Antibodies to Peptide Determinants in Transforming Growth Factor β and Their Applications†

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ABSTRACT: Polyclonal antibodies have been raised to a series of synthetic peptides which correspond to essentially all regions of the transforming growth factor β1 (TGF-β1) molecule. All antisera were evaluated for their abilities to react with TGF-β1 and TGF-β2 in either the native or reduced form in enzyme-linked immunosorbent assays, Western blots, and immunoprecipitation assays. While all antisera demonstrated some ability to recognize TGF-β1 in these systems, there was limited cross-reactivity with TGF-β2, suggesting that substantial sequence or conformational differences exist between the two growth factors. On Western blots 5–10 ng of purified human platelet TGF-β1 could be detected when probed with affinity-purified peptide antisera generated against peptides corresponding to residues 48–77, 50–75, and 78–109 of the 112 amino acid TGF-β1 monomer. Antisera raised against peptides 50–75 and 78–109 were most effective in immunoprecipitating reduced and native ^{125}I-TGF-β1, respectively. The antisera also were tested for their effectiveness in blocking the binding of ^{125}I-TGF-β1 to its receptor. Anti-peptide 78–109 and anti-peptide 50–75 blocked 80% and 40% of the binding, respectively, while antibodies against amino-terminal peptides were without effect. These data suggest that the carboxyl-terminal region of TGF-β1 may play a significant role in the binding of the native ligand to its receptor.

Transforming growth factor β is a multifunctional regulator of cell growth which can affect cellular proliferation and differentiation (Sporn et al., 1986; Roberts & Sporn, 1987). In general, TGF-β[1] inhibits the growth of most epithelial cells (Moses et al., 1985) but will either stimulate or inhibit proliferation of mesenchymal cells depending on the presence of other growth factors (Roberts et al., 1985). Effects on differentiation are also bifunctional; TGF-β induces the differentiation of bronchial epithelial cells (Masui et al., 1986) but inhibits differentiation of myoblasts to myotubules (Florini et al., 1986; Massagué et al., 1986; Olson et al., 1986) and 3T3 fibroblasts to adipocytes (Ignotz & Massagué, 1985). TGF-β also modulates cell function [for review, see Sporn et al. (1986) and Roberts and Sporn (1987)], including enhancing extracellular matrix production by fibroblasts (Ignotz & Massagué, 1986; Roberts et al., 1986), inducing chemotaxis of both fibroblasts (Postlethwaite et al., 1987) and monocytes/macrophages (Wahl et al., 1987), and suppressing the action of T and B lymphocytes (Kehrl et al., 1986a,b; Rook et al., 1986).

TGF-β is a dimer composed of two identical disulfide-linked chains. The cDNA for TGF-β (Derynck et al., 1985) shows that the mature 112 amino acid monomer is derived from the carboxyl-terminal portion of a 390 amino acid precursor. Formation of the biologically active dimer then involves both disulfide bonding of the monomers and proteolytic cleavage from the precursor. While all neoplastic and nonneoplastic cells seem to produce TGF-β, platelets (Assoian et al., 1983) and bone (Seyedin et al., 1985, 1986) seem to be the major sources from which TGF-β can be purified. Little is known about the biosynthesis of the growth factor or its packaging into a storage site such as platelets. Recently a second form of TGF-β, TGF-β2, has been isolated from bovine bone (Seyedin et al., 1985, 1987) and porcine platelets (Cheifetz et al., 1987). Even though there are 14 amino acid differences in the first 36 amino acids of the two forms (Cheifetz et al., 1987; Seyedin et al., 1987), their biological activities are similar (Cheifetz et al., 1987; Seyedin et al., 1985, 1987). The functional role of this second form is unknown.

Antibodies to TGF-β are needed to investigate its varied biological actions, to study its biosynthesis, and possibly to distinguish between the effects of the two forms. The generation of immunoprecipitating and neutralizing antibodies to native TGF-β has been extremely difficult due to the highly conserved nature of native TGF-β among different species. The human sequence (Derynck et al., 1985) is identical with the bovine (Van Obberghen-Schilling et al., 1987) and porcine (Derynck & Rhee, 1987; P. Kondaiah, unpublished results) sequences and differs from the murine sequence by one amino acid (Derynck et al., 1986). As an alternative approach, we have raised antibodies to peptides corresponding to various regions of TGF-β. These antibodies recognize TGF-β in a variety of immunological assays and have been useful in identifying regions of the molecule that affect receptor binding.

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[1] Abbreviations: TGF-β, transforming growth factor β; BSA, bovine serum albumin; PBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; KLH, keyhole limpet hemocyanin; SBTI, soybean trypsin inhibitor; MeBSA, methylated bovine serum albumin; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IgG, immunoglobin G.

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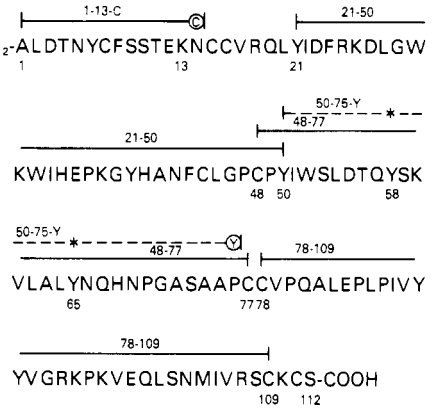

FIGURE 1: Amino acid sequence of the TGF-β monomer showing the peptides used to generate region-specific antisera. The peptide 1–13-C was synthesized with a cysteine at residue 14 instead of asparagine, while peptides in the region of 50–75 were synthesized with a tyrosine at position 76 instead of proline. The asterisks mark the start of two additional peptides that begin at residue 58 or 65 and end with residue 75 followed by tyrosine. The terminal cysteine residues of peptides 48–77 and 78–109 were disulfide-linked.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides P 1–13, P 21–50, and P 78–109 (Figure 1) were purchased from Peninsula Laboratories. P 1–13 was synthesized with an added carboxyl-terminal cysteine, while the terminal cysteine residues of P 78–109 were cyclized. In addition, a series of peptides of varying lengths were synthesized to the region of TGF-β between Cys-48 and Cys-77. P 48–77 was synthesized by Merrifield solid-phase methodology (Merrifield, 1963), and the terminal cysteine residues were cyclized with potassium ferricyanide (Ling et al., 1980). P 50–75, P 58–75, and P 65–75 also were synthesized by Merrifield solid-phase methods, each with an added carboxyl-terminal tyrosine. Peptides were purified by HPLC and quantitated by amino acid analysis.

Preparation of Immunogen and Production of Antisera. P 1–13 was coupled to soybean trypsin inhibitor through its carboxyl-terminal cysteine with *m*-maleimidobenzoyl sulfosuccinimide ester (Sigma) as the coupling agent in phosphate buffer as described by Green et al. (1982). P 50–75, P 58–75, and P 65–75 were coupled to BSA (Miles Laboratories) and P 21–50 was coupled to ovalbumin (Sigma) with stoichiometric amounts of diazotized benzidine (Guillemin et al., 1977). P 48–77 and P 78–109 were electrostatically coupled to methylated BSA (Sigma) (Benoit et al., 1982).

New Zealand white rabbits (generally two rabbits per peptide) were injected in 20–30 intradermal sites with 1–2 mg of conjugated peptide in an emulsion of 50% Freund's complete adjuvant in a total volume of 2 mL. Rabbits were boosted every 3–4 weeks with 0.5–1 mg of conjugated peptide in 50% Freund's incomplete adjuvant. Bleeds were taken 10 days after boosts. P 78–109 and P 21–50 were also injected without coupling to a carrier protein following this protocol. Production of antisera raised against TGF-β coupled to KLH has been previously described (Roberts et al., 1986).

Purification of Antisera. Serum was partially purified by 50% ammonium sulfate precipitation. In some cases IgG was purified with protein A–Sepharose (Pharmacia) as described (Goeding, 1978). Some peptide antisera were further purified by passage over a 1.5–mL column of Affi-Gel 10 (Bio-Rad) that had been cross-linked to the appropriate peptide according to the manufacturer's instructions. Briefly, 3 mL of gel slurry was washed with H_2O, and the resin was incubated with 1–2 mg of peptide for 4 h at 4 °C in 4 mL of 0.1 M Hepes, pH 7.5. Blocking of the unreacted active ester sites was accomplished by addition of 0.1 mL of 1 M ethanolamine hydrochloride followed by a 1-h incubation at room temperature. The resin then was washed extensively in PBS. A 50% ammonium sulfate fraction of the serum was applied, the column was washed as described by Curran et al. (1985), and specifically bound IgG was eluted with 0.1 M glycine hydrochloride, pH 2.5, into tubes containing 3.0 M Tris-HCl, pH 8.8. All IgG fractions were dialyzed against PBS.

ELISA. Antigens (250 ng of peptide or 25 ng of human platelet TGF-β1 or porcine TGF-β2 from R & D Systems) were solubilized in PBS and allowed to air-dry overnight on to wells of microtiterplates (Nunclon). Nonspecific binding was blocked with 0.2% gelatin in PBS, followed by washing of the plates with buffered 0.02% Tween-20 (Kirkegaard & Perry Laboratories). Antisera to be tested were added in PBS–gelatin at an initial dilution of 1:50 with subsequent 3-fold dilutions. After incubation for 2 h at room temperature and washing of the plate, alkaline phosphatase labeled affinity-purified goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) in PBS–gelatin (0.5 μg/mL) was added for an additional 2 h. The plates were washed and color developed for a maximum of 30 min with *p*-nitrophenyl phosphate substrate in diethanolamine (Kirkegaard & Perry Laboratories). Optical densities at 405 nm were determined on a Titertek Multiskan MC (Flow Laboratories).

Radioimmunoassay. Titration of antisera against ^{125}I-TGF-β and subsequent competition with unlabeled TGF-β under nonreducing conditions were performed as described by Assoian and Sporn (1986). Assays done under reducing conditions were performed in the same manner except that the buffer was composed of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 40 mM dithiothreitol, and the TGF-β was denatured by boiling in this buffer for 4 min before the addition of antibody.

Immunoblotting. Electrophoresis of TGF-β on SDS–polyacrylamide gels, electrophoretic transfer to nitrocellulose followed by incubation with test antibody, and detection of the immune complexes with gold-labeled goat anti-rabbit IgG and subsequent silver enhancement were carried out as described by Florini et al. (1986). Alternatively, immune complexes were detected with an avidin–biotin conjugate immunoperoxidase kit (Vector Laboratories) following the protocol recommended by the manufacturer.

Immunoprecipitation of Radiolabeled Cell Media. Immunoprecipitation of media conditioned by Ha-ras-transfected NIH-3T3 cells labeled for 16 h with [^{35}S]cysteine (250 μCi/60-mm dish) was performed as previously described (Knabbe et al., 1987; Robey et al., 1987). Briefly, the labeled media was boiled in immunoprecipitation buffer for 4 min to activate latent TGF-β. The sample was preprecipitated with normal rabbit serum IgG (Cappel) and fixed *Staphylococcus aureus* (Boehringer Mannheim Biochemicals) and then incubated overnight at 4 °C with test antibody. The specificity of the immunoprecipitation was determined by preincubating the antibody with either platelet-derived TGF-β or the appropriate peptide. The immunoreactive TGF-β was recovered by precipitation with *S. aureus* and eluted by boiling in a 2% SDS buffer. The samples were electrophoresed in a 10% polyacrylamide gel according to Laemmli (1970). The gels were fixed, enhanced with 2,5-diphenyloxazole dissolved in dimethyl sulfoxide, dried, and exposed to Kodak XAR-5 X-ray film at −70 °C for 2 weeks.

Inhibition of TGF-β Receptor Binding by Antisera. ^{125}I-TGF-β in binding buffer (MEM, no bicarbonate, containing

0.1% BSA and 25 mM Hepes, pH 7.4) was preincubated for 2 h at room temperature with an appropriate dilution of the IgG fraction of the antisera or control serum to be tested. Aliquots of the mixture were then assayed in duplicate for 2 h at room temperature, on A549 human lung carcinoma cells grown in 24-well cluster plates according to the binding protocol previously described (Frolik et al., 1984).

Inhibition of TGF-β-Induced Collagen Production in NRK Cells by Antisera. Similar to the binding assay, TGF-β (20–50 pM) was preincubated for 2 h at room temperature with appropriate dilutions of the IgG fractions of various peptide antisera or control serum in DMEM. The medium was then diluted 1:1 with MEM (containing glutamine, 20 mM Hepes, and 4% plasma-derived calf serum), and triplicate aliquots of 0.3 mL were added to NRK cells grown in 24-well cluster dishes. The incubation was continued for 18 h overnight in a 37 °C incubator, 5% CO_2. Incorporation of [^{3}H]proline into collagen was measured by a modification of the method of Peterkovsky et al. (1982), as previously described (Roberts et al., 1986).

RESULTS

Synthesis of Peptides and Production of Antisera. Immunization of rabbits with TGF-β coupled to KLH has produced antibodies that block TGF-β binding in only 2 of 25 rabbits, presumably because of the highly conserved nature of the molecule. To overcome this problem, we decided to raise antibodies to TGF-β with peptide antigens. Since the positions of the disulfide linkages in TGF-β and its tertiary structure are unknown, a complete set of peptides which encompass all regions of the molecule (Figure 1) was synthesized to gain information as to which regions of TGF-β might be exposed. Since antigenic determinants formed by free amino and carboxyl termini of synthetic peptides would have no counterpart in native TGF-β, an approach was taken to generate a looped structure for these peptides. In one approach P 21–50 and peptides in the region of amino acids 50–75 were synthesized with terminal tyrosine residues. Theoretically, coupling of both of these tyrosine residues to a carrier protein via a diazonium linkage anchors both ends of the peptide, making the structure of the peptide in the immunogen more like that of the peptide in the TGF-β molecule and possibly increasing the reactivity of an antibody with native TGF-β. Peptides of several lengths were synthesized in the amino acid region 50–75 to determine the correlation of length of the peptide antigen with reactivity of the resulting antibody to TGF-β. In another approach to generate a looped structure, the terminal cysteine residues of peptides 48–77 and 78–109 were disulfide linked, and these peptides were coinjected with methylated BSA as a carrier protein. In some instances rabbits were immunized with unconjugated peptides to investigate the effects of conjugation of longer peptides upon the reactivity of these antisera with the TGF-β molecule.

ELISA Reactivity. Antisera were initially screened by their reactivity to both the immunizing peptide and TGF-β in an ELISA assay. The antisera dilutions giving 50% maximal response are shown in Table I. The ED_{50} values of antisera generated to the same peptide in different rabbits agreed well, generally within a factor of 2 and the values reported in Table I are an average of the results from duplicate rabbits. Antisera raised to all peptides showed some reactivity to TGF-β. Peptide 21–50 has limited solubility, and conjugation to carrier proteins has been difficult, but immunizations of rabbits with the uncoupled peptide generated an antibody with some reactivity toward TGF-β. In a test of the effect of chain length on peptide antigenicity, the ELISA results demonstrate that in the region of amino acids 50–75 antiserum raised against the longer peptide (27 amino acids) was more reactive with TGF-β than that raised against the two shorter peptides of 12 and 19 amino acids. As a result, the antisera raised against P 58–75 and P 65–75 were not further characterized. Inclusion of a carrier protein increased the antigenicity of the peptides as demonstrated by the fact that coinjection of P 78–109 with methylated BSA more than doubled the reactivity of the resulting antiserum toward TGF-β compared to that produced against unconjugated peptide.

Table I: Antisera Titers by ELISA

antiserum generated against	carrier protein	ED_{50} (antiserum dilution)[a] vs peptide	vs TGF-β1	vs TFG-β2
TGF-β	KLH		1:400	0
1–13-C	SBTI	1:1000	1:300	0
21–50	ovalbumin	0	0	0
21–50		1:1000	1:700	0
48–77	MeBSA	1:2000	1:1000	1:400
50–75-Y	BSA	1:3000	1:2000	1:3000
58–75-Y	BSA	1:400	1:100	ND[b]
65–75-Y	BSA	1:200	1:150	ND
78–109	MeBSA	1:1400	1:2000	1:250
78–109		1:700	1:850	slight

[a] ED_{50} values given are representative of the average value for each antiserum over a number of bleeds. [b] ND, not determined.

When antisera were tested for reactivity against porcine TGF-β2, only antisera raised against P 50–75 showed significant reactivity with an ED_{50} of 1:3000, while anti-P 48–77 and anti-P 78–109 showed slight reactivity with an ED_{50} of approximately 1:400 (Table I). Antisera against native TGF-β1, P 1–13, and P 21–50 showed no reactivity, as might be expected since the available sequence data of the first 36 amino acids of TGF-β2 (Cheifetz et al., 1987) shows substantial sequence diversity in this region. These data suggest that additional sequence diversity should be expected in residues 50–112.

Immunoblots. The ability of the peptide antisera to detect TGF-β on an immunoblot was strongly dependent on the degree of purity of the antibody. TGF-β, either reduced or nonreduced, was electrophoresed on SDS–polyacrylamide gels and probed with the peptide antisera. A representative blot is shown in Figure 2. While a total IgG fraction of anti-P 48–77 prepared by passage of the serum over a protein A–Sepharose column barely detected 50–100 ng of reduced or nonreduced TGF-β, affinity purification of the antiserum greatly increased its reactivity giving a detection limit of approximately 5 ng of both reduced and nonreduced TGF-β. Similar results were obtained with antisera against P 50–75 and P 78–109 in that affinity purification of the antisera greatly increased the sensitivity of the immunoblot to approximately 10 ng of TGF-β. Both antisera to P 78–109 (one raised to peptide conjugated to methylated BSA and one raised to unconjugated peptide) showed similar reactivities. Anti-P 1–13 and anti-P 21–50 were not tested in immunoblots. None of the peptide-generated antisera reacted with TGF-β in immunoblots as well as the affinity-purified antiserum raised against native TGF-β (see Figure 2), which can detect 1 ng of nonreduced TGF-β.

The affinity-purified antisera were also tested for their immunoreactivity to 100 ng of TGF-β2, both reduced and nonreduced. The only affinity-purified antisera that showed any reactivity was anti-P 78–109 raised against the unconjugated peptide; this reacted only with reduced TGF-β2 (Figure 3). The reactivity of this antiserum with reduced TGF-β2 was judged to be approximately one-tenth of the reactivity with

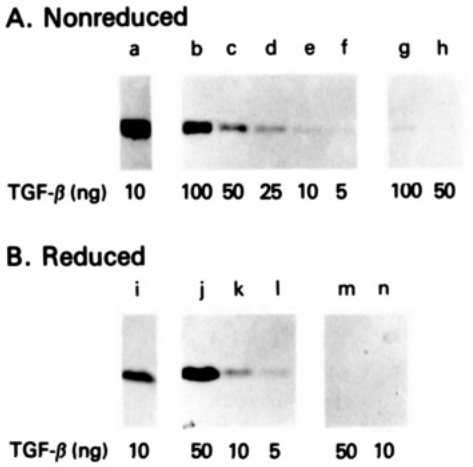

FIGURE 2: Detection of TGF-β on Western blots. The indicated amounts of purified human platelet TGF-β either nonreduced (A) or reduced (B) were electrophoresed on 12.5% SDS–polyacrylamide gels. Following transfer to nitrocellulose, the blots were probed with affinity-purified anti-TGF-β (1:50 dilution of original serum) (lanes a and i), affinity-purified anti-P 48–77 (1:75 dilution of original serum) (lanes b–f, j and l), or a total IgG fraction of anti-P 48–77 (1:50 dilution of original serum) (lanes g, h, m, and n). The immune complexes were detected with gold-labeled goat anti-rabbit IgG followed by silver enhancement. The band detected in nonreduced samples migrated with a molecular mass of approximately 24 kDa while the reduced band was detected at approximately 12 kDa. No other bands were detected on the blots.

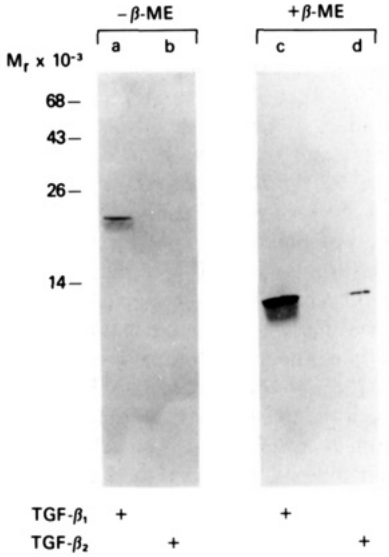

FIGURE 3: Detection of TGF-β2 in Western blots. TGF-β1 (100 ng) (lanes a and c) and TGF-β2 (100 ng) (lanes b and d) were electrophoresed on 15% polyacrylamide gels in the absence (lanes a and b) or presence (lanes c and d) of 2-mercaptoethanol. Following transfer to nitrocellulose, the blots were probed with affinity-purified anti-P 78–109 (1:50 dilution of original serum), and the immune complexes were detected with biotinylated goat anti-rabbit IgG by avidin-conjugated peroxidase.

TGF-β1. Even though the anti-P 50–75 serum showed strong reactivity to type 2 in an ELISA, neither affinity-purified anti-P 50–75 nor a total IgG fraction of anti-P 50–75 showed any reactivity toward TGF-β2 on immunoblots.

Radioimmunoassay. The immunoprecipitating abilities of the antisera were evaluated by incubating various dilutions of antisera (50% ammonium sulfate fraction) with ^{125}I-TGF-β under both reducing and nonreducing conditions (Figure 4). The titers of the antisera and the maximum amount of ^{125}I-TGF-β precipitated are given in Table II. The peptide antisera can be divided into two groups on the basis of comparison of their responses to immunoprecipitations done in the presence

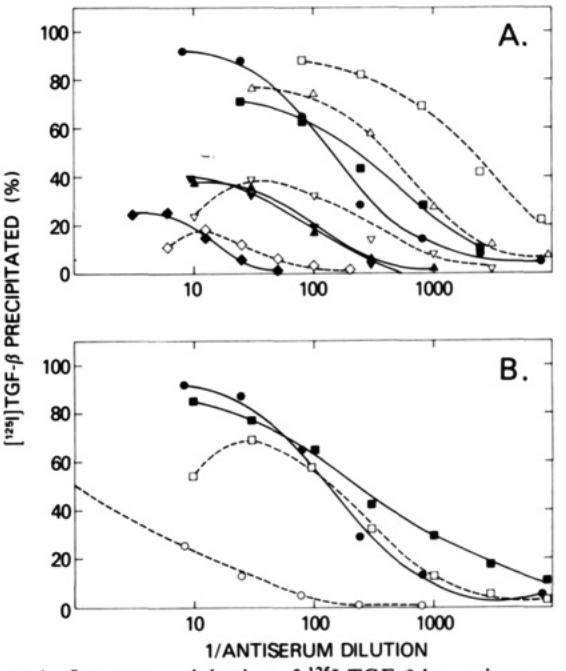

FIGURE 4: Immunoprecipitation of ^{125}I-TGF-β by antisera under nonreducing or reducing conditions. Varying dilutions of antisera (50% ammonium sulfate precipitable fraction) were incubated with ^{125}I-TGF-β in the absence (closed symbols) or presence (open symbols) of 40 mM DTT. Immune complexes were precipitated with *S. aureus*, and the pellet was counted in a Packard γ counter. The antisera used are as follows: (A) (●) anti-TGF-β, (■, □) anti-P 50–75-Y, (▲, △) anti-P 48–77, (◆, ◇) anti-P 1–13-C, and (▼, ▽) anti-P 21–50; (B) (●, ○) anti-TGF-β and (■, □) anti-P 78–109 raised against peptide conjugated to methylated BSA. Points are the mean of duplicate determinations.

Table II: Antisera Titers by Immunoprecipitation of ^{125}I-TGF-β1

antiserum generated against	ED_{50} (antiserum dilution)		maximum ^{125}I-TGF-β precipitated (%)	
	-DTT	+DTT	-DTT	+DTT
TGF-β	1:50	1:10[a]	90	50[a]
1–13-C	1:15	1:30	24	18
21–50	1:100	1:300	37	39
48–77	1:100	1:400	35	75
50–75-Y	1:500	1:2500	70	88
78–109	1:300	1:300	84	70
78–109 (unconjugated)	1:200	1:200	78	80

[a] Values determined by extrapolation.

and absence of DTT. The first group (shown in Figure 4A) consists of antisera to peptides 1–13, 21–50, 48–77, and 50–75; all of these show a substantial increase in both the maximum amount of ^{125}I-TGF-β precipitated and the ED_{50} (Table II) when the immunoprecipitations are done in the presence of 40 mM DTT. The reducing agent presumably opens the structure of TGF-β, making these regions of the molecule more accessible to the peptide antisera. The second group consists of antisera to P 78–109 and to native TGF-β; anti-P 78–109 shows little change in reactivity in the presence of DTT while the antiserum raised against the entire TGF-β molecule shows a dramatic decrease in reactivity under reducing conditions (Figure 4B). Anti-P 78–109 raised against conjugated or unconjugated peptide gave nearly identical results. It is to be expected that reduction would destroy the immunoprecipitating ability of anti-TGF-β since it was raised to the dimeric molecule. The similarity of reactivity of anti-P 78–109 in the presence and absence of DTT suggests that in the nonreduced, dimeric molecule this region may be relatively exposed and therefore accessible to the appropriate peptide antisera. None

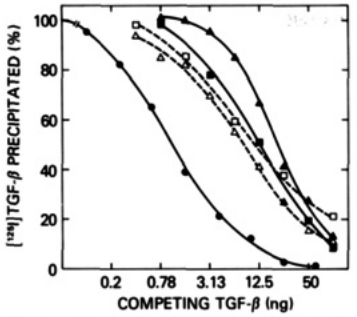

FIGURE 5: TGF-β radioimmunoassay. ^{125}I-TGF-β and varying amounts of unlabeled TGF-β were incubated with antiserum (50% ammonium sulfate precipitable fractions), and the immune complexes were recovered by precipitation with *S. aureus*. Assays were done in the presence (open symbols) or absence (closed symbols) of 40 mM DTT. The antisera used were (●) anti-TGF-β at a dilution of 1:1000, (■, □) anti-P 78–109 at a dilution of 1:200 without DTT and 1:50 in the presence of DTT, and (▲, △) anti-P 50–75-Y at a dilution of 1:400 without DTT and 1:2000 in the presence of DTT. Points are the mean of duplicate determinations.

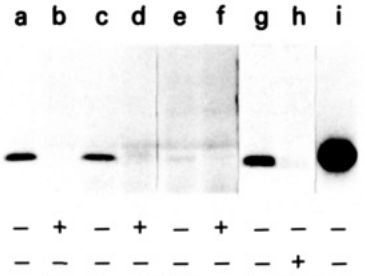

FIGURE 6: Immunoprecipitation of [^{35}S]cysteine-labeled media from Ha-ras NIH-3T3 cells by peptide antisera. Radiolabeled media (3 × 10^6 trichloroacetic acid precipitable counts) were precipitated with a 50% ammonium sulfate precipitable fraction of anti-TGF-β (lanes a and b), anti-P 78–109 raised against unconjugated peptide (lanes c and d), anti-P 48–77 (lanes e and f), or anti-P 78–109 raised to unconjugated peptide (lanes g and h). Some precipitations were done in the presence of unlabeled TGF-β (500 ng) (lanes b, d, and f) or peptide 78–109 (25 μg) (lane h). ^{125}I-TGF-β (lane i) was run for comparison.

of the antisera tested were able to immunoprecipitate bovine ^{125}I-TGF-β2 under reducing or nonreducing conditions.

A competitive radioimmunoassay using a representative antiserum from each group was performed in the presence or absence of DTT, and the results are shown in Figure 5. The antiserum raised to the TGF-β dimer was not tested under reducing conditions, but in the absence of DTT, 50% of the ^{125}I-TGF-β was competed by 1.2 ng of TGF-β. Anti-P 50–75 had an ED_{50} of 19 ng and 8.2 ng of TGF-β in the absence and presence of DTT, respectively, while the equivalent values for anti-P 78–109 raised to unconjugated peptide were 12.5 ng both in the presence and in the absence of DTT. No radioimmunoassay done with any peptide antiserum either with or without DTT was as sensitive as that done with the antiserum raised against the TGF-β dimer.

Immunoprecipitation of Radiolabeled Cell Media. To evaluate the usefulness of the peptide antisera for biosynthesis studies, media conditioned by Ha-ras NIH-3T3 cells grown in the presence of [^{35}S]cysteine were immunoprecipitated with the various peptide antisera. The immunoprecipitates were fractionated on SDS–polyacrylamide gels, and the resulting autoradiographs are shown in Figure 6. Antiserum to P 48–77 (lane e) and the antisera to unconjugated P 78–109 (lane c) or P 78–109 conjugated to methylated BSA (lane g) immunoprecipiated a 25-kDa protein that comigrated with ^{125}I-TGF-β (lane i). The specificity of this band is demonstrated by the fact that it is competed by unlabeled TGF-β (lanes d and f) or peptide (lane h). Furthermore, it comigrated with a species specifically immunoprecipitated by antiserum raised against the TGF-β dimer (lane a). No other specific bands were observed. Anti-P 50–75 also immunoprecipitated TGF-β from the media, but the background on the autoradiograph was very high; the immunoprecipitate resulting from anti-P 1–13 was barely detectable. Since the antisera were able to immunoprecipitate the murine TGF-β, it appears that position 75 in which the alanine in the human sequence is replaced by a serine in the murine sequence does not significantly alter the immunodetection of the molecule.

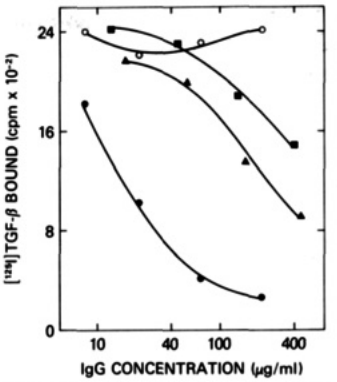

FIGURE 7: Inhibition of ^{125}I-TGF-β receptor binding to A549 cells by anti-peptide antisera. Increasing concentrations of antisera (total IgG fraction prepared by passage over protein A–Sepharose) were incubated with 100 pM ^{125}I-TGF-β prior to addition to cells, and a standard receptor binding assay was performed as described under Experimental Procedures. In the absence of added antiserum, 3000 cpm were bound. Results are representative of those obtained in three separate experiments and are the average of duplicate determinations. Antisera used were (●) anti-TGF-β, (■) anti-P 50–75-Y, (▲) anti-P 78–109, and (○) normal rabbit serum.

Inhibition of TGF-β Biological Activity by Region-Specific Antisera. Anti-P 50–75 and anti-P 78–109 (raised to unconjugated peptide) could partially block the binding of 100 pM ^{125}I-TGF-β to receptors on A549 cells (Figure 7). While antibodies raised to the TGF-β dimer blocked essentially all binding at an IgG concentration of approximately 250 μg/mL, anti-P 50–75 and anti-P 78–109 blocked approximately 40% and 80% of binding, respectively, at 450 μg of IgG/mL. Similar results were obtained with NRK cells. Anti-P 48–77 showed less blocking ability than anti-P 50–75, while anti-P 1–13-C and anti-P 21–50 showed essentially no ability to block receptor binding. This observation provides further evidence that the carboxyl-terminal region of TGF-β is exposed and affects receptor binding.

The abilities of the antisera to block TGF-β biological activity were evaluated in an assay that measures the TGF-β-induced production of collagen by NRK cells. Both the antiserum raised to the TGF-β dimer and that raised to P 78–109 at IgG concentrations of 80 and 500 μg/mL, respectively, blocked over 90% of the increase in collagen production induced by 50 pM TGF-β. Anti-P 50–75 at an IgG concentration of 500 μg/mL inhibited the increase by 40%. These results parallel those obtained when these antisera were tested for their abilities to block TGF-β receptor binding.

DISCUSSION

We have generated antibodies to peptides corresponding to regions of the TGF-β primary sequence in order to gain information about the tertiary structure of the TGF-β dimer and the areas of the molecule that can affect receptor binding. In addition, these antisera can be used to detect TGF-β in a variety of immunological assays, including enzyme-linked immunosorbent assays, immunoblots, and radioimmunoassays.

The region-specific antisera also have been used in immunohistochemical studies to investigate the role of TGF-β in embryonic development of the mouse (Heine et al., 1987).

Our results suggest that the carboxyl-terminal half of the TGF-β monomer is relatively more exposed in the native form of the protein and contributes either directly or indirectly to receptor binding, while the amino-terminal half is less accessible in the dimeric structure. Thus, antibodies raised to peptides 1–13 and 21–50 in the amino-terminal half of the molecule showed reactivity toward TGF-β in an ELISA and only slight reactivity in immunoprecipitation assays. Ellingsworth et al. (1986) have prepared an antiserum against the first 30 amino acids of TGF-β which has been reported to react with TGF-β either in an ELISA or on immunoblots; however, comparison of this antiserum with the peptide antisera described here shows that its immunoprecipitating ability is approximately equal to that of anti-P 1–13 (K. Flanders, unpublished results). These results suggest that antibodies directed against the amino-terminal portion of TGF-β recognize TGF-β bound to a solid support when it possibly is denatured but fail to recognize it in an immunoprecipitation assay where TGF-β would be in a more native configuration. The weaker reactivity to the native structure suggests that the amino-terminal region probably is inaccessible in the dimeric conformation.

Data obtained with antibodies raised against peptides in the carboxyl-terminal half of the molecule suggest that amino acids 48–77 are fairly exposed, as anti-P 48–77 and anti-P 50–75 reacted well with TGF-β in ELISA, immunoblots, and immunoprecipitation assays. This region also may have some role in receptor binding as anti-P 50–75 blocked 40% of ^{125}I-TGF-β receptor binding, although anti-P 48–77 was less effective in this regard. The difference between the reactivity of these two antisera suggests that the antisera might be generated to different epitopes of the peptides; this is likely as they were conjugated differently to carrier proteins. In contrast, anti-P 78–109 blocked 80% of TGF-β receptor binding, suggesting that this carboxyl-terminal region plays a significant role in the binding of native TGF-β to its receptor. This antiserum also most closely resembles antiserum raised against native TGF-β in its ability to immunoprecipitate TGF-β. Since the cysteines at positions 78 and 109 of the peptide used to generate the antiserum were disulfide linked, these cysteines also may be cross-linked to each other in the native TGF-β molecule, generating a loop that is exposed to the surface. These data suggest that the carboxyl-terminal region may be the major epitope involved in generating blocking antiserum to native TGF-β. It is interesting to note that forming a disulfide link between the cysteines at positions 78 and 109 brings together two domains that are conserved in all members of the TGF-β family (Padgett et al., 1987), suggesting that this region may be important for biological activity. The native, dimeric TGF-β molecule must also possess other minor epitopes that serve as antigenic determinants since the reactivity of anti-TGF-β is always somewhat greater than that of anti-P 78–109 in both immunoprecipitation and antireceptor binding assays.

Since peptide antisera were not generated in a large number of rabbits (two rabbits were used per peptide), some caution should be applied in interpreting these results as there may be variability in responses between individual rabbits. We do believe, however, that the general characteristics of the antisera described here result from the peptides used to generate these antisera. The ELISA and radioimmunoassay titers of antisera from duplicate rabbits agreed well. Furthermore, the antisera generated to P 78–109 were the only peptide antisera that did not exhibit a greater ability to immunoprecipitate ^{125}I-TGF-β under reducing conditions; this property was demonstrated whether the rabbit was presented with peptide alone or conjugated to methylated BSA and thus seems to be derived from the peptide itself. Therefore, we believe that our conclusions concerning the relative accessibility of the carboxyl-terminal region of TGF-β and its involvement in receptor binding are generally applicable and not the result of the response of an individual animal.

The region-specific antisera also provide further information on possible differences in amino acid sequence and three-dimensional conformation of TGF-β2 as compared to TGF-β1. Anti-TGF-β1 does not recognize the type 2 peptide, and as expected from available sequence data, antibodies against amino-terminal peptides 1–13 and 21–50 also did not react with type 2. Similar results have been obtained with anti-P 1–30 (Ellingsworth et al., 1986) and anti-P 21–38 (Cheifetz et al., 1987). In contrast, the antibodies against the carboxyl-terminal peptides 48–77, 50–75, and 78–109 did show some cross-reactivity with type 2 depending on the assay system. Anti-P 50–75 and 48–77 reacted against type 2 in an ELISA assay but showed no reactivity in immunoblots, while anti-P 78–109 reacted weakly in an ELISA assay and with reduced TGF-β2 on immunoblots. Anti-P 64–91 also did not recognize reduced or nonreduced TGF-β2 on Western blots (Cheifetz et al., 1987). None of the antisera immunoprecipitated ^{125}I-TGF-β2. The data showing limited cross-reactivity of TGF-β2 with the TGF-β1 peptide antisera when TGF-β2 is bound to a solid support suggest that the two forms of TGF-β might share partial carboxyl-terminal sequence homology. However, it cannot be ruled out that a change in primary structure, even in the amino-terminal region of type 2, may alter the tertiary structure of the dimeric protein, causing slight shifts in accessible regions. This alternative mechanism could also account for the lack of reactivity of type 2 with these peptide antisera in an immunoprecipitating assay. In the absence of appropriate sequence data, it is not possible to determine if the decreased reactivity of the carboxyl-terminal antibodies with type 2 as compared to type 1 is due to changes in the primary or tertiary structure.

It is interesting that porcine TGF-β2 has been found to interact equally well with one form of the TGF-β receptor as does type 1 (Cheifetz et al., 1987). If the region of amino acids 78–109 is the major domain that affects receptor binding for TGF-β1, it might be expected that there would be extensive sequence homology in this region between the two forms. Anti-P 78–109 did react with reduced TGF-β2 on Western blots; however, it did not immunoprecipitate or block its receptor binding to a significant extent. This suggests that possibly only a few, perhaps nonconsecutive, amino acids are critical for maximal receptor interaction and that this region was not in the epitope to which anti-P 78–109 was generated. The blocking of receptor binding of TGF-β1 by anti-P 78–109 would then result from antibody binding to TGF-β in a region near the amino acids important for receptor binding, making this sequence inaccessible to the receptor. Thus, although the antibody binding domain representing amino acids 78–109 of TGF-β1 may not be present in TGF-β2, the receptor binding domains of the two peptides might nonetheless be identical.

The identification of the region of TGF-β that seems to most strongly affect the interaction of TGF-β with its receptor establishes a region that also may be important for biological activity of TGF-β homologues. All members of the TGF-β family end at equivalent carboxyl-terminal positions, and since

seven of the nine cysteine residues, and presumably the basic tertiary structure, are highly conserved in this family (Padgett et al., 1987), the carboxyl-terminal regions of the other proteins may also be accessible to antibodies generated to this region. Production of antisera against the carboxyl-terminal regions of inhibin (Mason et al., 1985), activin (Ling et al., 1986; Vale et al., 1986), Müllerian inhibiting substance (Cate et al., 1986), or the product of the *Drosophila* decapentaplegic gene complex (Padgett et al., 1987) may generate useful immunoprecipitating and blocking antisera to these protens. Furthermore, this domain may be a candidate for site-directed mutagenesis of the cloned gene to determine which amino acids are critical to the ligand–receptor interaction. In addition, an antibody raised against anti-P 78–109 may bind to the receptor itself (Sege & Peterson, 1978; Schreiber et al., 1980; Marasco & Becker, 1982; Wasserman et al., 1982) and be useful in TGF-β structure–function studies. Such an anti-idiotype antibody could provide an alternative approach to isolate the TGF-β receptor or perhaps, if TGF-β receptor binding and subsequent biological activity require different domains, generate a TGF-β antagonist.

Added in Proof

Since submission of the manuscript, the complete amino acid sequence of human TGF-β2 has been published (Marquardt et al., 1987), which shows additional sequence diversity between the carboxyl-terminal regions of TGF-β1 and TGF-β2.

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Paramagnetic Probes of the Domain Structure of Histidine-Rich Glycoprotein†

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ABSTRACT: The interaction of Cu^{2+} and Fe^{3+}-mesoporphyrin with histidine-rich glycoprotein (HRG) from rabbit serum was examined spectroscopically. The first equivalent of Cu^{2+} binds to HRG producing a type II electron paramagnetic resonance (EPR) spectrum with $g_{\parallel} = 2.25$, $g_m = 2.05$, $A_{\parallel} = 0.019$ cm^{-1} (180 G), and superhyperfine along g_m. These spectral parameters suggest moderately covalent coordination of Cu^{2+} to the protein by nitrogens. With increasing Cu^{2+} the superhyperfine disappears; however, the g and A values change only marginally. The increase in EPR signal amplitude throughout the addition of 1-15 equiv of Cu^{2+} is linear and thereafter maximizes, suggesting 18-22 equiv are bound. In contrast, changes in the circular dichroism spectrum at 280 nm appear sigmoidal and can be interpreted as the binding of Cu^{2+} to two structurally distinct regions of the protein. Evidence for two structurally distinct binding domains is found by comparing EPR spectra of Cu^{2+} complexes of HRG with spectra from complexes of two of its major proteolysis products (peptides). After binding 1 equiv of Cu^{2+}, both the 30-kDa histidine-rich peptide and the native protein exhibit identical spectra including the pronounced superhyperfine. In contrast, the spectrum of the histidine-normal 45-kDa peptide with 1 equiv of Cu^{2+} bound lacks superhyperfine and parallels closely that of the native protein with 20 equiv bound. Finally, Fe^{3+}-mesoporphyrin binds to HRG exhibiting both high-spin ($g = 6.05$) and low-spin ($g_z = 2.94$, $g_y = 2.25$, $g_x = 1.50$) EPR resonances, and the latter imply bis(histidine) coordination. The amplitude of the low-spin g_z resonance decreases throughout the addition of 5-20 equiv of Cu^{2+}, and the Fe^{3+}-mesoporphyrin high-spin resonance increases and becomes rhombic at 10 equiv. The nitrogen superhyperfine is clearly visible even with Fe^{3+}-mesoporphyrin bound. Taken together, these data are consistent with Cu^{2+} binding to two structurally distinct metal-binding domains of HRG and a mostly sequential saturation of these domains. With increasing Cu^{2+} bound, Cu^{2+} coordination appears to progress from predominately histidine in the 30-kDa domain to aspartic acid and glutamic acid in the 45-kDa domain.

Although first isolated over 15 years ago (Heimburger et al., 1972), the physiological function(s) of the plasma glycoprotein HRG[1] is (are) yet to be determined. HRG interacts with plasminogen (Lijnen et al., 1980) and thrombospondin (Leung et al., 1984), with several divalent metals (Morgan, 1981, 1978), with hemes (Morgan, 1981; Tsutsui & Muller, 1982), and with organics such as rose bengal (Burch &

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[1] Abbreviations: HRG, histidine-rich glycoprotein; hemes, iron porphyrins; protoheme, iron protoporphyrin; mesoheme, iron mesoporphyrin; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; kDa, kilodalton(s); EPR, electron paramagnetic resonance; CD, circular dichroism.

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