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Betaglycan has Two Independent Domains Required for High Affinity TGF-β Binding: Proteolytic Cleavage Separates the Domains and Inactivates the Neutralizing Activity of the Soluble Receptor[†]

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ABSTRACT: Betaglycan is a coreceptor for members of the transforming growth factor β (TGF-β) superfamily. Mutagenesis has identified two ligand binding regions, one at the membrane-distal and the other at the membrane-proximal half of the betaglycan ectodomain. Here we show that partial plasmin digestion of soluble betaglycan produces two proteolysis-resistant fragments of 45 and 55 kDa, consistent with the predicted secondary structure, which indicates an intervening nonstructured linker region separating the highly structured N- and C-terminal domains. Amino terminal sequencing indicates that the 45 and 55 kDa fragments correspond, respectively, to the membrane-distal and -proximal regions. Plasmin treatment of membrane betaglycan results in the production of equivalent proteolysis-resistant fragments. The 45 and 55 kDa fragments, as well as their recombinant soluble counterparts, Sol Δ10 and Sol Δ11, bind TGF-β, but nonetheless, compared to intact soluble betaglycan, have a severely diminished ability to block TGF-β activity. Surface plasmon resonance (SPR) analysis indicates that soluble betaglycan has K_d's in the low nanomolar range for the three TGF-β isoforms, while those for Sol Δ10 and Sol Δ11 are 1−2 orders of magnitude higher. SPR analysis further shows that the K_d's of Sol Δ11 are not changed in the presence of Sol Δ10, indicating that the high affinity of soluble betaglycan is a consequence of tethering the domains together. Overall, these results suggest that betaglycan ectodomain exhibits a bilobular structure in which each lobule folds independently and binds TGF-β through distinct nonoverlapping interfaces and that linker modification may be an approach to improve soluble betaglycan's TGF-β neutralizing activity.

Betaglycan (BG)[1] is a membrane proteoglycan that serves as a coreceptor for diverse members of the transforming growth factor β (TGF-β) superfamily of autocrine and paracrine factors. These factors are involved in diverse biological functions that include, among others, embryonic development, cell differentiation and proliferation, control of the immune response, and wound repair (*1*). BG binds TGF-β superfamily factors with a characteristic selectivity: TGF-β2 > TGF-β1 > inhibin A, and establishes ligand-dependent complexes with several type II receptors (*2*). The TGF-β type II receptor:TGF-β:betaglycan complex, for example, positively regulates TGF-β effects, a function that is particularly important for TGF-β2, which binds TβRII weakly (*3*−*5*). On the other hand, the activin type II receptor:inhibin A:betaglycan complex negatively regulates activin effects, which is the only functionally relevant inhibin receptor identified to date (*6*, *7*). The BMP type II receptor: inhibin A:betaglycan complex has similar negative effects, providing a mechanism for the inhibin antagonism of some BMPs actions (*8*). In view of these regulatory interactions that modulate such powerful morphogens as TGF-βs, BMPs, and activins, it is not surprising that the betaglycan null mutant mice exhibit embryonic lethality (*9*).

The soluble form of betaglycan (Sol BG), which is generated under normal physiological conditions by ectodomain shedding of the membrane bound receptor (*10*−*12*), plays a different role; it is a very potent neutralizing agent of TGF-β actions (*13*, *14*) and is responsible for the betaglycan's tumor suppressor activity (*15*). This activity is instrumental in the suppression of tumor growth and angiogenesis in xenograft models of human cancer (*16*, *17*), raising the expectation of its eventual use as a human therapeutic agent in cancer and other diseases in which TGF-β plays a central pathophysiological role (*18*, *19*).

In order to better understand the structure and function of this important and versatile coreceptor, we have investigated the structure of the betaglycan ectodomain. Previous mutagenesis studies showed the existence of two ligand-binding regions within

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[1]Abbreviations: TGF-β, transforming growth factor β; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; TβRIII, TGF-β type III receptor; BG, betaglycan; Sol BG, soluble betaglycan; Sol Δ10, soluble betaglycan endoglin-like domain; Sol Δ11, soluble betaglycan uromodulin-like domain; SPR, surface plasmon resonance; GAG, glycosaminoglycan.

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the betaglycan ectodomain: the membrane-distal amino-terminal half (the endoglin-like or E-domain) and the membrane-proximal carboxy-terminal half (the uromodulin-like or U-domain) (*13*, *20*, *21*). However, because of the nature of this experimental approach, there is the possibility that deletion of discrete portions of the ectodomain could endow the resulting mutants with artificial binding properties distinct from those of the wild type receptor. This prompted us to further investigate the subdomain structure of the betaglycan ectodomain using limited proteolysis and binding studies. These indicate that the ectodomain of the native receptor is indeed formed by two independent subdomains and that these contact the ligand through distinct nonoverlapping sites. The separation of the subdomains reduces soluble betaglycan affinity for TGF-β and abolishes its TGF-β neutralizing activity.

MATERIALS AND METHODS

Materials. Recombinant Sol BG and the truncated mutants Sol Δ10 and Sol Δ11 were expressed and purified as described previously (*14*, *20*). Human plasmin (10 U/mL, at 8 U/mg, E.C. *3*.4.21.7) was from Roche Molecular Biochemicals (Indianapolis, IN). The BG polyclonal antiserum (#822), and the c-myc, Flag, HA epitope monoclonal antibodies were obtained as previously described (*12*−*14*). TGF-β1 was from R&D Systems, Inc., MN. The isoforms TGF-β2 and TGF-β3 were kind gifts from Dr. N. Cerletti (Ciba Geigy, Basel, Switzerland). Molecular weight standards were from Gibco BRL.

Secondary Structure Prediction. The secondary structure prediction was carried out using the Jpred algorithm, which uses hidden Markov modeling (*22*), as well as the PROF and SSPro algorithms, which utilize neural networks (*23*, *24*). Prediction accuracies for each of these algorithms are reported to be 75% or higher.

Cleavage of Soluble Betaglycan by Plasmin. Ten micrograms of recombinant soluble betaglycan was incubated at 30 °C with 2.5 μg of human plasmin (20 mU) in 100 mM NaH_2PO_4, 150 mM NaCl, pH 6.0 in a final volume of 200 μL. Digestion was stopped by addition of 1 mM of PMSF and its products were revealed by SDS−PAGE on 9% polyacrylamide slab reducing gels. After electrophoresis, gels were stained by the silver nitrate technique, or blotted onto nitrocellulose membranes for immunodetection. Nitrocellulose was blocked in 5% nonfat dry milk in PBS (pH 7.5), 0.05% Tween 20, and primary antibodies used at 1:2000 dilution. Secondary peroxidase-coupled antibodies (Sigma, Saint Louis, MO) were used at a 1:5000 dilution and revealed by chemiluminiscence (Amersham Pharmacia Biotech, NJ).

Plasmin Cleavage of Membrane Betaglycan. COS1 cells were transfected with the plasmid HA gag$^-$ Flag or with the empty pCMV5 expression vector using the DEAE dextran method (*13*). The HA gag$^-$ Flag plasmid encodes a double tagged BG incapable of attaching GAG chains (*12*). Two days after transfection, cells were metabolic labeled with ^{35}S-methionine/cysteine (120 min pulse, 135 min chase), washed 3 times with KRH buffer at room temperature, and then treated with plasmin (160 mU, 20 μg of plasmin in 600 μL of KRH for each 3.5 cm-diameter dishes) for 60 min at 37 °C. After incubation, the media were collected and plasmin inactivated by addition of 1 mM PMSF; the cells were washed twice in a saline Tris buffer containing 1 mM PMSF, then detached and lysed in presence of protease inhibitors. Media and cells lysates were immunoprecipitated with antiserum #822 or the anti-Flag or anti-HA monoclonal antibodies. Immune complexes were recovered with protein A-sepharose, separated by SDS−PAGE and revealed by Phosphorimager scanning.

Size Exclusion Chromatography Analysis of Soluble Betaglycan Proteolytic Fragments. Dimeric state of plasmin-cleaved soluble betaglycan was determined by gel filtration as described previously (*14*). Briefly, 30−50 μg of protein were analyzed in a column BioSep Sec-S3000 (300 × 7.8 mm) with the HPLC system Beckman Gold 126 using 25 mM NaH_2PO_4, 150 mM NaCl, pH 6.8 as mobile phase with a flow of 1 mL/min. Protein was followed at 280 nm. Fractions of gel filtration was collected manually and analyzed by SDS−PAGE and silver nitrate staining.

N-Terminal Sequencing of Soluble Betaglycan Proteolytic Fragments. The N-terminal sequence of plasmin-cleaved betaglycan products was done as described before (*14*). Briefly, protein samples were prepared by SDS−PAGE followed by electroblotting on PVDF membranes (Millipore, MA) and revealed by Coomassie staining. Sliced bands were subjected to automated Edman degradation on a gas-phase protein sequencer (LF 3000, Beckman Instruments) equipped with an on line Beckman system Gold high performance liquid chromatography system. The HPLC equipment included a model 126 pump and a 168 diode array detector setting at 268 and 293 nm for signal and reference, respectively. The HPLC column used was a Beckman Spherogel Micro PTH (2 × 150 mm). The standard Beckman sequencing reagents were used for the analyses.

Isolation of Soluble Betaglycan Proteolytic Fragments. Two hundred micrograms of recombinant betaglycan was incubated for 2 h at 30 °C with 150 mU of human plasmin in 100 mM NaH_2PO_4, 150 mM NaCl, pH 6.0 in a final volume of 2 mL. Digestion was stopped by addition of 1 mM of PMSF. Plasmin-cleaved betaglycan preparation was incubated with 50 μL of Sepharose-Ni^{2+} slurry (Amersham Pharmacia Biotech, NJ) for 1 h at room temperature, in presence of 0.01% of Triton X-100. Beads were pelleted by centrifugation (2 min at 6000 rpm at 4 °C) and the unbound supernatant material were collected. Beads were washed 3 times with 25 mm HEPES, 1 M KCl, 0.1% Triton X-100, pH 6.0 and one more time with plain PBS alone. The C-terminal fragment was eluted by incubation of beads with 400 μL of 100 mm EDTA in PBS, pH 7.5 for 30 min at 4 °C; the eluate of a second elution step was pooled with the first one. Finally, unbound material and eluates were dialyzed against PBS buffer and concentrated in a Speed-Vac (Sorvall) system. Isolation of fragments for TGF-β binding and luciferase assays was done replacing 0.1% Triton X-100 with 0.3 M urea and 0.002% Triton X-100 and concentration step was done with Centricon-10 tubes (Millipore, MA).

TGF-β Affinity Labeling Assays. Affinity labeling and TGF-β ^{125}I-iodination were performed as described before (*13*). Briefly, 50 ng of protein samples were incubated with 100 pM of ^{125}I-labeled TGF-β1, TGF-β2, or TGF-β3 for 3 h at 4 °C. For competition assays, this incubation was carried out in the presence of the indicated concentration of nonlabeled TGF-β. At the end of binding, proteins were cross-linked with 0.1 mg/mL disuccinimidyl suberate (DSS, Pierce Chem, Co., IL) for 15 min and then the reaction was stopped by adding 10 mM Tris. For immunoprecipitations, cross-linked samples were incubated with antiserum #822 (1:200 dilution) or with the c-myc monoclonal antibody (1:200 dilution) and immune complexes recovered with protein A-sepharose (Sigma Co., Mo). Affinity labeled proteins were analyzed by SDS−PAGE under reducing

conditions and phosphorimagining (Storm Instrument, Molecular Dynamics).

TGF-β Reporter Luciferase Assay. The p3TP-lux TGF-β reporter assay was performed as previously described (*14*). Briefly, Mv1Lu cells, at 25% of confluence, were transfected with 30 μg of the p3TP-lux plasmid by the DEAE-dextran method. The day after transfection, cells were plated in 24-well tissue culture dishes at 5×10^5 cells per well and incubated overnight. TGF-β and the 45 or 55 kDa fragments or the Sol Δ10 or Sol Δ11 recombinant proteins were added at the indicated concentrations in DMEM with 0.2% FBS and incubation was continued for 18 h a 37 °C. Cells were washed with PBS and prepared and assayed for luciferase activity using the Luciferase Assay System (Promega, MI). All assays were performed in triplicate with error bars representing the standard error of the mean of at least three samples.

Surface Plasmon Resonance measurements. Binding studies were performed with a Biacore 3000 surface plasmon resonance instrument (GE Healthcare) and were analyzed using the software package Scrubber 2 (Biologic Software). TGF-β1, TGF-β2, and TGF-β3 were biotinylated and captured at a density of 100 RU or less onto a carboxymethylated dextran chip (CM5, GE Healthcare) preimmobilized with about 5000 RU streptavidin on all flow cells. TGF-β2 was biotinylated in 25 mM MES (pH 4.8) by first activating it with a 10-fold molar excess of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; GE Healthcare) in the presence of a 25-fold molar excess of and N-hydroxy succinimide (NHS; GE Healthcare) and then by adding a 100-fold molar excess of the reagent amine-PEO_3-biotin (Pierce). Excess reagents were removed by dialysis. TGF-β1 and -β3 were biotinylated in 10 mM HEPES buffer at pH 7.5 in the presence of a 3-fold molar excess of TβRII ectodomain by adding a 2-fold molar excess of Sulfo-NHS-LC-LC-Biotin (Pierce). Biotinylated ligands were then separated from excess reagents, as well as TβRII ectodomain, by applying them to a Source S cation exchange column (GE Healthcare) in the presence of 30% isopropanol in 20 mM sodium acetate, pH 4.0. Receptors were prepared for analysis by dialyzing them into HBS buffer (10 mM HEPES, 3 mM EDTA, 150 mM NaCl) supplemented with 5 mM CHAPS, at pH 7.4. Binding assays were performed by injecting 2-fold serial dilutions of the receptors in duplicate or triplicate in HBS buffer (pH 7.4) supplemented with 5 mM CHAPS at a flow rate of either 50 μL/min (Sol Δ10 or Sol Δ11) or 5 μL/min (full-length Sol BG). To assess the effect of the Sol Δ10 on the binding of Sol Δ11, coinjection experiments were performed wherein 2 μM Sol Δ10 was first injected, followed by 2-fold dilutions of Sol Δ11 in the presence of 2 μM Sol Δ10. The flow rate for the coinjection experiments was 10 μL/min. Surfaces were regenerated between cycles by a brief (10 s) injection of 4 M guanidine hydrochloride at a flow rate of 100 μL/min. Instrument noise was removed by referencing the data against three or more buffer blank injections, while background signal, caused by nonspecific absorption of the receptors to the surface, was eliminated by referencing the data against a flow cell containing only immobilized streptavidin. Equilibrium analyses were performed by averaging the steady-state response near the end of the injection, RU_{max}, and by fitting this as a function of receptor concentration, [R], to eq 1

$$RU_{max} = \frac{R_{max}[R]}{K_d + [R]} \quad (1)$$

where K_d is equilibrium dissociation constant and R_{max} is the maximal response at a saturating concentration of receptor.

RESULTS

Secondary Structure Prediction of the Betaglycan Extracellular Domains Reveals Two Structured Regions Separated by an Unstructured Linker. Mutagenesis analysis of betaglycan has identified two independent regions in its ectodomain with ligand binding activity (*13*, *20*, *21*). The membrane distal region is known as the E-domain, based on its similarity to the protein endoglin, while the membrane-proximal region is known as the U-domain, based on its similarity with the protein uromodulin. To further investigate the subdomain structure of the betaglycan extracellular domain, we performed secondary structure analysis using hidden Markov model and neural network based methods (*22*−*24*). This analysis demonstrates that the betaglycan extracellular domain is comprised of two highly structured regions joined by an unstructured linker of roughly 50 amino acids (residues 380−430, Supplementary Figure 1, Supporting Information). As expected, the structured regions correspond to the previously characterized Δ10 and Δ11 mutants that is to the E and U domains, respectively. Also, the signal peptide and the transmembrane region were predicted as structured (alpha-helices), while the cytoplasmic region was virtually nonstructured. These predictions are consistent with the proposal that betaglycan ectodomain is folded into two independent ligand binding domains. As shown in the following section, plasmin partial proteolysis was chosen to test these predictions, since this protease has been shown to produce discrete BG fragments (*25*).

Partial Proteolysis of Recombinant Soluble Betaglycan Reveals Two Discrete Resistant Fragments. Previously, we characterized a recombinant form of rat soluble betaglycan that consists of betaglycan extracellular region, engineered with a c-myc epitope tag at the amino end, just seven residues after the signal peptide cleavage site, as well as with a hexa-histidine tail at its carboxyl end (*14*). This protein is expressed in insect cells as a 110 kDa secretory glycoprotein that forms noncovalent dimers of 220 kDa and is devoid of GAG chains. Now, we subjected this soluble betaglycan to digestion for different times with limiting concentrations of plasmin at 30 °C and the products of the reaction were displayed on SDS−PAGE and analyzed by Western blotting with specific antibodies. The intact protein is cleaved yielding two major fragments, one of 55 and another of 45 kDa (Figure 1A). At relatively short digestion times, the 45 kDa band appears as a doublet in which the amount of the smaller one (45 kDa) increases at the expense of the slightly larger one, which practically disappears at 120 min time. On the contrary, the 55 kDa band, which somehow stains poorly with silver, tends to vanish at longer incubation times. To better identify these bands, they were probed by immunoblotting with antibodies against the whole receptor ectodomain (#822) and the c-myc epitope (Figure 1B,C, respectively). While both of the 55 and 45 kDa fragments, as well as the intact soluble BG were recognized by antiserum #822, the monoclonal anti-c-myc antibody only recognized the intact protein and the 45 kDa fragment. Notice that the larger band of the early 45 kDa doublet also is recognized by the anti-c-myc antibody, suggesting that it is a precursor of the smaller band. Furthermore, at shorter times (not shown) the larger band appears before the smaller one and the amount of the latter increases concomitantly with the disappearance of the band above it. The 55 kDa band is clearly

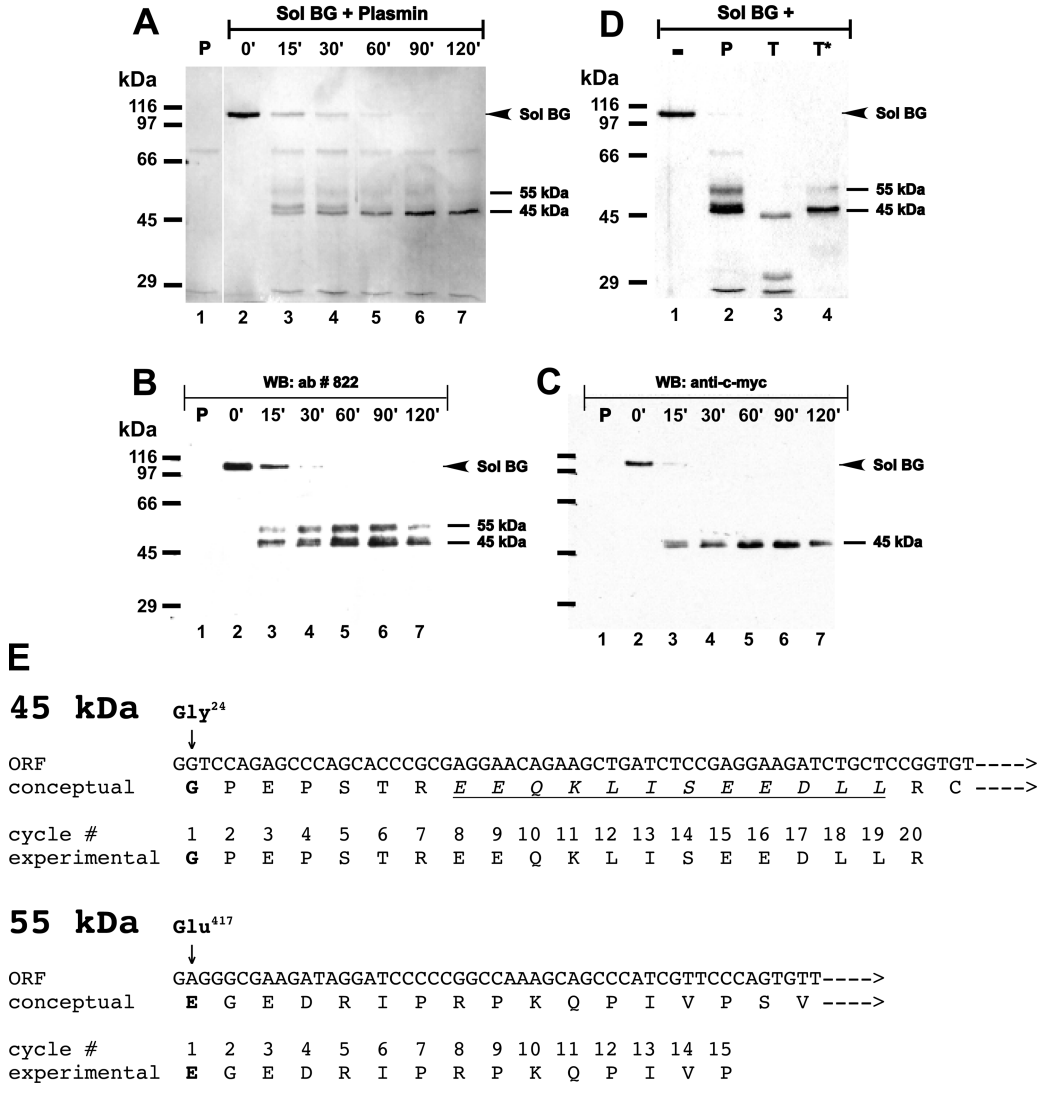

FIGURE 1: Plasmin treatment of recombinant soluble betaglycan reveals 2 proteolysis resistant fragments. (A) Soluble betaglycan (Sol BG) was digested with limiting concentrations of plasmin at 30 °C for the indicated times and the resulting fragments (of 55 and 45 kDa) were analyzed in a reducing SDS−PAGE stained with silver. Western blot analysis of these proteolytic fragments (probed with serum #822, against BG ectodomain, panel B, and probed with anti-c-myc monoclonal antibody, panel C). (D) Soluble betaglycan was digested with limiting concentrations of plasmin or trypsin at high (T) and low (T*) concentrations and the resulting fragments were analyzed in a reducing SDS−PAGE stained with silver. (E) Amino-end sequence results for the first 20 and 15 cycles for the 45 and 55 kDa fragments, aligned with the conceptual sequence predicted from the cDNA.

distinguished at early times; however, at later times it disappears, suggesting a low resistance to the protease. In addition to the 45 and 55 kDa bands, a couple of minor faint bands are revealed, one which migrates with the front of the dye in SDS−PAGE and other migrating above the 66 kDa marker. Very likely, these minor bands are contributed by the protease preparation (compare lanes 3−7 with lane 1 in Figure 1A) since they are not recognized by the polyclonal rabbit anti-BG antibody (see Figure 1B). Treatment with trypsin, that has the same substrate specificity of plasmin, produced the 45 and 55 kDa fragments (compare lanes 4 and 2 in Figure 1D). However, higher concentrations of this protease brought about degradation of the fragments, reducing the mass of the 45 kDa and eliminating the 55 kDa fragment (Figure 1D, lane 3).

The above data strongly suggest that the 45 kDa fragment corresponds to the amino-terminal half of the soluble receptor, while the 55 kDa one corresponds to its carboxyl-terminal half. To conclusively identify the 45 and 55 kDa fragments, they were resolved in SDS−PAGE and electroblotted on to PVDF membranes for sequencing by the Edman degradation method. The sequence of the 45 kDa fragment (Figure 1E) was identical to the already published amino terminal sequence of intact rat soluble BG (*14*), confirming that it corresponds to the endoglin-related ligand binding region. On the other hand, the 55 kDa fragment sequence started with Glu417, which along with the preceding lysine, constitutes a plasmin cleavage site motif (Lys/Arg-Xxx). This cleavage site is very close to Asp409, the aminoacid at the downstream limit of the residues 45−409, which are deleted in mutant Δ11 (*20*). Thus, the data in Figure 1 reveal the existence of two proteolytic resistant fragments in betaglycan ectodomain that match with the E and U ligand binding regions previously identified by deletion mutagenesis of membrane BG.

Partial Proteolysis of Membrane Betaglycan Also Reveals Two Resistant Fragments. The previous data are consistent with the secondary structure prediction (Supplementary Figure 1, Supporting Information) and indicated that recombinant Sol BG is folded into two discrete protein domains that can be separated by partial proteolysis, a property that should also be

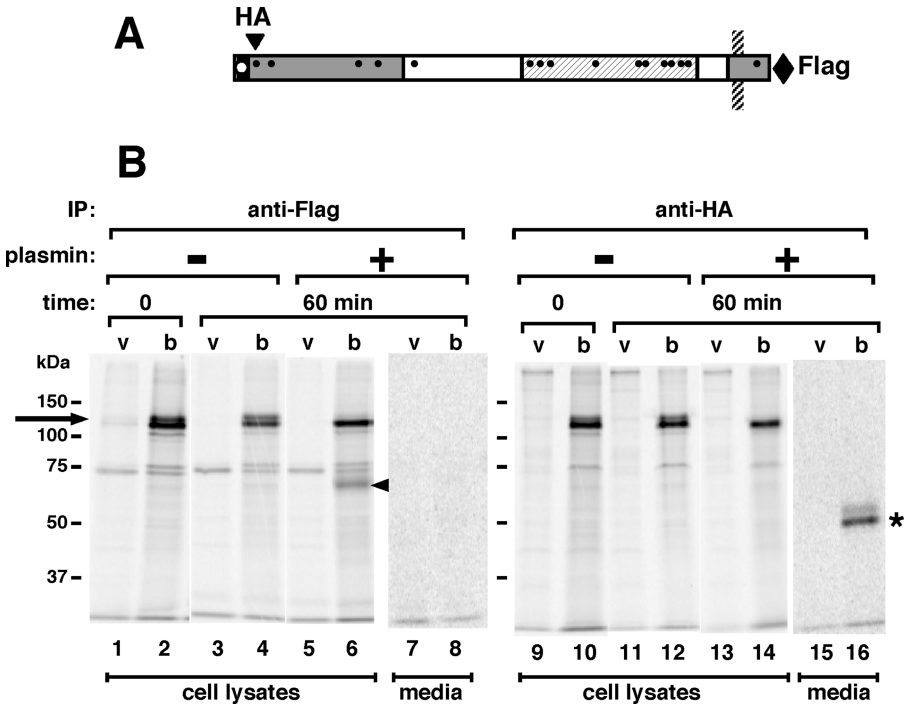

FIGURE 2: Membrane betaglycan also exhibits 2 plasmin resistant fragments. COS-1 cells were transfected with the HA gag$^-$ Flag (A), a GAG-less double tagged betaglycan construct (lane B) or the empty pCMV5 expression vector (lanes v). After being metabolically labeled with ^{35}S-methione/cysteine, cell monolayers were incubated at 37 °C for 60 min with digestion buffer alone (−) or added with 20 μg of plasmin (+). After this treatment, the cells lysates or conditioned media were immunoprecipitated with anti-Flag or anti-HA monoclonal antibodies (B). Lysates from untreated cells were also immunoprecipitated (0') as control for proper expression and labeling. HA gag$^-$ Flag plasmid transfected cells express a GAG-less betaglycan that is revealed by IP with both antibodies as a ~120 kDa doublet (arrow). Upon plasmin treatment novel ~70 kDa (arrowhead) and ~50 kDa (asterisk) bands are observed in the lysates and conditioned media, respectively.

exhibited by membrane bound BG. To test this possibility, a doubly epitope-tagged, GAG-less betaglycan (the HA gag$^-$ Flag construct, Figure 2A) was employed, which, because it is expressed solely as a core betaglycan, simplifies its analysis in SDS−PAGE (*12*). The BG construct HA gag$^-$ Flag was transfected in COS-1 cells which were metabolically labeled with ^{35}S-Methionine/Cysteine and then subjected to plasmin treatment. The resulting proteolysis fragments, in both the cells and the media, were identified by immunoprecipitation with specific antibodies as shown in Figure 2B. The ~120 kDa mature BG (Figure 2B, arrow) is lost after 60 min of plasmin treatment (lanes 6 and 14) yielding protease resistant fragments that are found in the media and cell lysates. The HA epitope is located at the same site as the c-myc epitope in the Sol BG used in previous experiments, while the flag epitope is engineered at the end of the BG cytoplasmatic tail. The anti-HA immunoprecipitation reveals a 50 kDa fragment in the media of the plasmin treated cells (lane 16), which is absent in the nondigested cells (not shown) and in the pCMV5 transfected cells (lane 15). This 50 kDa proteolytic resistant fragment released to the media is the cognate of the 45 kDa one produced by digestion of the soluble betaglycan. A 55 kDa cognate, membrane bound fragment, is also generated during the plasmin treatment and is revealed as a ~70 kDa band which is immunoprecipitated from cell lysates by the anti-Flag antibody (arrowhead, lane 6). These data indicated that, as expected, membrane BG ectodomain is folded also into two discrete protein domains that correspond to the 45 kDa fragment (its endoglin-related, membrane-distal region) and the 55 kDa fragment (its uromodulin-related, membrane-proximal region).

Loss of Quaternary Structure of Recombinant Soluble Betaglycan upon Proteolysis. Recombinant Sol BG is a homodimer held together by noncovalent interactions (*14*). In order to find out whether or not the 45 and/or 55 kDa fragments retain this quaternary structure, we separated the plasmin-treated soluble betaglycan by size exclusion chromatography, as we did before for the intact protein. While the native protein had an elution time of 7.4 min, which indicated a molecular size of 220 kDa (see Figure 3A in ref *14*), the digested protein eluted at 8.6 min indicating a size of 50 kDa (Figure 3A, inset). SDS−PAGE analysis of the fractions collected around this elution time (Figure 3B) revealed that 55 and 45 kDa fragments peaked separately, closer to the elution times expected for their sizes, at approximately 8.4 and 8.6 min, respectively. Thus, the apparent single peak at 8.6 min in the chromatograph of Figure 3A contains two similarly sized monomeric proteins, the 55 and 45 kDa fragments. Noticeably, there is a significant amount of 280 nm-absorbing material eluting as asymmetric peaks at 10.4 and 11 min. The elution time of this material indicates a mass of 3.5−2 kDa, suggesting that it may be formed by a heterogeneous mixture of small peptides, ranging up to about 30 residues in length.

The Purified 45 and 55 kDa Fragments Bind TGF-β. Assuming that the hexa-histidine tail of the 55 kDa fragment was not digested by plasmin, and considering that the 45 and 55 kDa fragments are not associated, then it would be possible to purify the 55 kDa fragment by IMAC. For that purpose soluble BG was treated with plasmin and run through chelating sepharose under conditions similar to those employed to purify soluble betaglycan. Lane 1 in Figure 4A shows the material loaded in the column, while lane 2 shows that the 45 kDa fragment

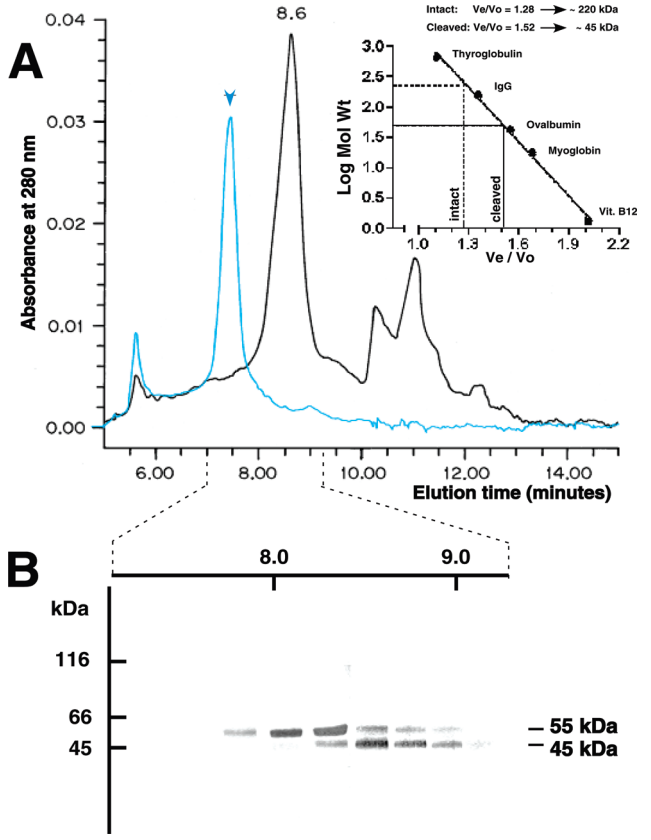

FIGURE 3: Plasmin treatment of recombinant soluble betaglycan loses its quaternary structure. HPLC gel filtration analysis of plasmin digested soluble betaglycan (blue tracing, A) and the calculated molecular weight of the 8.6 min main elution peak (inset, A). For comparison, the profile of intact soluble betaglycan (from ref *14*) in the same HPLC system is shown in gray tracing. (B) HPLC fractions collected between the 7 and 9 min were separated in a reducing SDS−PAGE and revealed by silver staining.

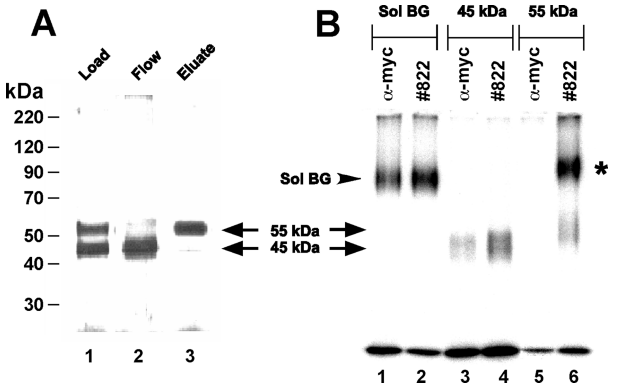

FIGURE 4: Purification and TGF-β binding activity of the 45 and 55 kDa fragments. (A) Plasmin digested soluble betaglycan was subjected to immobilized metal-ion affinity chromatography (IMAC) using chelating Sepharose. Samples of the loaded, the unbound flow-through, and EDTA eluted material were separated in a reducing SDS−PAGE (lanes 1, 2, and 3 in panel A, respectively) and affinity labeled with 100 pM ^{125}I-TGF-β2 (equivalent amount of intact soluble betaglycan was included as a positive control). The labeled materials were immunoprecipitated with serum #822, against BG ectodomain, or with the anti-c-myc monoclonal antibody, then separated in reducing SDS−PAGE and finally revealed by Phosphorimager scanning of the gel (panel B). The positions of the intact soluble betaglycan (Sol BG) as well as that of the 45 and 55 kDa fragments are indicated. The asterisk indicates the position of the affinity labeled 55 kDa fragment dimer which results from nonspecific DSS-cross-linking. This artifactual cross-linking is observed with some betaglycan truncated mutants (*20*).

was in the flow-through, whereas the 55 kDa fragment remained bound after washing the column, and is eluted with EDTA (lane 3) indicating that it retained a functional poly histidine tail.

The purified fragments and the intact recombinant soluble betaglycan, as a positive control, were affinity labeled with 100 pM ^{125}I-TGF-β2, immunoprecipitated with either the antiserum # 822 or the c-myc antibody, separated in SDS−PAGE and revealed by autoradiography. As shown in Figure 4B, the intact receptor, as well as the two fragments bound TGF-β2 and were recognized by the whole ectodomain antiserum # 822, while the anti-c-myc antibody immunoprecipitated only the intact protein and the 45 kDa fragment but not the 55 kDa fragment. It was also observed that the majority of the TGF-β affinity labeled 55 kDa fragment migrated at approximately 120 kDa (asterisk in Figure 4B, lane 6), suggesting that it was heavily cross-linked by disuccinimidyl suberate, the bifunctional cross-linking reagent used in this type of experiments.

In order to better define the binding properties of the purified 45 and 55 kda fragments, they were subjected to TGF-β binding under specific isoform competition. For that purpose, each one of the purified 45 and 55 kDa fragments was affinity labeled with a constant concentration of ^{125}I-labeled TGF-β (100 pM) in the presence of increasing amounts (0−2 nM) of one unlabeled isoform (Figure 5). Figure 5A shows the competition of ^{125}I-TGF-β1 against TGF-β1 and TGF-β2, Figure 5B shows that of ^{125}I-TGF-β2 against TGF-β2 and TGF-β1, and finally, Figure 5C shows that of ^{125}I-TGF-β3 against TGF-β3 and TGF-β2. Quantification of the data indicates that while each isoform competes well with itself, the degree of heterologous competition varies in a manner that is characteristic of betaglycan, namely, that TGF-β2 competes very efficiently with the other isoforms, and its binding is hardly affected by any of them. This indicates that the purified 45 and 55 kDa fragments bind the TGF-β isoforms with the following relative preference: TGF-β2 $\gg$ TGF-β3 $\sim$ TGF-β1, which is similar to that reported for the intact soluble receptor and the recombinant deletion mutants Sol Δ10 (soluble E domain) and Sol Δ11 (soluble U domain) (*14*, *21*).

Loss of TGF-β Neutralizing Activity of Recombinant Soluble Betaglycan upon Proteolysis. The most relevant property of recombinant soluble betaglycan is its capacity to neutralize TGF-β; therefore, we determined if either or both of the 45 and/or 55 kDa fragments retain this property. To this end, we determined the effect of each one of the purified 45 and/or 55 kDa fragments in the well characterized TGF-β assay, the p3TP-lux assay (*14*). In this assay, addition of 20 pM TGF-β2 to Mv1Lu mink lung epithelial cells transfected with the TGF-β reporter plasmid (p3TP-lux) induces maximal luciferase response. As reported before, the intact Sol BG completely blocks the activity of 20 pM TGF-β2 even at concentrations as low as 12.5 nM (Figure 6). However, concentration as high as 500 nM of the purified 45 kDa fragment decreased the reporter activity by $\sim$ 20%. The purified 55 kDa fragment had a slightly better TGF-β2 inhibitory activity than the 45 kDa (down to 60% with 500 nM); however, when compared to intact Sol BG, its effect was quite poor (Figure 6A). When both fragments were mixed together in an equimolar ratio, the anti-TGF-β2 activity of the mixture fared slightly better than the fragments alone (down to 20%). Nonetheless, it is evident that the TGF-β neutralizing activity of the purified betaglycan 45 and 55 kDa fragments is at

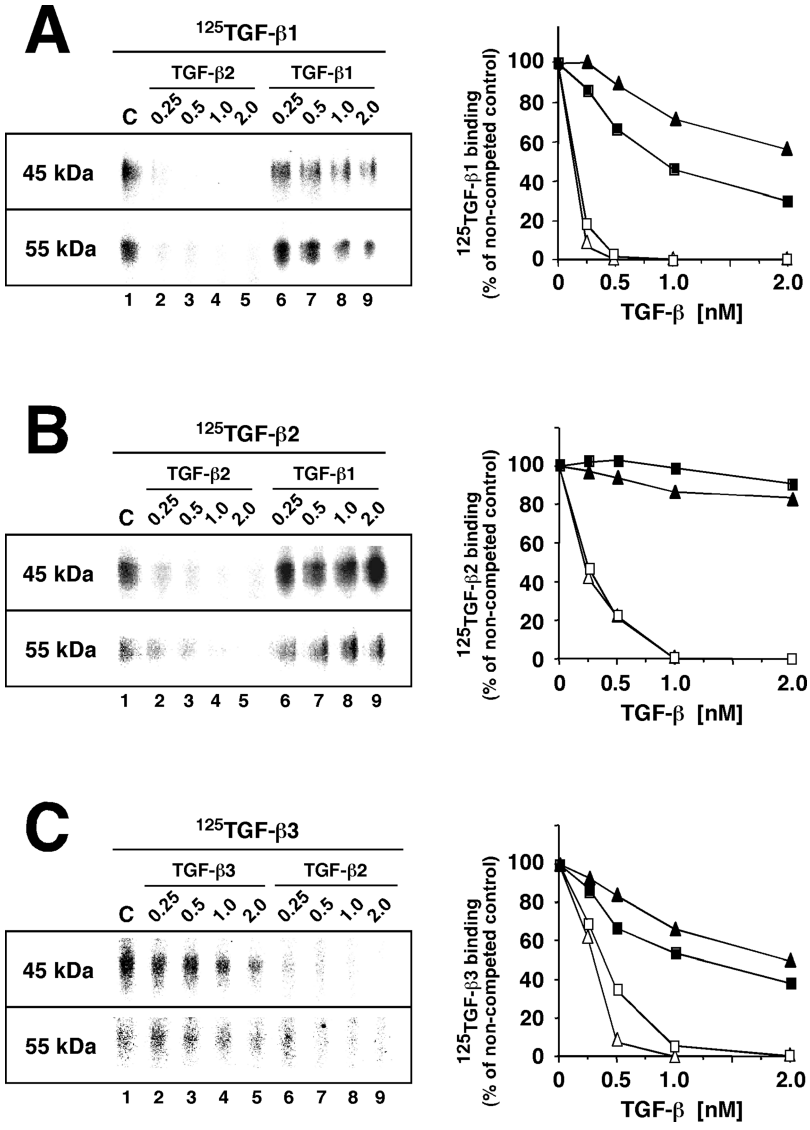

FIGURE 5: TGF-β isoform specific binding activity of the 45 and 55 kDa fragments. IMAC purified 45 kDa (squares) and 55 kDa (triangles) fragments were affinity labeled with 100 pM of the indicated ^{125}I-TGF-β isoform in presence of the indicated increasing nanomolar concentrations of unradioactive TGF-β isoform. Uncompeted controls (lanes 1) and homologous and heterologous competed samples were separated in reducing SDS−PAGE and revealed by Phosphorimager scanning of the gel (left panels). Densitographic analysis of the affinity labeled products were plotted, taking the signal of the uncompeted reaction as 100% (rigth panels: A and B, unlabeled TGF-β1 closed symbols, unlabeled TGF-β2 open symbols; C, unlabeled TGF-β3 closed symbols, unlabeled TGF-β2 open symbols).

least 100 times lower than that of the native soluble receptor. This significant drop of TGF-β neutralizing potency could be due to the lack of minor peptide(s) that could have been removed during the purification step (see peaks eluting at 10.4 and 11 min in Figure 3A). In order to rule out this possibility, we tested the TGF-β neutralizing activity of the plasmin digested, but unfractionated, recombinant Sol BG. In this experiment the digested but unfractionated protein also failed to neutralize either TGF-β1 or TGF-β2 (data not shown). Thus, compared to the intact soluble receptor, the plasmin cleaved soluble betaglycan, as well as the resulting 45 and 55 kDa fragments, lack significant anti-TGF-β activity.

Recombinant Soluble Betaglycan Ligand Binding Domains Also Have Decreased TGF-β Neutralizing Activity. Soluble betaglycan deletion mutants Sol Δ10 (E-domain) and Sol Δ11 (U-domain) contain regions of its ectodomain that closely match the 45 kDa and 55 kDa fragments generated by proteolysis (*20*). Since we showed previously that recombinant Sol Δ10 and Sol Δ11 have TGF-β binding properties similar to those presented here for the purified 45 kDa and 55 kDa fragments (*20*), we tested whether or not their ability to neutralize TGF-β also mimic those of the 45 and 55 kDa fragments. In the p3TP-lux assay at 125 nM, Sol Δ11 inhibited 50% of the activity of 20 pM TGF-β2, while Sol Δ10 did not have a significant effect (Figure 6B). The combination of both proteins at a final concentration of 125 nM did not increase the effect of Sol Δ11 alone. Sol Δ10 antagonized TGF-β2 activity by 50% only at concentrations as high as 1 μM, while under the same conditions, Sol Δ11 blocked the TGF-β2 response by 90% (data not shown). Thus, these results indicate that neither one of the 45 and 55 kDa betaglycan ectodomain fragments, nor their recombinant equivalents (Sol Δ10 and Sol Δ11), inhibit the access of TGF-β2 to the signaling receptors with the same potency as intact recombinant Sol BG.

Affinities of Recombinant Soluble Betaglycan Ligand Binding Subdomains for the TGF-β Isoforms are 20 to 100 Times Lower than Those of Full-Length Soluble Betaglycan. To explore the reasons for the decreased TGF-β neutralizing activity of soluble betaglycan deletion mutants Sol Δ10 and Sol

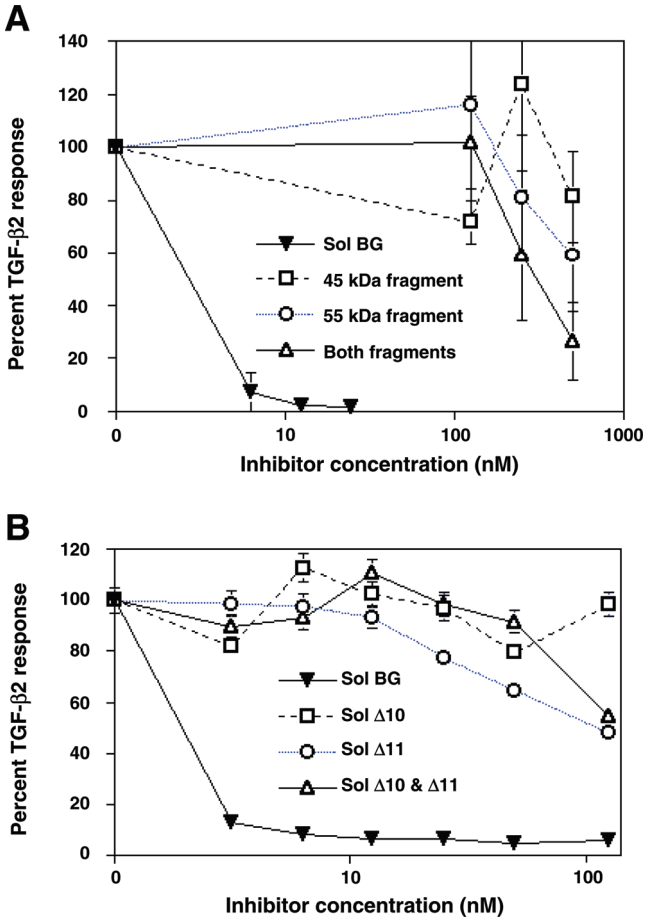

FIGURE 6: TGF-β2 neutralizing activity of the 45 and 55 kDa fragments and their cognate recombinant versions. Mv1Lu cells were transfected with the p3TP-lux and stimulated with 20 pM of TGF-β2 in the presence of intact soluble betaglycan (solid inverted triangles) or of the IMAC-purified 45 or 55 kDa fragments (panel A) or the recombinant Sol Δ10 or Sol Δ11 (panel B) added at the indicated nanomolar concentrations. When combined, the equimolar mixture had the indicated concentration. The luciferase activity (average of triplicates and its standard deviation) of a representative experiment are shown.

Δ11, we determined their TGF-β binding constants, as well as that of full-length Sol BG, by surface plasmon resonance (SPR) equilibrium analysis. To minimize heterogeneity in the surface, as well as to minimize artifacts due to rebinding, we prepared minimally biotinylated TGF-β ligands and captured them at low density (100 RU or less) onto streptavidin coated sensor chips. The surfactant CHAPS was also included in the buffer to minimize the gradual accumulation of surface bound receptor, which for Sol Δ11 was particularly pronounced. The sensorgrams obtained for TGF-β2 clearly showed that the full-length receptor (Sol BG) saturated the surface at concentrations that were 1−2 order of magnitudes lower than that of either Sol Δ10 or Sol Δ11 (Figure 7A). The dissociation constants (K_d), obtained by fitting the equilibrium response as a function of injected receptor concentration to a standard binding isotherm are listed in Table 1. Full-length Sol BG bound TGF-β2 with 17-fold higher affinity than Sol Δ10 and 72-fold higher than Sol Δ11. This accounts for the decreased effectiveness of these domains to block TGF-β induced receptor activation, as the reduction in affinity roughly correlates with the 50−100-fold reduced antagonistic potency. Notably, the antagonistic potencies of Sol Δ10 and Sol Δ11 are inversely correlated with their affinities, suggesting other factors, such as specific residues contacted in relation to those used by the signaling receptors, might also play in role in determining antagonistic potencies.

The affinities of Sol Δ10 and Sol Δ11, as well as Sol BG, for binding TGF-β1 and TGF-β3, were also measured using the methods outlined above (Supplementary Figures 2A and 3A, Supporting Information). The results, listed in Table 1, were similar to those obtained with TGF-β2, with Sol BG binding TGF-β1 with 20- and 102-fold higher affinity than either Sol Δ10 or Sol Δ11, respectively, and with Sol BG binding TGF-β3 with 41-fold higher affinity than either Sol Δ10 or Sol Δ11. The results further demonstrated that Sol BG, as well as Sol Δ10 and Sol Δ11, bind TGF-β2 better than TGF-βs 1 and 3, suggesting that both the E- and U-domains contribute to betaglycan's selectivity in binding TGF-β2.

To better understand the underlying mechanism for the increased affinity of full-length Sol BG for binding TGF-β, we performed a coinjection experiment wherein the TGF-β2 surface was first saturated with Sol Δ10, followed by a subsequent injection where Sol Δ11 was injected over a range of concentrations in the presence of the same saturating concentration of Sol Δ10 (2 μM). The results (Figure 7B, left and middle panels) showed a binding response similar to that obtained upon injection of Sol Δ11 alone. Thus, the derived K_d value in the presence of Sol Δ10 was hardly distinguishable from that measured in its absence (2.2 ± 0.4 μM vs 2.6 ± 0.3 μM, respectively) (Table 1). This same coinjection experiment was also carried out with TGF-β1 and TGF-β3 (Supplementary Figures 2B and 3B, Supporting Information) and as shown by the K_d values listed in Table 1 the results were similar. The fact that the K_d value for binding of Sol Δ11 is unchanged in the presence of Sol Δ10 suggests that the increased affinity of full-length soluble betaglycan is derived by a simple tethering of the two domains together, not induced conformational changes or mutual stabilization through receptor-receptor contacts.

DISCUSSION

Betaglycan has many functions (*2*), including the recent claim of its role as a tumor suppressor (*15*). The fact that mice null for BG gene are embryonic lethal due to heart and liver abnormalities indicates an important, albeit poorly characterized role during development (*9*). Furthermore, its ability to interact with many ligands and receptors of the TGF-β superfamily using two regions of its ectodomain illustrates the complexity of its tertiary structure (*2*). The ligand binding regions have been inferred from the analysis of deletions mutants (*13*, *20*, *21*) and suggest that the wild type receptor is endowed with two domains that fold independently during its synthesis. Here we present additional biochemical evidence, including limited proteolysis and binding studies, which lend support to the mutagenesis data.

Plasmin treatment of rat betaglycan, both as a soluble recombinant receptor produced in insect cells, and as a membrane bound core protein expressed in mammalian cells, clearly revealed two independent domains in the betaglycan ectodomain that nicely match the ligand binding regions identified by mutagenesis. The 45 kDa fragment produced by soluble betaglycan corresponds to the membrane distal region, or E-domain, while the 55 kDa to the membrane proximal region, or U-domain. These fragments roughly correspond to the previously reported soluble BG mutants Sol Δ10 (deleted residues: 410−853 of wild type rat BG) and Sol Δ11 (deleted residues: 45−409 and 783−853 of wild type rat BG), which encompass the so-called E- and U-domains, respectively (*20*).

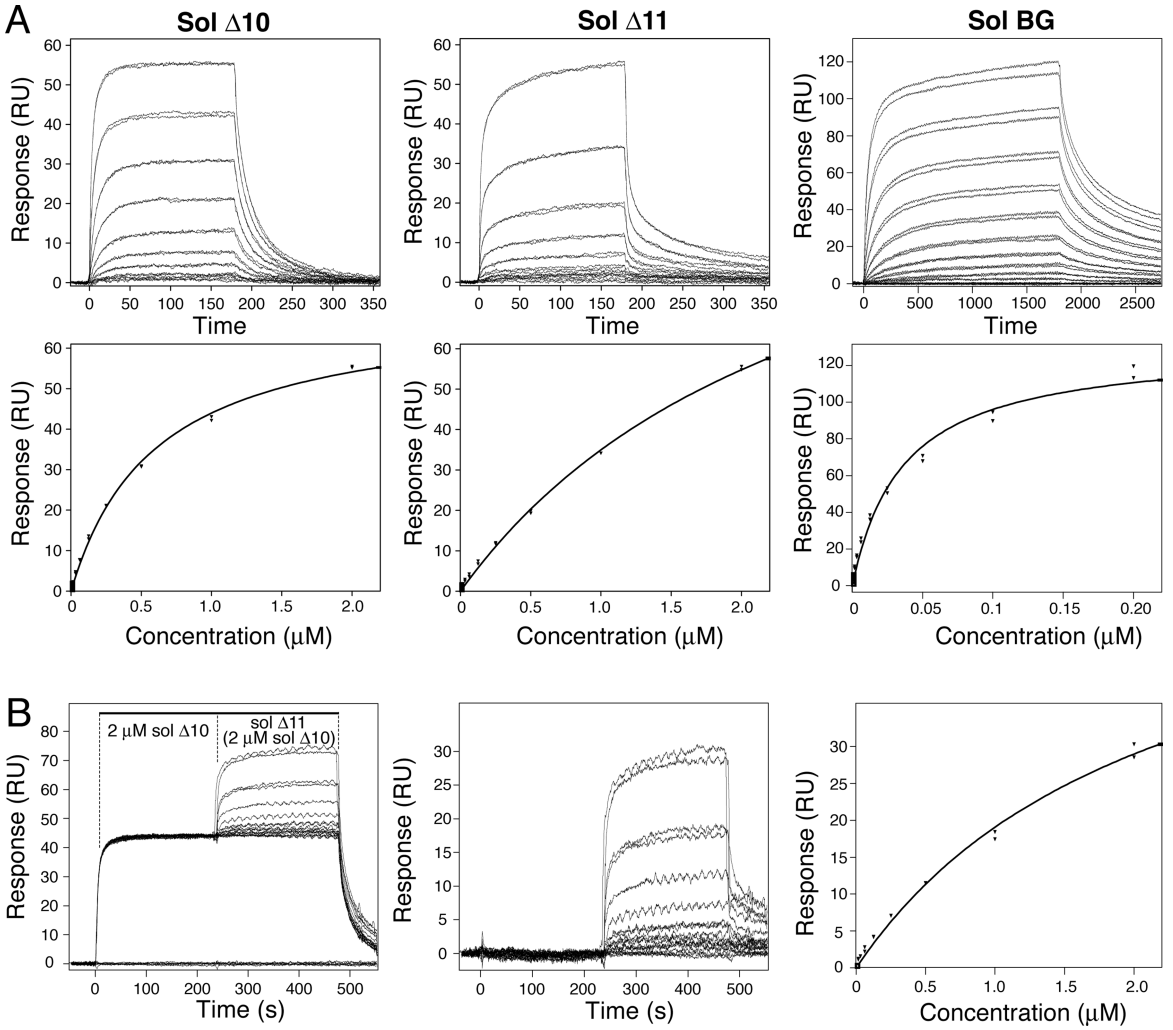

FIGURE 7: SPR analysis of TGF-β2 binding activity of the Sol Δ10, Sol Δ11, and Sol BG proteins. (A) Sensorgrams and binding isotherms obtained as Sol Δ10 (left panels), Sol Δ11 (middle panels) and recombinant soluble betaglycan (Sol BG, right panels) were injected. Traces correspond to replicate measurements of 2 fold serial dilutions of the receptors with concentrations 0−2 μM for both Sol Δ10 and Sol Δ11 and 0−0.2 μM for Sol BG. Binding isotherms correspond to plots of the response at equilibrium as a function of receptor concentration, which were fit to a hyperbolic equation using Scrubber 2 software. (B) Sensorgrams and binding isotherms obtained for coinjection experiments for Sol Δ11 binding to TGF-β2 in the presence of 2 μM Sol Δ10. Traces correspond to replicate measurements of 2 fold serial dilutions of Sol Δ11 with concentrations 0−2 μM. Left panel represents the original sensorgrams; middle panel represents the sensorgrams after subtracting the binding response of 2 μm Sol Δ10; right panel represents the binding isotherms corresponding to plots of the response at equilibrium (taken from middle panel) as a function of receptor concentration fit to eq 1.

Table 1: Dissociation Constants for Binding of TGF-β Isoforms to Recombinant Soluble Betaglycan (Sol BG) and Its Endoglin (Sol Δ10) and Uromodulin (Sol Δ11) Domains

		dissociation constant (μM)		
analyte	saturating receptor	TGF-β1	TGF-β2	TGF-β3
Sol Δ10	none	1.9 ± 0.1	0.60 ± 0.04	5.8 ± 0.9
Sol Δ11	none	9.7 ± 5.3	2.6 ± 0.3	5.8 ± 0.8
Sol BG	none	0.095 ± 0.008	0.036 ± 0.004	0.143 ± 0.010
Sol Δ11	Sol Δ10, 2 μM	2.9 ± 0.8	2.2 ± 0.4	4.4 ± 1.0

In the course of these experiments we found that the dimeric nature, as well as the TGF-β neutralizing ability of the soluble receptor, are lost upon proteolytic separation of the two domains: neither the 45 kDa nor the 55 kDa fragment are found as dimers after proteolysis, demonstrating conclusively that none of the possible intrachain disulfide bonds in BG ectodomain link the E and the U domains. The rat betaglycan ectodomain contains 15 conserved cysteines (*26*), suggesting that it has 7 disulfide intrachain bridges, 5 of them in the 55 kDa fragment and 2 in the 45 kDa fragment, and a free -SH_2 group in the latter.

More intriguing is the loss of the anti-TGF-β activity of the intact soluble receptor. Neither the major proteolytic resistant fragments of Sol BG nor their recombinant equivalents (Sol Δ10 and Sol Δ11) have the potent TGF-β neutralizing activity of the full-length ectodomain. Through SPR binding studies, the intact Sol BG was shown to bind the three TGF-βs 20 - 100 fold more tightly than either Sol Δ10 or Sol Δ11 alone, suggesting that its full antagonistic potency is predominantly due to its higher affinity for binding TGF-β. SPR coinjection studies further showed that the high affinity binding of Sol BG is due to a simple tethering of the two domains and their ability to simultaneously contact TGF-β, and not allosteric effects or receptor-receptor contact. Though high affinity binding is necessary for potent antagonistic activity, it alone is not sufficient, since the soluble receptor must bind the ligand in a manner that interferes with its binding to the cell surface receptors. This might explain

the fact that Sol Δ10 consistently bound the TGF-β isoforms with comparable (TGF-β3) or higher affinity (TGF-β1 and TGF-β2) than Sol Δ11, yet Sol Δ11 consistently functioned as a more potent TGF-β antagonist. This could be because the binding site for Sol Δ11 overlaps with that of either or both of the signaling receptors, TβRI and TβRII, whereas Sol Δ10 does not. This is partially supported by the recent observation that a recombinant E-domain:TβRII ectodomain fusion protein binds TGF-β with much higher affinity than either TβRII ectodomain or the E-domain alone (*27*). However, further analyses, most likely direct structural analyses, will be required to fully evaluate this hypothesis.

The data presented here pave the way to such structural analysis, as they show that the recombinant Sol Δ10 and Sol Δ11 are representative of the bona fide folding of the two TGF-β binding domains comprising the wild type receptor. Further support for this claim comes from the predicted secondary structure of the rat betaglycan ectodomain, which reveals two highly structured regions, corresponding to the 45 and 55 kDa fragments, joined by a linker segment of approximately 50 residues with little or no propensity to form helices or strands (Supplementary Figure 1, Supporting Information). Importantly, the site most susceptible to plasmin cleavage, K416-E417, lies near the center of this region. It is also noteworthy that partial digestion of the human recombinant soluble betaglycan also produced discrete fragments similar to those of its rat counterpart (data not shown).

Finally, the loss of the anti-TGF-β activity of soluble betaglycan upon proteolysis may be relevant for its ability to suppress tumor growth and metastasis. The pioneering work of Sun and co-workers (*16*, *17*, *28*, *29*) and the recent work of Blobe and co-workers (*15*) strongly indicate that betaglycan should be considered as a tumor suppressor, mainly because of the TGF-β neutralizing activity of Sol BG. Soluble betaglycan derives from the ectodomain shedding of the membrane bound receptor, a process that is carried by several metalloproteases in a regulated manner (*12*). Our present finding that TGF-β neutralizing activity of Sol BG is lost after digestion with plasmin, a protease commonly activated during inflammatory responses, could be envisioned as a physiological mechanism to attenuate this activity. In this light, it may be beneficial, in the clinical application of Sol BG as a suppressor of tumor growth and metastasis, to modify the linker so as to improve its resistance to proteolysis. These modifications, in principle, could improve its already proven and remarkable actions in the amelioration of diverse diseases in which TGF-β plays a pathophysiological role (*16*−*19*).

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SUPPORTING INFORMATION AVAILABLE

Supplementary Figures 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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