988*a*). This heterogeneity is also observed by surface-biotinylation of betaglycan core (this report). We have found no evidence for alternative betaglycan transcripts. Therefore, the basis for this heterogeneity remains unknown.

The NH_2-Terminal Endoglin-related Domain in Membrane Betaglycan Binds and Presents TGF-β

The NH_2-terminal region of betaglycan shows only 28% amino acid sequence similarity to the corresponding region of endoglin, another TGF-β-binding protein (Bork and Sander, 1992; Cheifetz et al., 1992). Our deletion analysis confirmed that in betaglycan this region, with a COOH-terminal extension, mediates TGF-β binding, and other regions are not critical for this function. Furthermore, in membrane-anchored betaglycan, the NH_2-terminal endoglin-related region is also sufficient to present bound TGF-β to the signaling type II receptor. The GAG chains, the uromodulin-related region which contains an additional short segment of similarity to endoglin, and the cytoplasmic domain which shows the highest level of sequence identity to endoglin (López-Casillas et al., 1991; Wang et al., 1991; see Fig. 1), are dispensable for TGF-β presentation to the signaling receptor. However, membrane anchorage of betaglycan seems critical for complex formation with receptor II and enhancement of ligand binding to this receptor. This conclusion is based on the observed inability of soluble betaglycan to perform these functions.

The preference of receptor II for TGF-β tethered to be-

taglycan over TGF-β free in the medium might be due to immobilization of the ligand at the cell surface. However, even accounting for the higher affinity of betaglycan for TGF-β2, this model seems too simple to explain the much stronger enhancement of TGF-β2 binding to receptor II by betaglycan. It is possible that TGF-β bound to betaglycan adopts a conformation that is more favorable for recognition by receptor II; unbound TGF-β1 might approximate such conformation better than unbound TGF-β2.

The present results predict that the TGF-β-binding site in endoglin is located in the NH_2-terminal betaglycan-related domain. The limited amino acid sequence similarity between these domains in betaglycan and endoglin correlates with a markedly different ability to bind TGF-β2. Betaglycan binds TGF-β2 somewhat better than it binds TGF-β1 or β3 whereas endoglin does not bind TGF-β2 (Cheifetz et al., 1992).

The Uromodulin-related Domain Is Not Critical for Interaction with TGF-β or Its Signaling Receptors

The region in betaglycan that is related to the proteins uromodulin, pancreatic zymogen granule protein GP-2, and sperm receptors Zp2 and Zp3, is characterized by a certain pattern of cysteines, hydrophobic, polar or turn-forming residues that are thought to generate related three-dimensional structures (Bork and Sander, 1992). The GAG attachment sites in betaglycan, serines 535 and 546, reside in this region. Except for the ability of the heparan sulfate GAG chains to bind bFGF (Andres et al., 1992), the function of this region remains unknown. Its deletion does not have a major effect on the TGF-β binding or presentation activities of betaglycan, although soluble betaglycan devoid of most of this region is unstable. It is noteworthy that all known proteins containing the uromodulin-related domain are either membrane proteins that yield soluble forms (betaglycan, uromodulin, and GP-2) (Andres et al., 1989; Hoops and Rindler, 1991; Rindler et al., 1990), or proteins that reside in the pericellular environment (Zp2 and 3) (Saling, 1991). This raises the possibility that the function of the uromodulin-related domain is to control the stability, diffusion, or interactions of these proteins in the pericellular environment. However, our truncated betaglycan form might be unstable for other reasons, and the present evidence is therefore insufficient to demonstrate a primary role of the uromodulin-related domain in the stabilization of these proteins.

Our results are in contrast to those reported by Fukushima et al. (1993) who localized TGF-β binding to a segment of the uromodulin-related domain. However, their studies used bacterially expressed fragments of betaglycan that bind TGF-β with a much lower affinity than we observed for the NH_2-terminal TGF-β-binding domain expressed in mammalian cells.

The Released Form of Betaglycan Is a TGF-β Antagonist

Several signaling cytokine receptors exist in soluble forms that are generated by cleavage of the membrane form or by exclusion of membrane and cytoplasmic-coding exons from their transcripts (Ehlers and Riordan, 1991). Some of these soluble forms may act as ligand antagonists, as has been shown with the TNF, IL-1, and IL-4 receptors (Howard et al., 1993; Loetscher et al., 1991; Maliszewski et al., 1990) whereas others such as the soluble CNTF receptor α still contribute positively to the assembly of a signaling receptor complex (Davis et al., 1993). The present results show that in contrast to membrane betaglycan, soluble betaglycan inhibits TGF-β binding to membrane receptors and acts as a TGF-β antagonist. Thus, betaglycan can play a dual role as a modulator of TGF-β access to signaling receptors.

Membrane anchorage is critical for the role of betaglycan as a regulator of TGF-β access to the signaling receptors. Membrane-anchored betaglycan binds TGF-β independently from the signaling receptors, and can form a ternary complex with receptor II through bound TGF-β. This phenomenon, observed in all cell lines examined, is thought to reflect a process of TGF-β presentation and transfer to the signaling receptor (López-Casillas et al., 1993). Membrane betaglycan enhances TGF-β binding to the signaling receptors and cell responsiveness to TGF-β, particularly to TGF-β2, suggesting that the signaling receptor binds TGF-β tethered to membrane betaglycan better than it binds TGF-β free in the medium (López-Casillas et al., 1993). The soluble form of betaglycan can also bind TGF-β independently of other receptors, however it does not form stable associations with membrane-anchored receptors. Rather, soluble betaglycan interferes with TGF-β access to these receptors and therefore inhibits TGF-β action. The ratio between soluble and membrane-anchored forms of betaglycan in the cell's periphery is probably an important determinant of TGF-β activity. It will be important to investigate whether the release of betaglycan ectodomain is a regulated process.

Given its high affinity and specificity for TGF-β, its nature as a physiological TGF-β receptor, and its potency as an inhibitor of TGF-β action, soluble betaglycan may prove advantageous as an anti-TGF-β agent in vivo. TGF-β is a regulator of cell proliferation, differentiation and extracellular matrix accumulation in many tissues (Massagué, 1990; Roberts and Sporn, 1990). Its excess is thought to contribute to pathological processes of inflammation, immune response (Kulkarni et al., 1993; Shull et al., 1992; Wahl, 1992), and fibrotic disorders (Border and Ruoslahti, 1992; Sellheyer et al., 1993). In animal model systems, administration of TGF-β antibodies or decorin, a small extracellular matrix proteoglycan that binds TGF-β, show beneficial effects in some of these disorders (Border et al., 1992; Border et al., 1990; Yamaguchi et al., 1990). Betaglycan could be useful for similar purposes. In addition to being able to bind TGF-β with a K_D in the subnanomolar range, betaglycan can act selectively on different TGF-β isoforms. TGF-β2 has a higher affinity for betaglycan than TGF-β1, and a higher dependence on betaglycan for access to the signaling receptors (Andres et al., 1991; López-Casillas et al., 1993; Mitchell et al., 1992; Segarini et al., 1987). TGF-β2 might therefore be the isoform most susceptible to the action of betaglycan in vivo, and the other isoforms might rank according to their binding affinity (Cheifetz et al., 1988*b*, 1990).

Our present work, using cell lines in culture, has revealed contrasting functions for soluble and membrane betaglycan as a modulator of TGF-β access to its signaling receptors. Experiments in animal model systems will be needed to establish whether or not betaglycan possesses similar dual functionality in vivo, and to prove its usefulness as a modulator of TGF-β activity.

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