Characterization of Mutated Transforming Growth Factor-βs Which Possess Unique Biological Properties

Su Wen Qian,*,†,§ James K. Burmester,§,‖ Peter D. Sun,⊥ Alison Huang,‡ Dennis J. Ohlsen,‖ Laurent Suardet,# Kathleen C. Flanders,‡ David Davies,⊥ Anita B. Roberts,‡ and Michael B. Sporn‡

Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892, Marshfield Medical Research and Education Foundation, Marshfield, Wisconsin 54449, Laboratory of Molecular Biology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Laboratory of Radiobiology, Harvard School of Public Health, Boston, Massachusetts 02115

Received April 11, 1994; Revised Manuscript Received July 25, 1994®

ABSTRACT: Transforming growth factor-β (TGF-β) is a potent regulator of cell growth and differentiation. On the basis of the crystal structure of TGF-β2, we have designed and synthesized two mutant TGF-βs, TGF-β1(71 Trp) and TGF-β1(Δ69–73). Although both of these molecules inhibited the growth of Mv1Lu mink lung epithelial cells and LS1034 colorectal cancer cells, which are affected equally by TGF-β1 and TGF-β2, TGF-β1(Δ69–73) was much less potent than TGF-β1 or TGF-β1(71 Trp) at inhibiting the growth of LS513 colorectal cancer cells which are growth-inhibited by TGF-β1 but not TGF-β2. Both TGF-β1(71 Trp) and TGF-β1(Δ69–73) increased levels of mRNAs for fibronectin and plasminogen activator inhibitor with Mv1Lu cells, whereas only TGF-β1(71 Trp) and not TGF-β1(Δ69–73) up-regulated the mRNA level of carcinoembryonic antigen in LS513 cells. The expression level of carcinoembryonic antigen mRNA in LS1034 cells was not altered by either wild-type or mutant TGF-βs. Receptor labeling experiments demonstrated that TGF-β1(71 Trp) bound with high affinity to the cell-surface receptors of Mv1Lu, LS1034, and LS513 cells while TGF-β1(Δ69–73) bound effectively to the receptors of Mv1Lu and LS1034 cells but much less to the receptors on LS513 cells. In contrast, binding of TGF-β1(71 Trp) and TGF-β1-(Δ69–73) to endoglin and the type II receptor of human umbilical vein endothelial cells (HUVECs) was similar to TGF-β1, not TGF-β2. These results demonstrate the feasibility of synthesizing TGF-β mutants with unique biological properties.

Transforming growth factor-β (TGF-β)[1] is a 25 kDa homodimeric protein which has potent growth and differentiation effects on virtually all cells (Sporn & Roberts, 1990; Massagué, 1990; Roberts & Sporn, 1990). Considerable potential exists for the use of TGF-β or its analogs in the treatment of common diseases (Sporn & Roberts, 1989; Border & Ruoslahti, 1992). Recent reports suggest that particular TGF-β isoforms can potentially be used for the treatment of scarring (Beck et al., 1990), retinal tears (Smiddy et al., 1989), or full thickness macular holes (Glaser et al., 1992), myocardial infarction (Lefer et al., 1990), and soft tissue inflammation (Brandes et al., 1991). In addition, analogs of TGF-β may be useful in the treatment of diseases such as cancer and osteoporosis. Antagonists of TGF-β could impact in the treatment of diseases such as glomerulonephritis, liver cirrhosis, pulmonary fibrosis, keloids, and other fibrotic diseases (Sporn & Roberts, 1989; Border & Ruoslahti, 1992).

To investigate the structure/function relationships of TGF-β, we have previously utilized chimeric TGF-β proteins, in which selected regions of the TGF-β isoforms have been interchanged, and assays which respond selectively to the bioactivities of the TGF-β isoforms (Qian et al., 1992; Burmester et al., 1993). By replacing amino acids 45 and 47 of TGF-β2 with the corresponding amino acids of TGF-β1, sequestration of the TGF-β2 molecule by α_2M was reduced to that of TGF-β1 (Burmester et al., 1993). Progress has also been made toward identifying a second set of amino acids which are involved in determining isoform specificity for inhibition of growth of LS513 colorectal cancer cells. Growth of these cells is inhibited by moderate concentrations of TGF-β1, whereas substantially greater concentrations of TGF-β2 are required to achieve the same amount of inhibition (Suardet et al., 1992).

Examination of the crystal structure of TGF-β2 revealed an unusual protein fold which can be characterized as an outstretched hand with two slightly curled fingers (Daopin et al., 1992; Schlunegger & Grutter, 1992). The hydrophobic regions on both the heel of the hand and near the finger tips allow the monomers to come together in the opposite orientation, forming the active dimer. All nine cysteines of each monomer form disulfide bonds with eight involved in intrachain bonds and residue 77 forming a single interchain disulfide bridge. Postulated receptor binding sites include the surfaces of the exposed loop consisting of amino acids 1–13, 23–35, 67–73, and 91–96.

To investigate the role of the exposed loop centered at residue 71, we have synthesized two mutant TGF-β molecules. Here we describe some of the unique biological properties of these molecules and demonstrate that changes within the region around amino acid 71 can affect binding of TGF-β to its receptors.

* To whom correspondence should be addressed. Telephone: 301-496-5391. Fax: 301-496-8395.
‡ National Cancer Institute.
§ Co-senior authors since they contributed equally to this project.
‖ Marshfield Medical Research and Education Foundation.
⊥ National Institute of Diabetes, Digestive and Kidney Diseases.
Harvard School of Public Health.
® Abstract published in *Advance ACS Abstracts,* September 15, 1994.
[1] Abbreviations: TGF-β, transforming growth factor-β; α_2M, α_2-macroglobulin; CHO, Chinese hamster ovary; HUVECs, human umbilical vein endothelial cells; PCR, polymerase chain reaction; ATCC, American Type Culture Collection; FBHE, fetal bovine heart endothelial.

0006-2960/94/0433-12298$04.50/0

MATERIALS AND METHODS

Synthesis and Purification of TGF-β Mutant Proteins. cDNA constructs for TGF-β1(71 Trp) and TGF-β1(Δ69–73) were generated using a two-step PCR protocol as previously described (Qian et al., 1992; Higuchi et al., 1988). The resulting cDNAs were then ligated to the prepro region of TGF-β1, in which Cys-33 had been changed to Ser, in order to prevent aberrant disulfide bond formation. This created a full-length coding region for TGF-β expression. This cDNA was cloned into the unique *Xho*I site of the plasmid pMSXND (Lee & Nathans, 1988) which contains the metallothionein promoter and used to transfect CHO cells by the calcium/phosphate precipitation method. Transfectants were selected by culturing the cells in 400 μg/mL G418. Expression of the recombinant proteins was induced by culturing the cells in 100 nM $CdCl_2$. Expression levels were quantitated by measuring inhibition of growth of the Mv1Lu cell line as described (Danielpour & Sporn, 1990) and by immunoblotting. For immunoblotting, the crude recombinant medium was dialyzed against 1 M acetic acid, lyophilized to dryness, and resuspended in sample buffer for separation through a 10% Tricine–SDS polyacrylamide gel (NOVEX, San Diego). Levels of expression were quantitated by comparing the intensity of the immunoblot of the test samples to the intensity of 100 ng of TGF-β1. For each construct, a single clone was expanded and used to produce expression media for purification of the recombinant TGF-β protein. Protein was purified as described (Qian et al., 1992) using C18, cation-exchange and C4 chromatography. Purified protein was quantitated by silver-staining serial dilutions of the protein and comparing the band intensities to those of TGF-β1 standards.

Growth Inhibition Assays. Inhibition of growth of Mv1Lu cells (Danielpour & Sporn, 1990) and FBHE cells (Qian et al., 1992) by TGF-β molecules was determined as described. Inhibition of LS1034 and LS513 cell growth was determined using a modification of the Mv1Lu assay. In this modified assay, cells were first plated in Dulbecco's modified Eagle's medium/F-12 supplemented with 0.2% fetal bovine serum in 24 well Falcon dishes at 3×10^5 cells/0.5 mL per well. After 7 h at 37 °C to allow attachment, TGF-βs were added, and the cells were cultured an additional 17 h, at which point, 0.5 μCi/well of [^{3}H]thymidine was added. Cells were cultured in the presence of [^{3}H]thymidine for an additional 24 h and then processed as described (Danielpour & Sporn, 1990).

Receptor Cross-Linking. Mv1Lu cells were purchased from ATCC. HUVECs were purchased from both ATCC and Clonetics (San Diego, CA). Purified TGF-β isoforms were iodinated as described (Frolik et al., 1984) and used for labeling of the cell-surface binding proteins of Mv1Lu, LS513, and HUVECs as described (Massagué & Like, 1985; Geiser et al., 1992). Deglycosylation experiments were performed as described (Cheifetz et al., 1988). In these experiments, confluent monolayers of cells in a 35 mm dish were treated for 2.5 h at 37 °C with 1 mL of binding buffer containing chondroitinase ABC (0.1 unit/mL), heparinase II (0.5 unit/mL), and heparinase III (0.25 unit/mL). Enzymes were purchased from Sigma. Cells were then washed 3 times with binding buffer and cross-linked as described using 250 pM ^{125}I-TGF-β1. Proteins were reduced by adding β-mercaptoethanol, electrophoresed through an 8% acrylamide gel, and visualized by autoradiography using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and quantitated with the Image Quant software.

Northern Blot. Mv1Lu cells were treated with 0.5 ng/mL TGF-β for 24 h. LS1034 cells were treated with 5 ng/mL TGF-β for 24 h, and LS513 cells were treated with 12.5 ng/mL TGF-β for 24 h. Total RNA was isolated by the guanidine isothiocyanate method (Chirgwin et al., 1979). Twelve micrograms of RNA was separated on a 1% agarose/2.2 M formaldehyde gel, and transferred to a nylon membrane. Hybridizations were performed as described (Church & Gilbert, 1984) using ^{32}P-labeled cDNA for fibronectin, plasminogen activator inhibitor, or carcinoembryonic antigen.

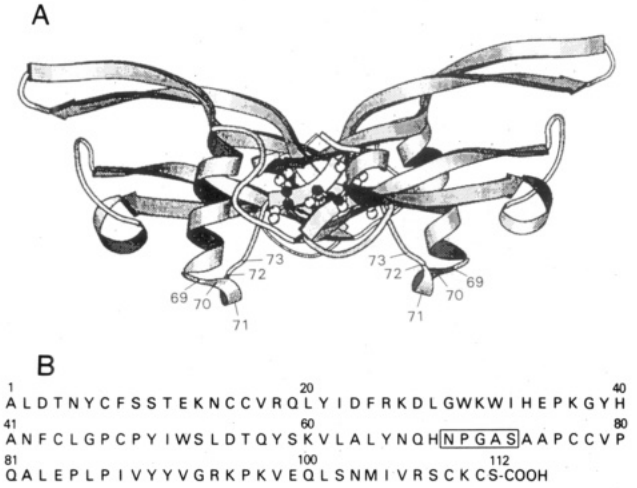

FIGURE 1: Summary of mutation sites. (*A*) Ribbon diagram of a dimer of TGF-β2. The representation of the structure of TGF-β2 is from Daopin et al. (1993). Positions of amino acids 69–73 are indicated. The cysteines of the disulfides are shown in the form of ball and stick models with carbon atoms shown in white and sulfur atoms in shaded balls. (*B*) Sequence of TGF-β1. Amino acids 69–73 have been boxed.

RESULTS

To investigate the role of the exposed loop of TGF-β which is centered at amino acid 71, we designed two mutated TGF-β molecules. In the first mutant, TGF-β1(71 Trp), Gly(71) which is located at the tip of an exposed loop of TGF-β has been changed to Trp in order to maximize the difference in size of these amino acids. In the second mutant, TGF-β1-(Δ69–73), amino acids 69–73 have been deleted from the TGF-β1 molecule (Figure 1A). These changes have the potential of changing the patterns of receptor binding of the mutant TGF-βs. The sequence of TGF-β1 is shown in Figure 1B. TGF-β1 was chosen for mutation in these experiments since this allows for determination of activity in a broad range of biological systems, including those which discriminate between the potencies of the isoforms. The mutation of TGF-β2 would have restricted the use of assays, such as the LS513 assay, in which TGF-β2 is much less potent than TGF-β1 (Suardet et al., 1992).

TGF-β1(71 Trp) and TGF-β1(Δ69–73) were expressed in CHO cells using the inducible metallothionein promoter of the plasmid pMSXND and were confirmed by immunoblotting using the antibody LC 1–30 which reacts with TGF-β1 but not TGF-β2 (Flanders et al., 1989). Expression levels of TGF-β1(71 Trp) and TGF-β1(Δ69–73) were approximately 100 ng/mL as demonstrated by comparing immunoblots of conditioned media to known concentrations of TGF-β (Figure 2A). In contrast, neither TGF-β2 (100 ng) nor 1 mL of conditioned media from cells transfected with the expression vector alone produced a positive signal. The basal level of TGF-β production by control CHO cells, quantitated by the Mv1Lu growth inhibition assay, is 0.5 ng/mL (data not shown), suggesting that the level of the recombinant TGF-βs is approximately 200 times that of endogenous TGF-βs. Both

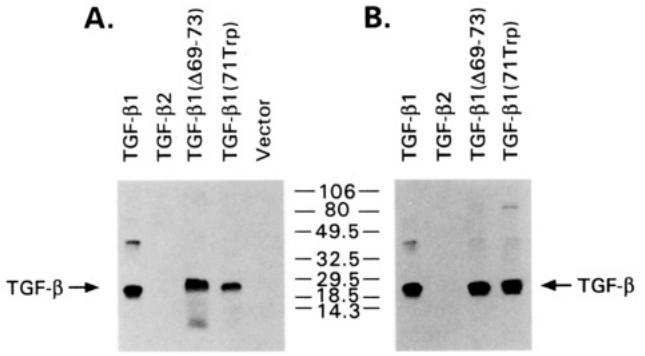

FIGURE 2: Immunoblot of TGF-β expression media. (*A*) TGF-β1 (100 ng), TGF-β2 (100 ng), and media collected from cells stably transfected with the expression plasmids TGF-β1(71 Trp) or TGF-β1(Δ69–73) or with the control plasmid pMSXND (vector) were electrophoresed through a 10% Tricine–SDS–polyacrylamide gel in the absence of reducing agents and immunoblotted using antibody LC 1–30, which reacts with TGF-β1 but not TGF-β2. (*B*) Purified TGF-β1, TGF-β2, TGF-β1(71 Trp), and TGF-β1(Δ69–73) were immunoblotted using antibody LC 1–30. Each lane contained 100 ng of TGF-β isoform.

TGF-β1(71 Trp) and TGF-β1(Δ69–73) were purified as described (Qian et al., 1992) to greater than 95% purity as determined by silver-staining electrophoresed protein (data not shown). Figure 2B confirms that purified TGF-β1(71 Trp) and TGF-β1(Δ69–73) react with antibody LC 1–30 similarly to TGF-β1, but not TGF-β2, as expected.

TGF-β1(71 Trp) and TGF-β1(Δ69–73) were assayed for their abilities to inhibit growth of Mv1Lu, LS1034, LS513 and FBHE cells (Figure 3). Both Mv1Lu and LS1034 cells were used for the assay of the mutant proteins since TGF-β1 and TGF-β2 are equivalent in inhibiting the growth of these cells (Suardet et al., 1992; Danielpour & Sporn, 1990). Figure 3A shows that TGF-β1 and TGF-β1(71 Trp) have nearly equivalent potencies on Mv1Lu cells. TGF-β1(Δ69–73) was approximately 4-fold less active in each of three independent assays. Similarly, TGF-β1, TGF-β2, and both of the mutant TGF-βs inhibited the growth of LS1034 cells equivalently (Figure 3B). These results demonstrate that the overall conformation of the recombinant proteins has not been destroyed and that they have retained biological activity. The observed variation in activity of TGF-β1(Δ69–73) between Mv1Lu and LS1034 cells may reflect subtle differences between receptor or signaling pathways in mink and human cells.

In contrast to LS1034 and Mv1Lu cells, LS513 cells are growth-inhibited by moderate concentrations of TGF-β1 but are refractory to TGF-β2 (Suardet et al., 1992). The difference between the growth response to TGF-β of LS1034 and LS513 cells is presumed to be due to expression of selective cell-surface receptors for TGF-β on these cells since these assays are performed using identical conditions (Suardet et al., 1992). TGF-β1 and TGF-β1(71 Trp) were nearly equivalent in inhibiting the growth of LS513 cells, but TGF-β1(Δ69–73) was relatively inactive, producing a growth inhibition curve similar to that of TGF-β2 (Figure 3C). Thus, although TGF-β1, TGF-β1(71 Trp), and TGF-β1(Δ69–73) had similar activities in Mv1Lu and LS1034 assays, TGF-β1(Δ69–73), like TGF-β2, was ineffective at inhibiting growth of LS513 cells. Inhibition of the growth of these cells by TGF-β1(Δ69–73) was observed only at very high concentrations. Similarly, the morphology of LS513 cells changed by flattening and enlarging when the cells were treated with TGF-β1 and TGF-β1(71 Trp) at 2 ng/mL, whereas 20 ng/mL TGF-β2 or TGF-β1(Δ69–73) did not affect morphology (data not shown).

To investigate the possibility that TGF-β1(Δ69–73) is less active in some assays because of isoform-specific inactivation by α_2M, TGF-β1(Δ69–73) and TGF-β1(71 Trp) were tested for their ability to inhibit the growth of FBHE cells. We have

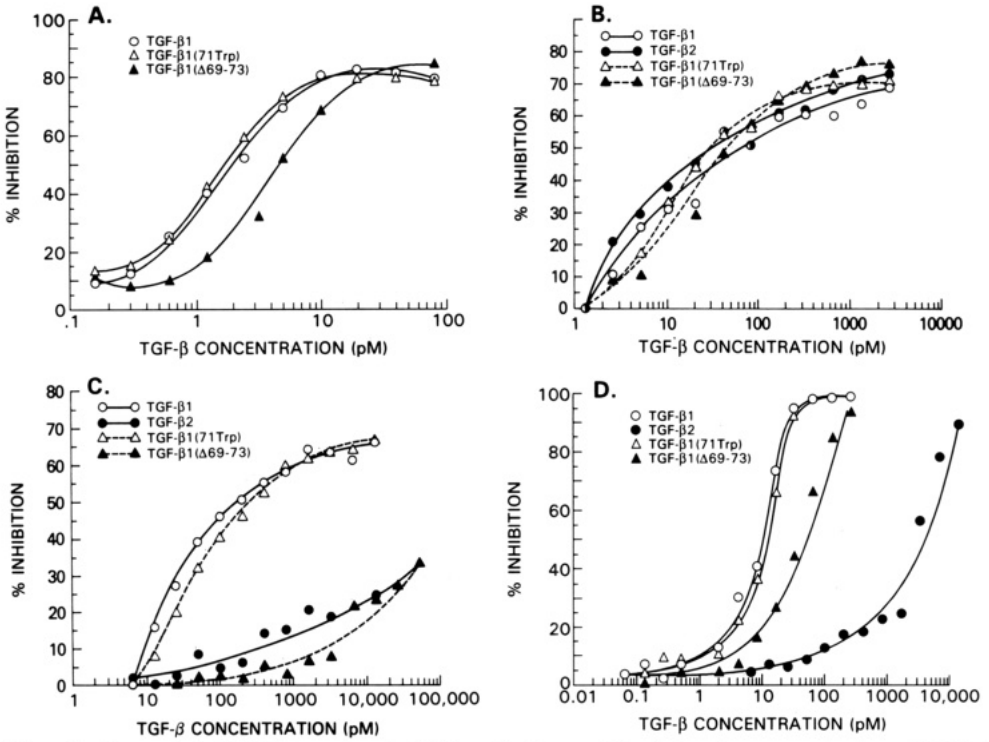

FIGURE 3: Inhibition of cell growth by TGF-β mutants. Inhibition of cell growth by increasing concentrations of TGF-β1 (○), TGF-β2 (●), TGF-β1(71 Trp) (△), and TGF-β1(Δ69–73) (▲) is shown for Mv1Lu cells (A), LS1034 cells (B), LS513 cells (C), and FBHE cells (D). Mv1Lu, LS1034, and LS513 assays were performed in media supplemented with 0.2% fetal bovine serum, whereas the FBHE assay was performed in medium supplemented with 10% calf serum. Incorporation of [^{3}H]thymidine into DNA was measured and expressed as percentage inhibition relative to that of untreated cells.

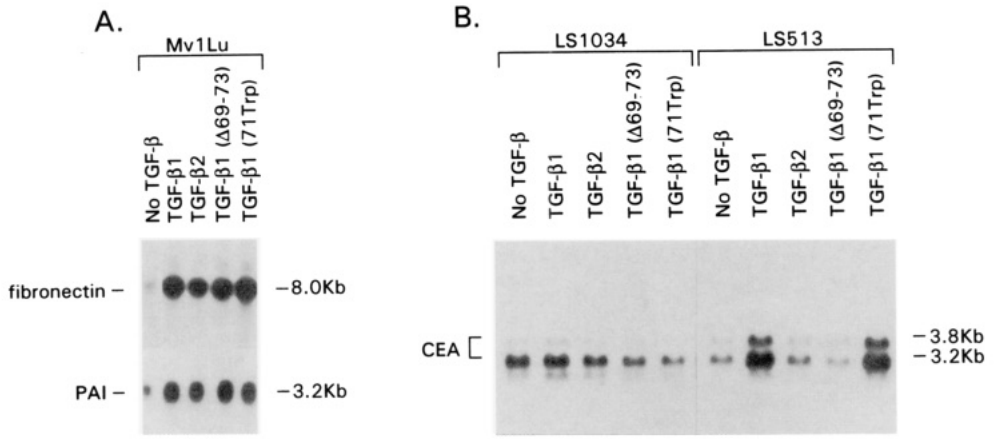

FIGURE 4: Changes in mRNA level following treatment of cells with TGF-β isoforms. (*A*) Levels of fibronectin and plasminogen activator inhibitor mRNAs in Mv1Lu cells following treatment with TGF-β isoforms. Mv1Lu cells were treated with 0.5 ng/mL TGF-β1, TGF-β2, TGF-β1(71 Trp), or TGF-β1(Δ69–73) for 24 h, or left untreated, and then total RNA was isolated. Twelve micrograms of each RNA was electrophoresed through a 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled cDNA for fibronectin or plasminogen activator inhibitor. (*B*) Levels of carcinoembryonic antigen in LS1034 and LS513 cells following treatment with TGF-β isoforms. LS1034 cells were treated with 5 ng/mL TGF-β isoforms, and LS513 cells were treated with 12.5 ng/mL TGF-β isoforms for 24 h, or left untreated, and used for blotting as described above using a ^{32}P-labeled cDNA from carcinoembryonic antigen. Equal loading of RNA samples was shown by ethidium bromide staining of the gels (data not shown).

previously used this assay to demonstrate that TGF-β1 is approximately 100-fold more active than TGF-β2 when the assay is performed in medium supplemented with 10% calf serum, because of the preferential sequestration of TGF-β2 by α_2M and other components present in serum (Burmester et al., 1993). Figure 3D shows that TGF-β1(71Trp) was nearly as potent as TGF-β1, whereas TGF-β1(Δ69–73) was about 4-fold less active than TGF-β1 at inhibiting the growth of FBHE cells, a result that is consistent with inhibition of Mv1Lu growth by both of these mutants (Figure 3A). In contrast, TGF-β2 was about 100-fold less effective than TGF-β1 at inhibiting the growth of FBHE cells. These results demonstrate that TGF-β1(Δ69–73) is not efficiently sequestered by α_2M or other serum components and eliminate the possibility that inactivation of TGF-β1(Δ69–73) is the cause of the decreased activity of this mutant in the LS513 assay.

TGF-β1(71 Trp) and TGF-β1(Δ69–73) were also assayed for their ability to regulate fibronectin and plasminogen activator inhibitor mRNA levels in Mv1Lu cells and carcinoembryonic antigen mRNA levels in LS1034 and LS513 cells (Figure 4). These genes have been shown previously to be regulated by TGF-β (Massagué, 1990; Chakrabarty et al., 1988). Both TGF-β1(71 Trp) and TGF-β1(Δ69–73) increased the level of fibronectin and plasminogen activator inhibitor mRNA in Mv1Lu cells (Figure 4A) similar to TGF-β1 and TGF-β2. The level of carcinoembryonic antigen mRNA in LS1034 cells did not change significantly when cells were treated with TGF-β1, TGF-β2, TGF-β1(71 Trp), or TGF-β1(Δ69–73) (Figure 4B). In contrast, TGF-β1 and TGF-β1(71 Trp) but not TGF-β2 or TGF-β1(Δ69–73) increased the level of carcinoembryonic antigen mRNA in LS513 cells (Figure 4C).

To investigate potential differences in binding of TGF-β1(Δ69–73) to receptors of Mv1Lu, LS1034, and LS513 cells, cross-linking experiments were performed. Figure 5A shows the results from cross-linking experiments using 250 pM ^{125}I-TGF-β1 or 1000 pM ^{125}I-TGF-β1(Δ69–73) with Mv1Lu and LS513 cells, and 250 pM ^{125}I-TGF-β1, ^{125}I-TGF-β2, ^{125}I-TGF-β1(Δ69–73), or ^{125}I-TGF-β1(71Trp) with LS1034 cells. As expected, TGF-β1 bound equally well to the receptors of Mv1Lu, LS1034, and LS513 cells (Figure 5A) and was markedly reduced by the addition of a 20-fold excess of TGF-β1 (data not shown), demonstrating that this is specific labeling of the TGF-β receptors. The difference in migration of the human and mink receptors is due to differences in the amount of N-linked carbohydrate attached to the receptors (Cheifetz et al., 1988; Wrana et al., 1992). Confirmation that the high molecular weight binding protein on LS513 cells is betaglycan was obtained using a deglycosylation experiment. Figure 5B shows the cross-linking pattern for deglycosylated receptors of Mv1Lu and LS513 cells. Significantly, the pattern for both cell lines is similar to the pattern which has been previously shown to be characteristic of betaglycan (Cheifetz et al., 1988). TGF-β1(Δ69–73) bound efficiently to each of the receptors of Mv1Lu and LS1034 cells. An enhanced intensity with the type I receptor of Mv1Lu and LS1034 cells was observed, although it is currently not known if this represents increased binding to this receptor, or a higher efficiency of cross-linking. In contrast, TGF-β1(Δ69–73) bound only slightly to the receptors of LS513 cells despite the higher concentration used. These cross-linking results are consistent with the biological data of Figure 3, and they suggest that the differential response of these cells to TGF-β1(Δ69–73) is due to differences in the specificity of the TGF-β receptors on these cells.

TGF-β1(71 Trp) and TGF-β1(Δ69–73) were also assayed for their ability to bind to endoglin on HUVEC cells. Endoglin is a dimeric membrane glycoprotein which is expressed on pre-erythroblasts, macrophages, leukemic cell of lymphoid and myeloid lineage, syncytiotrophoblasts, and human vascular endothelial cells (Cheifetz et al., 1992). Endoglin binds TGF-β1 and TGF-β3 with high affinity, but not TGF-β2 (Cheifetz et al., 1992), and it shares 27% overall sequence identity with betaglycan (López-Casillas et al., 1991; Wang et al., 1991). The most similar sequences are located in the transmembrane and cytoplasmic domains (74% identity), with two regions of greater than 40% sequence identity in the extracellular domain of the molecule (Morén et al., 1992). The biological function of endoglin is currently not known. Significantly, growth of HUVECs was not affected by the addition of TGF-β1 or TGF-β2 (Qian, unpublished results). Figure 6 shows the binding of ^{125}I-TGF-β1, ^{125}I-TGF-β2, ^{125}I-TGF-β1(71 Trp), and ^{125}I-TGF-β1(Δ69–73) to the cell-surface binding proteins on HUVEC and Mv1Lu cells. As expected, TGF-β1, but not TGF-β2, bound to endoglin and the type II receptor of HUVECs; both TGF-β1(71 Trp) and TGF-β1(Δ69–73) also bound to these proteins. Each of the TGF-β forms bound to

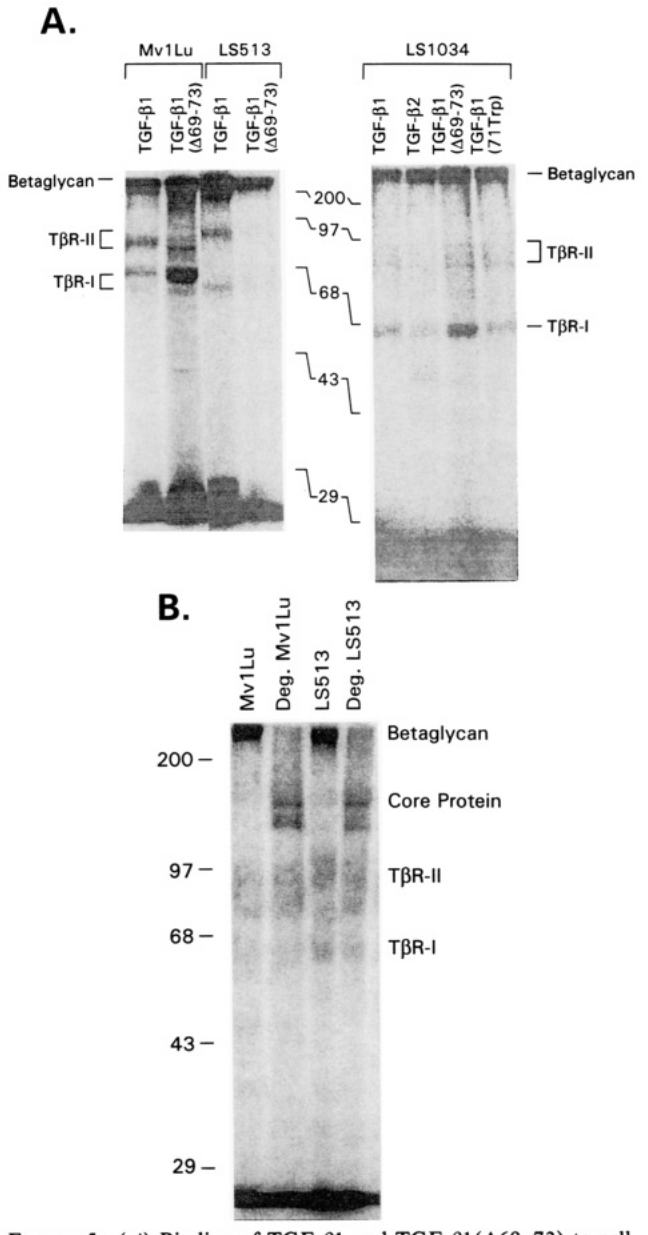

FIGURE 5: (*A*) Binding of TGF-β1 and TGF-β1(Δ69–73) to cell-surface proteins of Mv1Lu, LS1034, and LS513 cells. Mv1Lu and LS513 cells were cross-linked with 250 pM ^{125}I-TGF-β1 or 1000 pM ^{125}I-TGF-β1(Δ69–73). LS1034 cells were cross-linked with 250 pM ^{125}I-TGF-β1, ^{125}I-TGF-β2, ^{125}I-TGF-β1(Δ69–73), or ^{125}I-TGF-β1-(71Trp). Proteins were electrophoresed through an 8% acrylamide gel and visualized by autoradiography. The positions of the type I and II receptors and betaglycan are shown. (*B*) Deglycosylation of cell-surface TGF-β binding proteins. Confluent Mv1Lu and LS513 cells were incubated in the absence or presence of a mixture of chondroitinase ABC and heparinase to remove carbohydrate and then affinity-labeled with ^{125}I-TGF-β1. Cross-linked proteins were reduced and electrophoresed through an 8% acrylamide gel. Positions of the molecular weight markers are shown at the left.

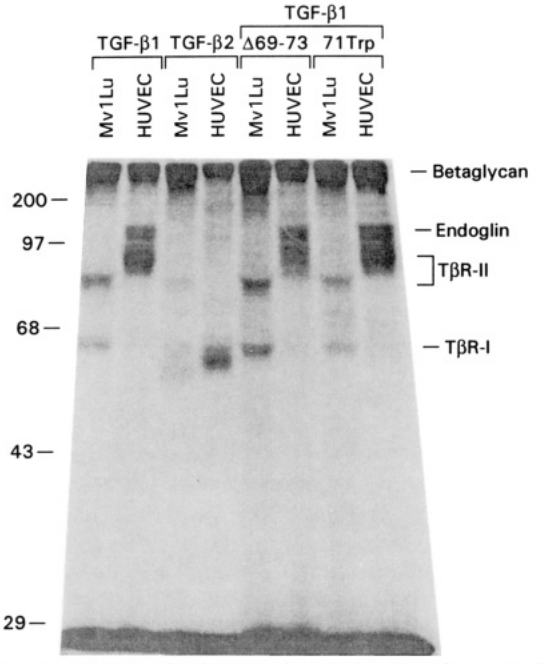

FIGURE 6: Binding of TGF-β isoforms to cell-surface proteins of HUVEC and Mv1Lu cells. Cells were cross-linked with 250 pM ^{125}I-TGF-β1, ^{125}I-TGF-β2, ^{125}I-TGF-β1(71 Trp), or ^{125}I-TGF-β1-(Δ69–73). Proteins were reduced by adding β-mercaptoethanol, electrophoresed through a 6% acrylamide gel, and visualized by autoradiography. Positions of the type I and II receptors and that of endoglin and betaglycan are labeled.

betaglycan on HUVECs, and, in agreement with Figure 5, each TGF-β form bound effectively to the cell-surface proteins of Mv1Lu cells (the control). Enhanced binding of TGF-β2 to a protein on HUVECs resulted in a labeled band of less than 68 kDa. The identity of this TGF-β binding protein is currently unknown, although it may be a form of the type I TGF-β receptor. Although our results for binding of the TGF-β isoforms to endoglin and the type II receptor are consistent with those of Cheifetz et al. (1992), they observed little or no betaglycan on HUVECs, in contrast to the cross-linking pattern of Figure 6. Our cross-linking to betaglycan was reproducible despite using cells purchased from two independent sources. The nature of this difference is unknown.

DISCUSSION

To investigate the possibility that the surface of the exposed loop of TGF-β centered at amino acid 71 participates in the binding of TGF-β to its receptors, we have synthesized two TGF-β1 mutant proteins. TGF-β1(71 Trp) was equivalent to TGF-β1 when assayed for both inhibition of growth and induction of target gene expression in the three test cell lines, Mv1Lu, LS1034, and LS513. In contrast, TGF-β1(Δ69–73) effectively inhibited the growth of both Mv1Lu and LS1034 cells, but not LS513 cells, which show a selective response to TGF-β1 compared to TGF-β2. Similarly, TGF-β1(Δ69–73) was as active as TGF-β1 or TGF-β2 at regulating expression of mRNAs for fibronectin and plasminogen activator inhibitor in Mv1Lu cells and carcinoembryonic antigen in LS1034 cells, whereas neither TGF-β2 nor TGF-β1(Δ69–73) altered expression of carcinoembryonic antigen mRNA with LS513 cells. Consistent with these biological results, receptor labeling experiments demonstrated that TGF-β1(71 Trp) bound efficiently to the receptors on Mv1Lu, LS1034, and LS513 cells whereas TGF-β1(Δ69–73) bound to the receptors of Mv1Lu and LS1034 cells much better than to the receptors of LS513 cells. Significantly, the ability of TGF-β1(Δ69–73) to bind to endoglin and the type II receptor on HUVECs was similar to TGF-β1, not TGF-β2. These results demonstrate that TGF-β1(Δ69–73) is a unique form of TGF-β, the activity of which is distinct from TGF-β1 or TGF-β2. As we learn more about the *in vivo* differences of the TGF-β isoforms, there may be important therapeutic applications of TGF-β1(Δ69–73) since it targets a unique set of TGF-β receptors and binding proteins.

The differential response of Mv1Lu, LS1034, and LS513 cells to TGF-β1(Δ69–73) is likely due to differences in the cell-surface receptors or binding proteins on these cells. It

has been suggested that type I and type II TGF-β receptors associate as interdependent components of a heteromeric complex: type I receptor requires type II receptor to bind TGF-β, and type II receptor requires type I to signal (Wrana et al., 1992). Since binding of TGF-β1(Δ69–73) to both type I and type II receptors is lost in the LS513 cells, one possible mechanism would be that the deletion mutant is not able to bind to the type II receptor, which would also result in the loss of type I receptor binding.

Considerable cross-linking evidence has accumulated demonstrating the existence of at least two forms of the type I TGF-β receptor and two forms of the type II TGF-β receptor (Cheifetz et al., 1990; Cheifetz & Massagué, 1991). To date, three distinct type I receptors which bind TGF-β have been cloned (Ebner et al., 1993; Franzén et al., 1993; Bassing et al., 1994; Attisano et al., 1993). In general, the prominent form of the type I or type II receptors binds TGF-β1 and TGF-β3 better than TGF-β2, and it has been suggested that signaling through these prominent TGF-β isoform-specific receptors can account for the differences in isoform potency on certain cells (Cheifetz et al., 1990; Cheifetz & Massagué, 1991). In contrast, both TGF-β1 high-affinity receptors and TGF-β2 high-affinity receptors have been detected on Mv1Lu cells, providing a potential explanation for why TGF-β1 and TGF-β2 have similar potencies with these cells (Cheifetz et al., 1990). The substantially greater potency of TGF-β1 than TGF-β2 at inhibiting growth of LS513 cells may suggest that only TGF-β1-specific high-affinity receptors are expressed on these cells.

An alternate hypothesis about isoform specificity suggests that betaglycan (type III receptor) is necessary to present TGF-β2 to the prominent type I and II signaling receptors, and that cells which fail to express betaglycan show a relative resistance to TGF-β2 (López-Casillas et al., 1993). When membrane-bound betaglycan was overexpressed in L_6E_9 myoblasts, biological differences between the TGF-β isoforms were eliminated, suggesting that betaglycan directly regulates access of TGF-β2 to the signaling receptors. However, it does not appear that this mechanism is responsible for the observed isoform differences with LS1034 and LS513 cells since both of these cell lines express betaglycan which binds TGF-β (Figure 5). It is possible, however, that mutations within betaglycan of LS513 cells prevent presentation of TGF-β2 to type I and type II receptors of these cells. Experiments are currently in progress to investigate this possibility. In addition, fetal bovine heart endothelial cells which respond equivalently to TGF-β1 and TGF-β2, when assayed in low-serum medium, do not express an appreciable amount of betaglycan (Burmester et al., 1993). Additional investigations are necessary in order to determine if betaglycan presentation to the signaling receptors is a widely utilized mechanism.

We have previously constructed chimeric TGF-βs to investigate the regions of the TGF-β molecule that determine isoform specificity in assays which discriminate between the TGF-β isoforms (Qian et al., 1992; Burmester et al., 1993). Using this approach, we have demonstrated that exchange of amino acids 45 and 47 of TGF-β1 into TGF-β2 specifies a TGF-β1-like affinity for the abundant serum protein α_2M. Additional experiments using chimeric TGF-β molecules are in progress to identify the exact amino acids that affect isoform specificity in the LS513 assay. Since a chimera containing amino acids 1–39 of TGF-β2, 40–82 of TGF-β1, and amino acids 83–112 of TGF-β2 was inactive in the LS513 assay (Burmester et al., 1993), amino acids 69–73 were not sufficient to specify the TGF-β1-like activity. In contrast a chimera comprised of amino acids 1–82 of TGF-β2 and 83–112 of TGF-β1 was equal to TGF-β1 in this assay, suggesting that all of the amino acids necessary to specify a TGF-β1-like activity reside between amino acids 83 and 112 (Burmester et al., in preparation). However, this chimera did not resemble TGF-β1 when assayed for binding to endoglin, suggesting that distinct functional domains specify these activities of TGF-β. Taken together, the results using chimeric TGF-βs and results presented in this paper suggest that TGF-β1(Δ69–73) may have lost its activity in the LS513 assay due to a modest conformational change which alters the exact location of the amino acids which interact directly with the TGF-β receptors on LS513 cells.

A model of the 69–73 deletion has been constructed based on the crystal structure of TGF-β2 since the crystal structure of TGF-β1 has not yet been reported. The solution structure of TGF-β1 (Archer et al., 1993a,b) is a composite of incomplete NMR data and the TGF-β2 crystal structure framework. The structural superposition of the 69–73 deletion mutant on the wild-type TGF-β2 did not show any second-order conformational perturbances beyond the mutation site. Further characterization of the structure of TGF-β1(Δ69–73) will likely involve solving its crystal structure.

It has recently been suggested that the type I TGF-β receptors are responsible for signaling effects on extracellular matrix, whereas the type II TGF-β receptors, possibly in conjunction with type I receptors, mediate growth inhibitory effects (Geiser et al., 1992; Chen et al., 1993). In the future, it may be possible to design TGF-β analogs capable of selectively activating one of these pathways. The results presented in this report represent a first step toward achieving this goal.

ACKNOWLEDGMENT

The plasmid pMSXND was generously provided by See-Jin Lee. We are grateful to Robert Lechleider for valuable discussion and Alice Stargardt for excellent secretarial assistance.

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