Identification and Subcellular Localization of a 21-Kilodalton Molecule Using Affinity-Purified Antibodies against α-Transforming Growth Factor†

Parul Hazarika,* Robert L. Pardue, Rosa Earls, and John R. Dedman
Departments of Physiology and Cell Biology and of Internal Medicine, University of Texas Medical School, Houston, Texas 77030

Received April 17, 1986; Revised Manuscript Received November 13, 1986

ABSTRACT: Monospecific antibodies were generated against each of six different peptide sequences derived from rat and human α-transforming growth factor (α-TGF). The affinity-purified antibody to the 17 amino acid carboxyl-terminal portion of the molecule proved most useful in detecting α-TGF. When used in a peptide-based radioimmunoassay, it was possible to measure nanogram quantities of native α-TGF in conditioned cell culture media. When used to analyze cell lysate, these antibodies specifically recognized a 21-kilodalton protein species. Indirect immunofluorescence localization procedures revealed a high concentration of α-TGF in a perinuclear ring with a diffuse cytoplasmic distribution. These results suggest that a precursor form of α-TGF has a cellular role beyond that of an autocrine growth factor.

Transforming growth factors (TGFs) are a family of acid-stable polypeptides which induce reversible phenotypic transformation of nontransformed cells in tissue culture. At present, there are two defined members of the transforming growth factor family, α-TGF and β-TGF. TGF-induced anchorage-independent cell growth has been measured by colony formation in soft agar assays (DeLarco & Todaro, 1978; Roberts et al., 1983a,b). α-TGF has been isolated from several sources, including urine samples of tumor-burdened patients, conditioned media from virally transformed cells in tissue cultures, and mouse embryos (Roberts et al., 1980). α-TGF competes with epidermal growth factor (EGF) for binding to the plasma membrane EGF receptor (Todaro, 1980; Carpenter, 1983; Massague, 1983) and activates the receptor-associated tyrosine kinase activity (Reynolds et al., 1981) which then is associated with cell growth. Detection and purification of α-TGF have been difficult as purification yields from natural sources are exceptionally low (1 μg/120 L of conditioned media; Todaro et al., 1980). To overcome this scarcity issue of obtaining pure α-TGF, a series of peptides were synthesized corresponding to different regions of the α-TGF molecule (Marquardt et al., 1984) which were used to generate monospecific antibodies for immunological analyses.

Materials and Methods

Preparation of Antipeptide Antibodies. Peptides were obtained from Peninsula Laboratories (San Carlos, CA) and further purified by C-18 reverse-phase high-performance liquid chromatography (HPLC) as described by Tam (1984). The synthetic peptides (10 mg) were coupled to an equal mass of keyhole limpet hemocyanin (KLH) using 3% glutaraldehyde (Rittenberg & Amkrout, 1966).

Immunization schedules for sheep were as follows: (1) 500 μg of KLH-coupled peptide in Freund's complete adjuvant (1:1) injected at multiple subcutaneous sites on days 0, 10, and 14; (2) 250 μg of KLH-coupled peptide in Freund's incomplete adjuvant (1:1) injected subcutaneously on days 21 and 35; (3) 100 μg of alum-absorbed peptide injected subcutaneously on days 49, 63, and 80. Animals were bled every 2 weeks beginning on day 60. The ability of the antisera to react with the peptides was initially determined by an enzyme-linked immunosorbent assay.

The synthetic peptides (10 mg) were coupled to 2.5 g of CNBr-activated Sepharose (Pharmacia) following the precedure outlined by the manufacturer. Antisera were purified by affinity chromatography on peptide–Sepharose 4B (Pharmacia) columns; specifically bound antibodies were eluted with 200 mM glycine, pH 2.7. Antibodies were then dialyzed against 100 mM borate and 75 mM NaCl, pH 8.4, supplemented with 1 mg/mL bovine serum albumin and stored at 4 °C.

Gel Electrophoresis and Immunoblot Analysis. TGF peptides and cell culture media were separated by sodium dodecyl sulfate–polyacrylamide gel (15%) electrophoresis (Laemmli, 1970), transferred to 0.22-μm pore nitrocellulose paper (Schleicher & Schuell), and detected with a biotin–avidin rabbit–anti-sheep peroxidase system (Vector Laboratories, Burlingame, CA).

Indirect Immunofluorescence. Human melanoma A-2058 cells and Swiss mouse 3T3 fibroblasts were grown to 60–70% confluency on 11 × 22 coverslips in Dulbecco's modified Eagle's media containing 10% fetal calf serum. Cells were fixed with 3% formaldehyde for 20 min and permeabilized with −20 °C acetone (7 min). The coverslips were incubated with 25 μL of affinity-purified TGF-3 antibody (1 h at 37 °C), followed by several phosphate-buffered saline (PBS) washes and incubation for 1 h at 37 °C in fluorescein-conjugated rabbit–anti-sheep IgG (Cappel Labs). Cells were viewed on a Nikon Optiplan epifluorescence microscope using the 40× and 60× dry objectives. Photomicrographs were recorded on Kodak Tri-X pan (ASA 400) film and developed in Kodak HC-110 (dilution B) for 10 min.

Radioiodination of Peptide. One microgram of TGF-3 peptide labeled with 200 μCi of $Na^{125}I$ using Iodogen (Pierce Chemical Co.) as the oxidative agent. Labeled peptide was separated from free iodine by Sephadex G-10 column chromatography (0.7 × 20 cm). The specific activity of the ^{125}I-TGF-3 was 35 000 dpm/ng.

RIA Procedures. Reaction mixtures contained 100 μL of sample, 1–5 μg of anti-TGF-3 IgG in 50 μL of RIA buffer [0.125 M borate, 75 mM NaCl, 1 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and

† This work was supported by grants from Triton Biosciences, Inc. (Alameda, CA), and by NIH Grant GM39323.

* Address correspondence to this author at the Department of Physiology and Cell Biology, University of Texas Medical School.

0006-2960/87/0426-2067$01.50/0

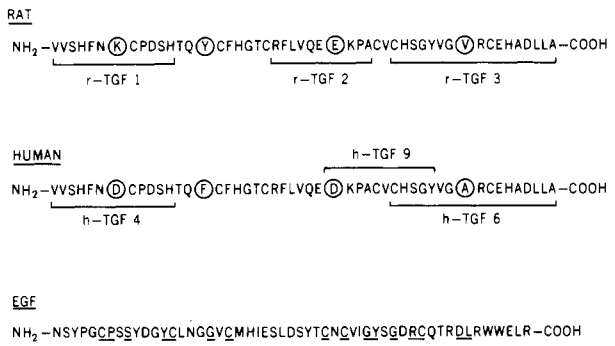

FIGURE 1: Sequence analysis of rat and human α-transforming growth factor (α-TGF) and human epidermal growth factor. Circled single-letter amino acid codes indicate differences in primary sequence (D). Bracketed areas represent peptides which were synthesized and used as antigens for the production of antibodies. Underscored amino acids in EGF represent conserved residues with α-TGF.

20 μg/mL bovine serum albumin, pH 8.4], and 50 000 dpm of ^{125}I-TGF-3 in 50 μL of RIA buffer with a final volume of 200 μL in an Eppendorf tube. Nonspecific binding was determined by substituting 50 μL of preimmune serum or an unrelated antipeptide IgG for the primary anti-TGF-3 antibody. Samples were incubated for 2 h at room temperature and then overnight at 4 °C. Twenty microliters of a 1:20 dilution of rabbit-anti-sheep antibody (Cappel Labs) and 20 μL of 30% poly(ethylene glycol) (M_r 6000) were added to the previously incubated assay mixtures. After 2 h of incubation, 10 μL of a 10% (w/v) suspension of Pansorbin (Calbiochem) was added, and the mixtures were incubated for an additional 30 min. The tubes were then centrifuged at 10000g for 1 min. Resultant pellets were washed twice by resuspension in 1-mL aliquots of RIA buffer. Radioactivity was determined on a Beckman Model 8500 γ counter.

RESULTS

The amino acid sequences for rat and human α-TGF and human epidermal growth factor (EGF) are shown for comparison (Figure 1). Note the lack of direct homology between EGF and the α-TGFs and the striking homology between the rat and human α-TGFs (46 of 50 residues). Peptides were synthesized against predetermined sequences of the α-TGFs. These sequences (TGF-1, -2, -3, -4, -6, and -9) were cross-linked to keyhole limpet hemocyanin and used as primary antigens in sheep. The antisera titers were monitored by ELISA assay. High-titer antisera were dialyzed and purified by the respective TGF-peptide-Sepharose affinity chromatography. No cross-reactivity was seen between affinity-purified antisera generated against TGF-1, -2, -4, or -9 or against EGF as evidenced by ELISA testing while antibodies against peptides 3 and 6 demonstrated an equivalent degree of cross-reactivity. Antibodies generated against the carboxy-terminal 17 amino acid sequence, TGF-3, demonstrated the highest titer and lowest cross-reactivity and were therefore selected for further studies. Elution of specific antipeptide immunoglobulins from TGF-3-Sepharose affinity columns yielded 70–100 μg of anti-TGF-3-specific immunoglobulin (IgG) per milliliter of antisera.

For quantitation of α-TGF levels, the purified anti-TGF-3 was employed for the development of a peptide-based radioimmunoassay (Figure 2). The synthetic TGF-3 peptide was iodinated by using the Bolton-Hunter (Amersham) or the Iodogen (Pierce) procedure and then reacted with purified antibody. Iodination of the peptide with the Bolton-Hunter reagent resulted in a maximum precipitation of 5–10% of the total counts whereas labeling by the Iodogen method produced

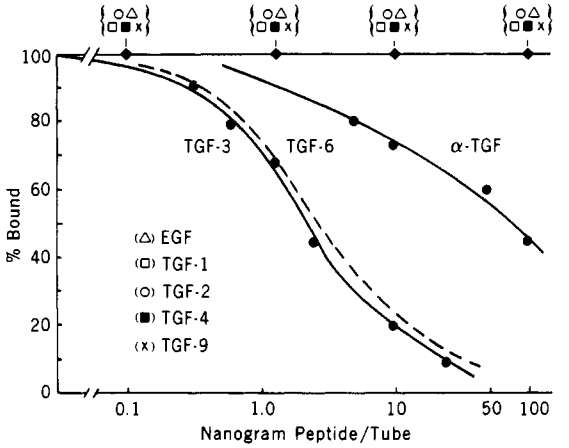

FIGURE 2: Immunoreactivity of the synthetic peptides and EGF in competition radioimmunoassay. Various concentrations of peptides were mixed with ^{125}I-TGF-3 (50 000 dpm) and anti-TGF-3 (1 μg). After incubation for 2 h at room temperature and then overnight at 4 °C, rabbit-anti-sheep IgG (20 μL, 1:20 dilution) and 30% poly(ethylene glycol) (20 μL) were added to the mixture. Following 2 h of incubation, 10% (w/v) Pansorbin (10 μL) was added and incubated for 30 min. The tubes were centrifuged, the pellets were washed, and the radioactivity of the pellets was determined. The degree of competition is expressed as a percentage of the radioactivity bound in the absence of competition.

a greater than 50% precipitation of the total counts. Serial dilution of antibody against a constant amount of ^{125}I-TGF-3 (50 000 dpm) demonstrated a linear relationship between IgG concentration and immunoprecipitation.

Specificity of the peptide-based RIA was tested by using peptides derived from distinct regions of the rat and human α-TGF sequences [TGF-1, -2, -3, -4, -6, and -9, full-length synthetic rat TGF, and human EGF (Figure 1)]. Only the corresponding carboxy-terminal 17 amino acid sequences of rat and human TGF (TGF-3 and TGF-6) and full-length rat TGF competed with the ^{125}I-labeled TGF-3 peptide. There is a single residue difference between the rat and human C-terminal 17 amino acid sequence (alanine to valine at position 41). Interestingly, peptide TGF-9 which overlaps five residues of TGF-3 at the N-terminus did not compete. In addition, treatment of TGF-3 peptide with trypsin which cleaves the sequence at arginine (position 42) into nine- and eight-residue fragments did not affect the competition (data not shown). Several thousandfold excess (1 μg) of human epidermal growth factor did not compete in the precipitation of iodinated TGF-3 peptide.

To measure natural α-TGF using our peptide-based RIA, supernatant media from nontransformed and TGF-producing cell lines were assayed. Media from nontransformed lines revealed a low concentration of TGF while conditioned media from feline sarcoma virus transformed CL-10 cells, Harvey and Kirsten transformed normal rat kidney cells, and human melanoma cells contained a 4–6-fold elevation of transforming growth factor (Figure 3).

The specificity of the antisera was determined by Western immunoblot analysis against synthetic peptide sequences and cell lysates. As shown in Figure 4, affinity-purified anti-TGF-3 recognizes the synthetic 17 amino acid peptide (the original antigen) and the full-length synthetic α-TGF. Due to the low molecular weight of the peptide (1800), the TGF-3 lane produced a diffusely stained pattern (lane 3). Western immunoblots of lysates obtained from TGF-producing cells revealed only the high molecular weight precursor molecule (lane 2) while lysates obtained from nonproducing TGF cells revealed an absence of immunoreactive bands (lane 4). Im-

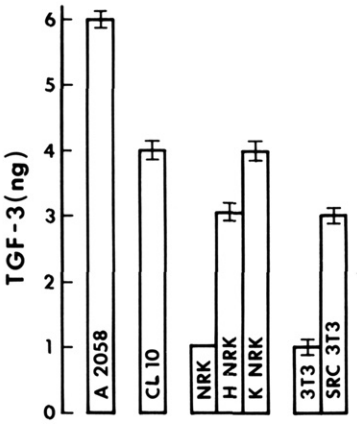

FIGURE 3: Radioimmunoassay of conditioned media. Conditioned (concentrated 10-fold) media from human melanoma cells (A 2058), feline sarcoma infected rat cells (CL 10), Harvey transformed normal rat kidney cells (H NRK), Kirsten transformed normal rat kidney cells (K NRK), Rous sarcoma transformed 3T3 fibroblasts (SRC 3T3), and nontransformed 3T3 fibroblasts were analyzed for the presence of α-TGF using the ^{125}I-TGF radioimmunoassay as described in Figure 2.

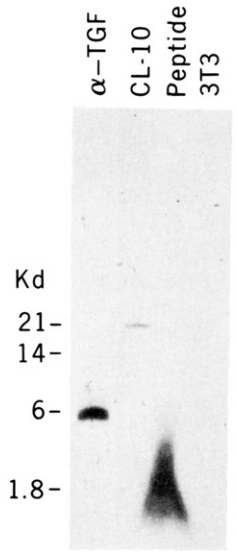

FIGURE 4: Immunoblot analysis using affinity-purified anti-TGF-3. Lane 1 represents 1 μg of refolded synthetic α-TGF. Lane 2 is a cell lysate (50 μg) from the feline sarcoma cell line (CL-10). Lane 3 represents 1 μg of TGF-3. Lane 4 represents cell lysate (50 μg) from the Swiss mouse 3T3 cell line.

munoblot staining was abolished when anti-TGF-3 was preincubated with a 100-fold molar excess of synthetic peptide (data not shown).

To elucidate the subcellular localization of the 21-kilodalton precursor molecule, indirect immunofluorescence was performed. As shown in Figure 5, the affinity-purified antibody diffusely stained the cytosol and decorated a well-defined perinuclear ring. This staining could be eliminated by preabsorption of the antibody with a 100-fold excess of TGF-3 peptide (panel B). The antibody did not stain nontransformed cells (3T3, PTK-2, NRK) used as controls (data not shown).

Discussion

The development of specific immunological probes for use in the detection of biologically scarce α-TGF required a systematic production and analysis of site-specific polyclonal antibodies. The antibody generated against the carboxy-terminal third of the rat α-TGF molecule (TGF-3) elicited the highest titer and was the most reactive against full-length rat α-TGF. Monospecificity required the use of affinity purification. The purified TGF-3 antibodies cross-reacted only with the carboxy-terminal 17 amino acid sequence of human TGF. The peptide, TGF-9, which has a five amino acid overlap region on the N-terminus of the TGF-3 sequence, did not compete. In addition, trypsin digestion of the TGF-3 peptide suggests that the antigen determinant resides in the C-terminal eight amino acid portion of α-TGF. Similar data have been suggested by other investigators (Linsley et al., 1985). In fact, we have demonstrated by epitope mapping that this region contains an immunodominant domain (P. Hazarika, R. L. Pardue, J. P. Tam, and J. R. Dedman, unpublished results).

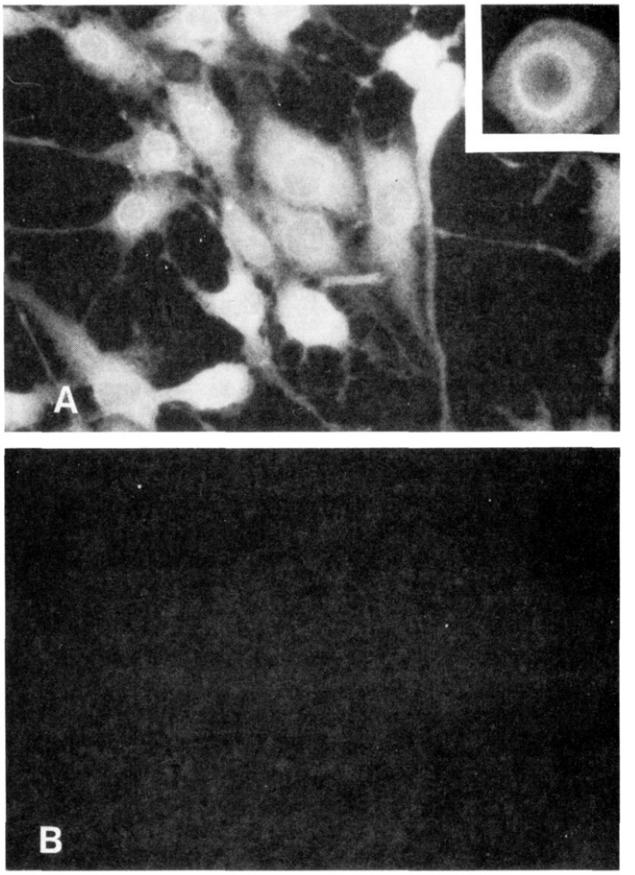

FIGURE 5: Intracellular localization of α-TGF. Human melanoma cells (A-2058) grown in culture, gently fixed (3% formaldehyde), and stained with affinity-purified TGF-3 antibodies (A) or antibodies preabsorbed with 100 μg of TGF-3 (B). (Panels A and B, 595×; insert, 840×).

The TGF-3 peptide-based radioimmunoassay was sensitive to 200 pg of peptide. The sensitivity of the assay to the full-length α-TGF molecule was 10-fold less than to the peptide. This difference may possibly be due to protein folding and the physical blocking of epitopes. Treatment of the full-length molecule with 10 mM 2–mercaptoethanol and heating at 90 °C for 5 min markedly improved competition (data not shown). The radioimmunoassay, when used in cell supernatants from normal cells, demonstrated a basal level of 1 ng/mL of α-TGF. In transformed cells (CL-10, A-2058, H-NRK, and K-NRK), there was a 4–6-fold increase in levels of TGF. The values obtained by using the RIA are 10–50 times greater than that reported by Marquardt et al. (1983) using the EGF radioreceptor assay. This difference may reflect detection of immunoreactive material which is not capable of receptor binding. Whether the low concentration of α-TGF is required for normal growth is yet to be determined. We have begun to investigate the TGF levels in fetal tissues to determine the normal concentration of α-TGF. When com-

pleted, these data can be compared to amounts found in virally transformed cells and primary tumors which should then permit the correlation of α-TGF in biological fluids with the degree of malignancy and during treatment regimens.

Immunoblot data from the CL10 cells did not demonstrate a mature 7000-dalton α-TGF but a 20–22-kilodalton precursor. Linsley et al. (1985) using rabbit serum reported that three possible precursors may exist (M_r 24 000, 40 000, and 42 000). However, Twardzik (1985) has suggested that the fetal mouse produces M_r 10 000 and 20 000 precursor molecules. Twardzik (1985) assumes that the M_r 10 000 peak represents the mature 50 amino acid α-TGF. In addition, the cloned α-TGF cDNA from human (Derynck et al., 1984) and rat (Lee et al., 1985) suggests only the possibility of a 20-kilodalton molecule. Out data demonstrate that the predominant cellular precursor for α-TGF is approximately 21 kilodaltons. The larger species detected by Linsley et al. (1985) remains unexplained.

Our purified, monospecific antibody localized α-TGF precursor in transformed cells as a distinct perinuclear pattern with diffuse cytoplasmic staining. The perinuclear staining indicates a fibrillar array and suggests cytoskeleton association. These data imply that the pre-TGF molecule has a cellular role beyond that of an autocrine growth factor. This line of experimentation is now being pursued in our laboratory to determine the degree and types of interactions between precursor α-TGF, the nuclear matrix, and cytoskeletal elements.

ACKNOWLEDGMENTS

We thank Dr. James Tam (Rockefeller University) for his generous gift of synthetic rat α-TGF and Dr. Martin Cline (UCLA) for supplying conditioned media. We appreciate the efforts of Libby Bajusz for preparing the manuscript and James Pastore for photographic reproduction of the figures.

REFERENCES

Carpenter, G., Stroscheck, C. M., Preston, Y. A., & DeLarco, J. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5627–5630.

DeLarco, J. E., & Todaro, G. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4001–4005.

Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., & Goeddel, D. V. (1984) *Cell (Cambridge, Mass.) 38*, 287–297.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–683.

Lee, D. E., Rose, T. M., Webb, N. R., & Todaro, G. J. (1985) *Nature (London) 313*, 489–491.

Linsley, P. S., Hargraves, W. R., Twardzik, D. R., & Todaro, G. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 356–360.

Marquardt, H., & Todaro, G. J. (1982) *J. Biol. Chem. 257*, 5220–5225.

Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., DeLarco, J. E., Stevenson, J. R., & Todaro, G. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4684–4688.

Marquardt, H., Hunkapiller, M. W., & Todaro, G. J. (1984) *Science (Washington, D.C.) 223*, 1079–1081.

Massague, J. (1983) *J. Biol. Chem. 258*, 13614–13620.

Massague, J. (1983) *J. Biol. Chem. 258*, 13603–13613.

Reynolds, R. H., Jr., Todaro, G. J., Fryling, C., & Stevenson, J. R. (1981) *Nature (London) 292*, 259–262.

Rittenberg, M. B., & Amkraut, A. A. (1966) *J. Immunol. 97*, 421–428.

Roberts, A. B., Lamb, L. C., Newton, D. L., Sporn, M. B., DeLarco, J. E., & Todaro, G. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3494–3498.

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacker, R., Pan, Y. C., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C., & Sporn, M. B. (1983a) *Biochemistry 22*, 5692–5698.

Roberts, A. B., Frolik, C. A., Anzano, M. A., & Sporn, M. B. (1983b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2621–2615.

Tam, J. P., Marquardt, H., Rosberger, D. F., Wong, T. W., & Todaro, G. J. (1984) *Nature (London) 309*, 376–378.

Todaro, G. J., Fryling, C., & DeLarco, J. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5258–5262.

Twardzik, D. R. (1985) *Cancer Res. 45*, 5413–5416.

CORRECTION

Spectroscopic Studies of the Interactions of Coenzymes and Coenzyme Fragments with Pig Heart, Oxidized Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase, by Maria T. Mas and Roberta F. Colman*, Volume 24, Number 7, March 26, 1985, pages 1634–1646.

Page 1638. Equation 9 should read

$$[L]_{bound} = [L]_{total}\frac{F/F_0 - 1}{Q}$$

Although eq 9 was printed incorrectly in the paper, the data presented were calculated correctly according to the equation given here.