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Articles

Purification and Properties of a Type β Transforming Growth Factor from Bovine Kidney†

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ABSTRACT: Type β transforming growth factor (TGF-β) has been purified 200000-fold from bovine kidneys. This peptide is characterized by its ability to induce anchorage-dependent normal rat kidney cells to grow in soft agar in the presence of epidermal growth factor (EGF); TGF-β is not mitogenic for cells grown in monolayer culture. Purified TGF-β does not compete with EGF for binding to membrane receptors. The concentration of TGF-β required to elicit a half-maximal response for formation of colonies >3100 μm^2 in the soft agar assay is 2-3 pM (55 pg/mL) when assayed in the presence of 0.8 nM EGF (5 ng/mL). The four-step purification procedure which includes chromatography of acid-ethanol tissue extracts on polyacrylamide sizing gels, cation exchange, and two steps of high-pressure liquid chromatography results in a 10% overall yield of colony-forming activity with a recovery of 3-4 μg/kg. Amino acid analysis of purified TGF-β shows 16 half-cystine residues per mole. Analysis of the purified polypeptide by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels indicates that TGF-β is composed of two closely related polypeptide chains cross-linked by disulfide bonds. In the absence of β-mercaptoethanol, the colony-forming activity is associated with a single silver-staining band of molecular weight 25 000; in the presence of β-mercaptoethanol, the TGF-β is converted to an inactive species that migrates as a single band of molecular weight 12 500-13 000. Sequence analysis indicates that at least the first 15 N-terminal amino acids of the two TGF-β subunits are identical.

Two years ago, a new set of acid-stable polypeptide growth factors was described that had the property of acting together with epidermal growth factor (EGF)[1] to induce normal anchorage-dependent indicator cells to grow under anchorage-independent conditions (Roberts et al., 1981, 1982b). The ability to induce anchorage-independent growth had previously been ascribed to crude preparations of sarcoma growth factor by De Larco & Todaro (1978). More recently, it has become clear that these polypeptides all belong to a larger family of transforming growth factors (TGFs) (Roberts et al., 1982a, 1983b) operationally defined by their ability to reversibly induce certain cells to express the transformed phenotype as measured by loss of density-dependent inhibition of growth, overgrowth, and acquisition of both a transformed morphology and the ability to form progressively growing colonies under anchorage-independent conditions (De Larco & Todaro, 1978; Roberts et al., 1981).

Recently, evidence has demonstrated that the transforming activity of these polypeptides cannot be ascribed to any one TGF acting alone on the cell but rather requires the combined action of two TGF subsets which we have designated type α and type β TGFs (Anzano et al., 1982; Roberts et al., 1983b). These two subsets of the TGF family are distinguished by both biological and biochemical properties. Members of the first group, designated TGF-α, are characterized by their ability to compete for binding to the EGF receptor (De Larco & Todaro, 1978, 1980; Todaro et al., 1980). Polypeptides belonging to this class are all single-chain polypeptides of molecular weight 5000-7000, and all contain three intrachain disulfide bonds (Marquardt et al., 1983). Type α TGFs have recently been purified to homogeneity from the conditioned media of both human and rodent transformed cell lines (Marquardt & Todaro, 1982; Twardzik et al., 1982). Since EGF shares extensive amino acid sequence homology with type α TGFs (Marquardt et al., 1983) and can also fully potentiate the soft agar colony-forming response in the presence of type β TGFs (Anzano et al., 1982; Roberts et al., 1982b), it may also be classified as a type α TGF. A second TGF subset, designated TGF-β, does not bind to the EGF receptor (Roberts et al., 1982b). Recent reports from our laboratory and data presented in this paper have demonstrated that type β TGFs from both neoplastic and nonneoplastic sources are of higher molecular weight (25 000) than the type α TGFs and appear to contain disulfide bonds that are necessary for biological

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[1] Abbreviations: EGF, epidermal growth factor; NRK, normal rat kidney; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TGF, transforming growth factor; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

activity (Roberts et al., 1983a).

Type β TGFs from three different nonneoplastic tissue sources have recently been purified to homogeneity in our laboratory: from human placenta (Frolik et al., 1983), from human platelets (Assoian et al., 1983), and, as reported here, from bovine kidney. Amino acid composition and behavior on $NaDodSO_4$–polyacrylamide gels indicate that these polypeptides are closely related in terms of structure as well as biologically. In this paper, we describe for the first time the purification, properties, and partial N-terminal amino acid sequence of TGF-β from a bovine source.

Experimental Procedures

Extraction of TGFs from Bovine Kidney. TGFs were extracted from kilogram quantities of bovine kidney by an adaptation of the previously described acid–ethanol extraction procedure (Roberts et al., 1980, 1983a). The kidneys were obtained fresh from the slaughterhouse, trimmed free of fat, immediately frozen on dry ice, and stored over liquid nitrogen. Before extraction, they were defrosted overnight at −20 °C and chopped into small pieces. The tissue was then allowed to defrost further in an acid–ethanol solution (4 mL/g of tissue) containing 792 mL of 95% ethanol, 192 mL of water, and 16 mL of concentrated HCl plus 53 mg of phenylmethanesulfonyl fluoride and 3 mg of pepstatin A as protease inhibitors. Following homogenization in a commercial Waring blender, the mixture was stirred for 2–3 h at room temperature and centrifuged at 11000*g* for 15 min. The pH of the supernatant was adjusted to 3.0 by addition of concentrated ammonium hydroxide, whereupon the crude TGF was precipitated by addition of 4 volumes of cold anhydrous ether and 2 volumes of cold ethanol. The precipitate was recovered by filtration after standing overnight at 4 °C. The residue was redissolved in 1 M acetic acid (1 mL/g of tissue), centrifuged to remove insoluble material, and lyophilized to dryness.

Purification of Type β TGF. The bovine kidney TGF-β was purified to homogeneity by the successive application of four chromatographic steps consisting of gel filtration, cation exchange, and two different steps of HPLC. All chromatography was carried out at room temperature, and fractions from each column were stored at 4 °C until ready for further processing. In every case, aliquots were lyophilized to dryness before being assayed for colony-forming activity. Protein analyses were done by the dye-binding method of Bradford (1976) using bovine γ-globulin as standard or, after final purification, by amino acid analysis. The purification steps are described below.

Gel Filtration. The lyophilized acid–ethanol extract of 7–8 kg of bovine kidney was redissolved in 1.5–2 L of acetic acid and applied to a sectional column (Pharmacia) comprised of five sections (37 × 15 cm) each containing 16 L of Bio-Gel P-30 in 1 M acetic acid. Chromatography was accomplished by upward flow at a rate of 1.2–1.5 L/h. Fractions of 1 L were collected, and aliquots of 50 μL were assayed for TGF-β activity. The colony-forming activity eluted in a single peak with an apparent molecular weight of 7000–10000 (Figure 1A).

Cation Exchange. The pooled fractions from the gel filtration column (6–12 L) were concentrated to approximately 0.1 volume by using a M_r 10000 cutoff filter (Millipore Pellicon cassette system) and lyophilized to dryness. The residue was redissolved in 0.1 M acetic acid (10–20 mL/g of protein) and diluted with an equal volume of 0.1 M sodium acetate; the pH was adjusted to 4.5. The sample was applied to a cation-exchange column (CM-Trisacryl M, 5 × 10 cm, LKB) equilibrated in 0.05 M sodium acetate, pH 4.5. After the sample was washed with several hundred milliliters of starting solvent, the column was developed with a linear salt gradient of 0.85 M sodium chloride in 0.05 M sodium acetate, pH 4.5, at a flow rate of 100–150 mL/h, collecting 20–30-mL fractions. The column was stripped with 200 mL of 1 M sodium chloride, pH 2. Aliquots of 5 μL were assayed for

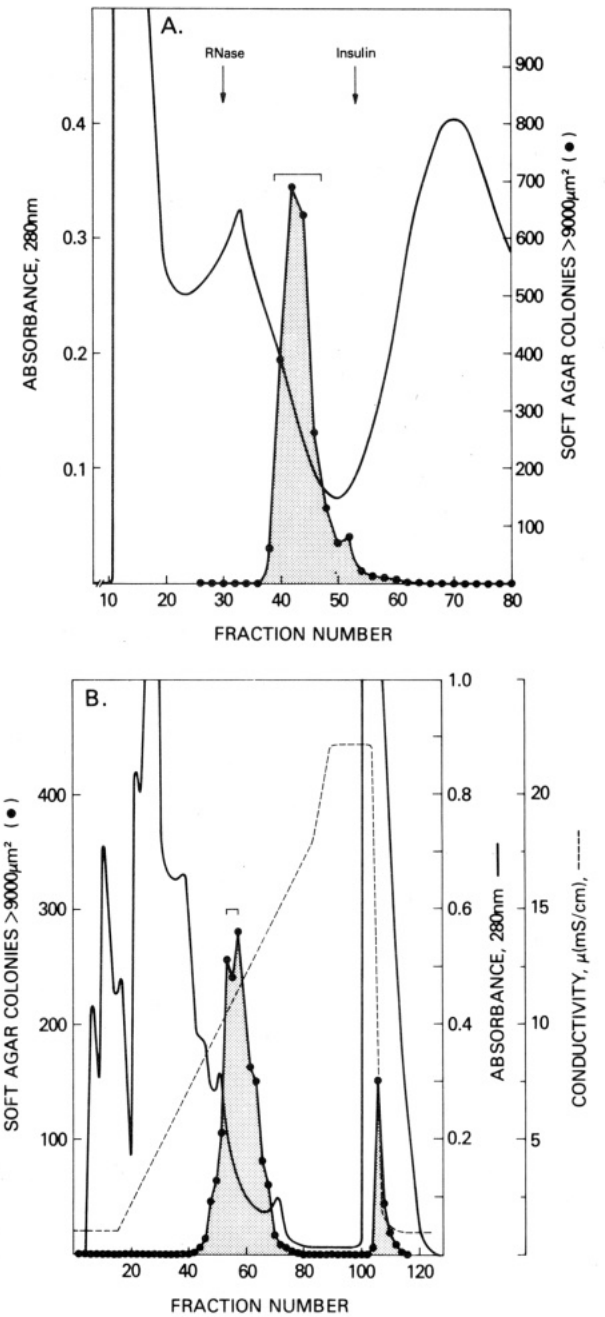

FIGURE 1: Purification of bovine kidney type β TGF by chromatography on Bio-Gel P-30 (A) and cation-exchange (B) columns. (A) The lyophilized acid–ethanol extract of 5–7 kg of kidney (45 g of protein) was dissolved in 1500 mL of 1 M acetic acid and applied to an 80-L column of Bio-Gel P-30 equilibrated in 1 M acetic acid. The column was developed by upward flow at 1.2 L/h, and fractions of 1 L were collected. The elution positions of RNase (M_r 13800) and insulin (M_r 6000) are indicated by arrows. Aliquots (50 μL) of alternate fractions were assayed for colony-forming activity in the soft agar assay in the presence of EGF (●). Fractions 38–48 were pooled, concentrated, lyophilized, and redissolved in 0.05 M sodium acetate, pH 4.5. (B) The sample (3 g) was applied to a column of CM-Trisacryl M equilibrated in the sample solvent. The TGF was eluted with a gradient of NaCl (0–0.85 M) at a flow rate of 150 mL/h. Aliquots (5 μL) of alternate fractions (30 mL) were assayed for colony-forming activity in the presence of EGF (●). Fractions 54–58 were pooled for further purification on HPLC.

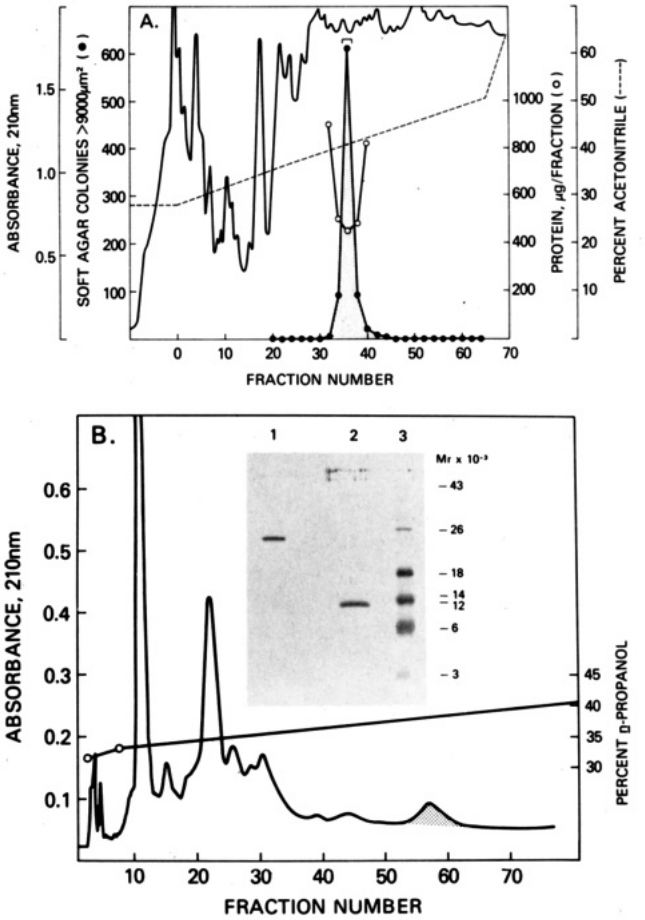

FIGURE 2: Final purification of bovine kidney TGF-β by HPLC on μBondapak C18 (A) and μBondapak CN (B). (A) The pooled fractions (150 mL) from the cation-exchange column were adjusted to pH 2, made 0.1% in TFA, and pumped onto the μBondapak C18 column at a flow rate of 1.5 mL/min. The TGF was eluted with a linear gradient of acetonitrile in 0.1% TFA (0.3% per min) at a flow rate of 0.8 mL/min; fractions of 0.8 mL were collected. Aliquots of 10 μL were assayed for protein (○) and aliquots of 0.5 μL for colony-forming activity (●). Fractions 35–37 were pooled and diluted with an equal volume of 0.1% TFA. (B) The diluted pooled fractions from the previous step were applied to a μBondapak CN column and eluted with a linear gradient of 1-propanol in 0.1% TFA (0.05% per min). All colony-forming activity eluted in the shaded area. (Inset) 86 ng of CN-purified TGF-β was subjected to electrophoresis on 15% $NaDodSO_4$–polyacrylamide gels in the absence (lane 1) or the presence of β-mercaptoethanol (lane 2); gels were then fixed with formaldehyde (Steck et al., 1980) and stained with silver (Sammons et al., 1981). Molecular weights standards (lane 3) were ovalbumin (M_r 43 000), α-chymotrypsinogen (M_r 25 700), β-lactoglobulin (M_r 18 400), lysozyme, (M_r 14 300), cytochrome *c* (M_r 12 300), bovine trypsin inhibitor (M_r 6200), and insulin A and B chains (M_r 3000).

colony-forming activity. The major peak of colony-forming activity eluted at a conductivity of 10–12.5 mS/cm, although some activity was recovered in the strip of the column (Figure 1B).

HPLC. HPLC was carried out by using an Altex system coupled to a variable-wavelength Schoeffel Spectroflow Model SF 770 detector. Solvents used were HPLC grade. The pooled fractions from the cation-exchange step (140–200 mL) were adjusted to pH 2, made 0.1% in TFA and 10% in acetonitrile, and pumped directly (flow rate 1.5 mL/min) onto a semi-preparative Waters μBondapak C18 column (10-μm particle size, 0.78 × 30 cm) equilibrated in 10% acetonitrile and 0.1% TFA. The sample was eluted with a linear gradient of acetonitrile at a flow rate of 0.8 mL/min as shown in Figure 2. Aliquots of 0.25–0.5 μL of alternate fractions (0.8 mL/fraction) were assayed for colony-forming activity. All of the colony-forming activity eluted in a single peak at approximately 40% acetonitrile (Figure 2A).

The pooled fractions from the C18 column (2.4–4.0 mL) were diluted with an equal volume of water–0.1% TFA and injected directly onto an analytical Waters μBondapak CN column (10-μm particle size, 0.38 × 30 cm) equilibrated with 31% 1-propanol–0.1% TFA. The sample was eluted with a linear gradient of 1-propanol as shown in Figure 2B. Flow rate and fraction size were as described above. TGF-β was eluted at approximately 38% 1-propanol and could be shown to be homogeneous after this step of the purification.

Assay for Colony-Forming Activity of Type β TGFs. Lyophilized samples were assayed for colony-forming activity in soft agar is described previously (Roberts et al., 1980). Briefly, samples were dissolved in 200 μL of 4 mM HCl containing 1 mg/mL bovine serum albumin as carrier at 10 times the final concentration in the assay. Activity was measured by counting the number of colonies of normal rat kidney cells (NRK-2B, clone 49F provided by Drs. De Larco and Todaro) >3100 μm^2 formed in 0.3% agar in the presence of 5 ng/mL EGF. Colonies were stained (Schaeffer & Friend, 1976) after 7 days of incubation and counted by using a Bausch and Lomb Omnicon image analysis system as described previously (Roberts et al., 1981). A unit of TGF-β colony-forming activity has been defined as the ED_{50} of the dose–response curve of colonies >3100 μm^2 when assayed in the presence of 5 ng/mL EGF.

Other Bioassays for Type β TGFs. For assays of [^{3}H]-thymidine uptake, NRK cells were seeded at 1 × 10^4 cells per 1 mL in 24-well multidishes (16-mm diameter) in 10% calf serum in Dulbecco's modified Eagle's medium containing 100 units/mL penicillin and 100 μg/mL streptomycin. Four days later, samples to be assayed were added to the confluent cell layer in 50 μL of medium containing 50 μg/mL ascorbate. [^{3}H]thymidine (1–2 μCi/well) was added 16 h later in 20 μL of ascorbate buffer, and uptake was measured after an additional 6 h.

For assays of cell growth in the monolayer, NRK cells were seeded into 35-mm petri dishes at (1–8) × 10^4 cells per 2 mL of medium containing 2% calf serum. Samples to be assayed were added 4 h later in 25 μL of 4 mM HCl containing 1 mg/mL bovine serum albumin. Cells were trypsinized and counted in a Coulter counter 2.5 and 4.5 days later.

Extraction of Colony-Forming Activity from $NaDodSO_4$–Polyacrylamide Gels. Electrophoresis of type β TGFs on $NaDodSO_4$–polyacrylamide gels (12.5–15%) was carried out as described by Laemmli (1970). Gels were fixed in formaldehyde (Steck et al., 1980) and stained with silver (Sammons et al., 1981). When the gels were being extracted for recovery of colony-forming activity, samples were not heated in the sample buffer but applied directly to the gel. Following electrophoresis, the gels were cut into 5-mm slices and crushed with glass wool in sealed pipet tips; the crushed gel was extracted for 24 h at room temperature in a solution of 1 M acetic acid containing 200 μg of bovine serum albumin per mL (Roberts et al., 1983). Eluates were dialyzed for 24 h in a microdialyzer (Bethesda Research Labs) against 8 L of 1 M acetic acid, lyophilized, and assayed for colony-forming activity in the soft agar assay.

Amino Acid Analysis. Samples (20–50 pmol) were taken to dryness and hydrolyzed in sealed, evacuated tubes at 150 °C for 2 h in 100 μL of constant-boiling HCl (Pierce) containing 0.1% liquid phenol (Westall & Hesser, 1974; Tsugita & Scheffler, 1982). Half-cystine and methionine were determined by performic acid oxidation followed by acid hy-

Table I: Purification of Bovine Kidney TGF-β[a]

purification step	amount of protein recovered	ED_{50} (ng/mL)	activity (units × 10^{-3})	sp act. (units/μg)	degree of purification (each step)	% recovery of activity (each step)
acid–ethanol extract	89 g	13000	5100	0.057		100
Bio-Gel P-30	5.6 g	1700	2400	0.43	7.5	47
cation exchange	99 mg	200	350	3.5	61 (8.1)	6.9 (15)
μBondapak C18	3.7 mg	5.8	460	120	2100 (34)	9.0 (130)
μBondapak CN	40 μg	0.056	510	13000	230000 (110)	10 (110)

[a] From 14 kg of bovine kidney.

drolysis. Analyses of *o*-phthalaldehyde derivatives of the amino acids were done on a modified Beckman 121MB amino acid analyzer (Barbarash & Quarles, 1982) equipped with a Gilson Model 121 fluorometer and an Autolab System I computing integrator.

NH_2-Terminal Sequencing by Edman Degradation. For amino-terminal sequence analysis, approximately 500 pmol (M_r 25000) of TGF-β was reduced and S-carboxymethylated with dithiothreitol (Pierce) and iodo[^{14}C]acetic acid (New England Nuclear) in the presence of 6 M guanidine hydrochloride in 1 M Tris-HCl buffer, pH 8.4, according to Allen (1981). Excess reagents were separated from carboxymethylated protein by HPLC on a 5-μm LC-18DB (50 × 4.6 mm) column (Supelco, Inc.) eluted with a gradient of 0–90% acetonitrile (1% per min) in 0.1% TFA (unpublished results). Overall recovery of the procedure was 96%, based on estimating the amount of protein by amino acid analysis using fluorescamine detection as described by Stein et al. (1973).

Automated Edman degradation was performed on about 500 pmol (M_r 12500) of the S-carboxymethylated protein with a gas-phase sequencer, Model 470A (Applied Biosystems, Inc.) (Hewick et al., 1981). PTH-amino acids were identified by using an HPLC system equipped with a Beckman Ultrasphere ODS column (Hawke et al., 1982). Initial yield was about 30% and repetitive yield about 90%.

Results

Previous investigations had demonstrated the presence of type β TGFs in a wide variety of both neoplastic and nonneoplastic tissues of several different genomes, including mouse, bovine, and human (Roberts et al., 1982a). Since it was apparent that these polypeptides were present in only very small amounts and that large quantities of tissues would have to be processed for the eventual characterization of the type β TGFs, it was decided to pursue the characterization of TGF-β from bovine kidneys which could be obtained inexpensively in sufficient quantity. As described in detail below and summarized in Table I, the yield of purified TGF-β was 3–5 μg/kg of bovine kidney. However, the TGF-β had high biological activity; a concentration of less than 2×10^{-12} M (50 pg/mL) was required to induce the formation of colonies in soft agar in the presence of EGF.

As shown in Figure 1A, a large bed-volume column (80 L) was employed for the first step of purification on Bio-Gel P-30. The acid–ethanol extract of bovine kidney showed a single peak of colony-forming activity with an apparent molecular weight of 7000–10000. Little colony-forming activity was detected in the absence of EGF nor was there any EGF receptor-competing activity associated with this peak (data not shown), confirming the presence of type β TGF. The recovery of colony-forming units of TGF-β activity after this first step of purification was 47% (Table I).

The pooled fractions of crude TGF-β from the gel filtration step were concentrated, lyophilized, reapplied to a cation-exchange column, and eluted with a salt gradient (Figure 1B). Two peaks of colony-forming activity were detected; one eluted at a conductivity of 10–12.5 mS/cm, and the other eluted with a prominent protein peak in the strip of the column. Upon rechromatography, approximately 25–30% of the activity of the late-eluting peak chromatographed in the position of the earlier eluting peak. Only the earlier eluting peak was pooled for further purification on HPLC. Although an 8-fold purification was achieved by this step, the recovery of TGF-β activity from the cation-exchange step was only 15% (Table I). It is currently being investigated whether this loss of activity results from separation of the TGF-β from an activator substance or whether it represents an actual physical loss of TGF-β.

Final purification of the TGF-β to homogeneity was achieved by two successive steps of HPLC: the first on a μBondapak C18 column using an acetonitrile gradient and the second on a μBondapak CN column using a gradient of 1-propanol (Figure 2). In contrast to the two previous steps of purification, recovery of TGF-β from the HPLC columns was quantitative (Table I). It was essential to avoid lyophilization in these final steps of purification as it was found to result in irreversible association of the TGF-β with a contaminating protein of approximate M_r 13000. Therefore, the pooled fractions of TGF-β activity from the cation-exchange column were pumped directly onto the C18 column, and the pooled fractions from that column were diluted and injected directly onto the CN column (see Experimental Procedures). A single peak of TGF-β activity eluted from the C18 column at approximately 40% acetonitrile (Figure 2A). After chromatography on the CN column, all the colony-forming activity eluted at approximately 38% 1-propanol (Figure 2B). The colony-forming activity was stable to storage at −20 to 4 °C for at least several weeks in the HPLC solvents.

As shown in Figure 2B (inset), further analysis of the CN-purified type β TGF on $NaDodSO_4$–polyacrylamide gels revealed a single silver-staining protein band at M_r 25000 under nonreducing conditions: in the presence of β-mercaptoethanol, the peptide stained as a single band at approximately M_r 12000–13000. Extraction of gel slices showed that all of the soft agar colony-forming activity was associated with the M_r 25000 band and that no activity could be recovered from the gel when the peptide was run under reducing conditions (data not shown). This is in agreement both with earlier observations that the TGF activity of crude preparations was destroyed by treatment with dithiothreitol (Roberts et al., 1981) and with experiments with the purified TGF-β showing that similar treatment destroyed all colony-forming activity (data not shown).

The purified type β TGF from bovine kidney was found to elicit an ED_{50} colony-forming response in the soft agar assay at only 2×10^{-12} M, or 56 pg/mL, when assayed in the presence of 0.8×10^{-9} M EGF (5 ng/mL) (Table I, Figure 3). In multiple analyses of the purified TGF-β from two

Table II: Effects of TGF-β and EGF on Anchorage-Dependent and Anchorage-Independent Growth of NRK Cells

addition	monolayer growth[a] [(cells/plate) × 10^{-4}]			[^{3}H]thymidine uptake[b] (cpm × 10^{-3})	growth in soft agar,[c] colonies >3100 μm²
	64 h[d]	112 h[d]	64 h[e]		
none	2.2 ± 0.4	3.8 ± 0.1	28 ± 1	190 ± 17	0
TGF-β	1.5 ± 0.1	1.9 ± 0.2	27 ± 0.5	240 ± 14	0
EGF	7.0 ± 0.1	25 ± 2	62 ± 6	490 ± 60	24
TGF-β + EGF	8.8 ± 0.4	29 ± 2	64 ± 2	630 ± 36	1400

[a] NRK cells were seeded in 2% calf serum; EGF (2.5 ng/mL) or TGF-β (3 units/mL) was added 4 h after plating. Similar results were obtained by using 10 units of TGF-β. Each point represents the mean ± SD of triplicate determinations. [b] NRK cells were seeded in 10% calf serum; after 72 h, EGF (0.5 ng/mL) or TGF-β (4 units/mL) was added. After 16 h, [^{3}H]thymidine (1 μCi/mL) was added; uptake was determined 8 h later. Values represent the mean ± SD of four plates. [c] NRK cells were incubated for 7 days according to the standard soft agar assay procedure; EGF (5 ng/mL), TGF-β (10 units). [d] Initial cell count 1.0×10^4. [e] Initial cell count 8.0×10^4.

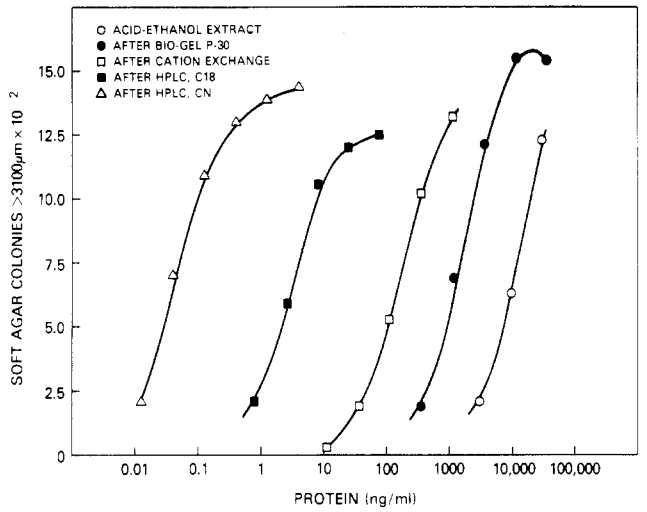

FIGURE 3: Soft agar dilution curves of bovine kidney TGF-β at various stages of purification. Appropriate aliquots of TGF pools were assayed in 1:3 dilutions in the presence of 5 ng/mL EGF: crude acid–ethanol extract (○); after Bio-Gel P-30 (●); after cation exchange (□); after HPLC on μBondapak C18 (■); after HPLC on μBondapak CN (△).

separate preparations of 7–8 kg of bovine kidney, the specific activity ranged from 6000 to 20 000 units/μg, values in excellent agreement with the specific activities of 11 000 units/μg for human placental TGF-β (Frolik et al., 1983) and of approximately 6000 units/μg for human platelet TGF-β (Assoian et al., 1983). The maximum activity attainable upon assay of higher concentrations of TGF-β was equal to that obtainable from less pure preparations, indicating that full colony-forming activity required the presence of only EGF and the purified type β TGF (Figure 3). Assayed in the absence of added EGF, TGF-β had no detectable colony-forming activity, in agreement with previous observations (Anzano et al., 1982).

The ability of a type β TGF to act together with EGF or another type α TGF to induce anchorage-independent growth of the NRK cells in soft agar (Anzano et al., 1982) is in marked contrast to its bioactivity assayed under anchorage-dependent conditions in monolayer cultures. As shown in Table II, purified bovine kidney type β TGF had only a weak effect on [^{3}H]thymidine uptake in NRK cells assayed in either the presence or the absence of added EGF. In separate experiments measuring the effects of both EGF and type β TGF on the proliferation of NRK cells grown in monolayer cultures in the presence of 2% calf serum, it was shown that addition of the TGF-β alone either had no effect or slightly increased the dividing time of the cells and that the combined action of EGF and TGF-β on the cells did not further increase the mitogenic response of the cells to EGF. However, the TGF-β did have striking effects on the morphology of the cells. Cells cultured in the presence of both EGF and TGF-β, but not in the presence of either peptide alone, displayed the morphology characteristic of transformed cells (not shown).

Table III: Amino Acid Composition of Bovine Kidney Type β TGF[a]

amino acid	residues/mol[b] (mean ± range)	amino acid	residues/mol[b] (mean ± range)
aspartic acid	25 ± 1	methionine[c]	3 ± 0
threonine	7 ± 1	isoleucine	11 ± 1
serine	16 ± 2	leucine	25 ± 1
glutamic acid	26 ± 1	tyrosine	17 ± 2
proline	ND[d]	phenylalanine	8 ± 1
glycine	15 ± 3	histidine	8 ± 1
alanine	18 ± 1	lysine	22 ± 2
half-cystine[c]	16 ± 2	tryptophan	ND
valine	17 ± 1	arginine	12 ± 1

[a] Amino acid composition of purified bovine kidney type β TGF determined after 2-h hydrolysis in 6 N HCl at 150 °C. Values are based on three separate determinations of three different preparations. [b] The number of residues per mole of each amino acid is based on an apparent molecular weight of 25 000. [c] Determined by performic acid oxidation and acid hydrolysis. [d] ND, not determined.

5 10 15
Ala-Leu-Asp-Thr-Asn-Tyr-CMC-Phe-Ser-Ser-Thr-Glu-Lys-Asn-CMC-

FIGURE 4: Determined N-terminal amino acid sequence of reduced and S-carboxymethylated bovine kidney TGF-β. CMC = *S*-(carboxymethyl)cysteine.

Amino acid analysis of the purified bovine kidney type β TGF revealed a content of 16 half-cystine residues per mole (Table III). The data from Figure 3 suggest that some of these disulfide bonds are interchain. The results reported have been obtained from separate determinations on three different preparations of bovine kidney TGF-β. The amino acid composition is significantly different from that of human platelet-derived growth factor, which has a molecular weight slightly higher than TGF-β and behaves similarly to TGF-β upon electrophoresis under reducing conditions (Deuel et al., 1981; Raines & Ross, 1982). Particularly striking is the difference in the tyrosine content of these two peptide growth factors: platelet-derived growth factor has three tyrosine residues per mole (Deuel et al., 1981) while TGF-β is estimated to have 17. On the other hand, comparisons of these results with those obtained from analyses of the human TGF-β's reveal no significant differences between the amino acid compositions of the type β TGFs from bovine kidney and from human placenta (Frolik et al., 1983) or human platelets (Assoian et al., 1983).

Amino acid sequence analysis of the reduced and S-carboxymethylated TGF-β by automated Edman degradation using a gas-phase sequencer (Hewick et al., 1981) revealed a single N-terminal amino acid sequence as shown in Figure 4. Initial and repetitive yields were found to be equal to the yields calculated when myoglobin was used as the standard

protein. This would seem to rule out the possibility that one of the chains might have a blocked N-terminus and suggests instead that the sequence of at least the first 15 N-terminal amino acids of each of the two subunits of TGF-β is identical. This interpretation is consistent with the observation of a single protein band of the reduced TGF-β on $NaDodSO_4$–polyacrylamide gels (Figure 2B, inset). In addition, the N-terminal sequence of the bovine kidney TGF-β is identical with the partial sequence of the type β TGF from human placenta (unpublished results), suggesting a high degree of relatedness of type β TGFs from different species and different tissue sources.

Discussion

A type β TGF has been purified over 200 000-fold from the acid–ethanol extract of bovine kidney. The biologically active form of TGF-β (M_r 25 000) consists of two polypeptide chains cross-linked by disulfide bridges. These two TGF-β subunits appear to be of nearly equal molecular weight (M_r 12 500) and share a common N-terminal amino acid sequence, suggesting that the two TGF-β subunits might be identical.

The recovery of TGF-β from the bovine kidney (3 μg/kg) is in close agreement with its occurrence in another nonneoplastic tissue, the human placenta (Frolik et al., 1983). In marked contrast to these low levels of tissue-derived TGF-β, human platelets have been found to contain almost 100 times the level of TGF-β on a weight basis (Assoian et al., 1983). The known role of the platelets in wound healing, together with both the apparent ubiquitous occurrence of TGF-β's in all tissues examined thus far (Roberts et al., 1982a) and the recent demonstration that bovine TGF-β can promote wound healing in rats (Sporn et al., 1983), suggests that these polypeptides may play a benign role in tissue repair and wound healing. The idea that transformation and wound healing might be related is not a new one; Haddow (1972) has suggested that tumor formation might be compared to an "overhealing" response. Elucidation of the physiological role of these type β TGFs should improve our understanding of this proposed relationship.

Although more data are needed in other cell systems, experiments directed at the effects of type β TGFs on NRK cells suggest that these polypeptides are not mitogenic for growth of cells in monolayer cultures even though they do promote transformation of the cells to a phenotype that can grow in soft agar in the presence of both type α and type β TGFs (Table II). In the simplest interpretation of the data, cells in an anchorage-independent environment, but not in an anchorage-dependent environment, require type β TGF for conversion to a phenotype permissive for the expression of the mitogenic effects of type α TGFs. This might be compared to the ability of platelet-derived growth factor to induce cells to a "competent" state responsive to the mitogenic effects of serum "progression factors" (Stiles et al., 1979). However, in contrast to TGF-β, these effects of platelet-derived growth factor are observed in monolayer cultures.

It is clear from the properties of the purified TGF-β (M_r 25 000) that this peptide is retarded on the Bio-gel P-30 support. In fact, it has been shown that the human platelet TGF-β is eluted from Bio-Gel P-60 with an apparent molecular weight of 15 000–18 000 (Assoian et al., 1983), and the human placental TGF-β is eluted from Bio-Gel P-30 with an apparent molecular weight of 3000–5000 (Frolik et al., 1983). Yet, when purified to homogeneity, each of these type β TGFs and the bovine TGF-β have an identical molecular weight of 25 000, as determined by electrophoresis on $NaDodSO_4$–polyacrylamide gels (Roberts et al., 1983a). In addition, recent experiments with Moloney murine sarcoma virus transformed mouse 3T3 cells have demonstrated that type α and type β TGF activities of both the cells and the conditioned medium coelute as a single peak of apparent TGF activity on Bio-Gel P-60, even though the molecular weight of TGF-α has been determined to be approximately 6000 and that of the TGF-β approximately 25 000 (Anzano et al., 1983). Thus, it should be clear that the physical properties of these polypeptides cannot be determined from chromatography on polyacrylamide sizing gels.

The yield of TGF-β activity from this purification scheme was only 10%, a value that is in agreement with the yields of approximately 6% and 5% for the purification of the TGF-βs from human placenta and human platelets, respectively (Frolik et al., 1983; Assoian et al., 1983). Although the purification steps for the placental TGF-β were similar to those described here, the platelet TGF-β, derived from a more enriched source, was purified after only two chromatographic steps. Since the latter procedure involved only sizing gel purification, it would appear that the apparent low yield associated with the cation-exchange step of this purification [see Table I and Frolik et al. (1983)] may be intrinsic to the chosen method of assay and that other, unrelated factors may be contributing to the colony-forming response at earlier steps of the purification. A similar phenomenon has been described for the yields of platelet-derived growth factor as determined by an assay for mitogenic activity as compared to estimates of yield based on receptor binding competition (Bowen-Pope & Ross, 1982). More realistic estimates of the relative TGF-β content of various tissues await the development of receptor binding and antibody binding assays.

The purification to homogeneity of the type β TGFs will now permit investigations of both the chemistry of this new family of TGFs and the biochemistry of its interaction with type α TGFs to induce cells to express an altered phenotype. Whether or not this mechanism will have elements in common with the mechanism whereby oncogene expression leads to cellular transformation [for a review, see Bishop (1982)] is an important question for future research. The recent discovery that the *sis* oncogene encodes a protein homologous to platelet-derived growth factor (Doolittle et al., 1983; Waterfield et al., 1983), together with the finding that the platelets carry TGF-β as well as platelet-derived growth factor (Assoian et al., 1983), suggests that common elements will be found.

Acknowledgments

We thank Drs. Richard Assoian and Charles Frolik for valuable discussions relating to the purification of the TGFs, Gary Barbarash for help in the modification of the amino acid analyzer, and Ruth Morsillo and Natalie Wilson for assistance in the preparation of the manuscript.

Registry No. Epidermal growth factor, 62229-50-9.

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Interaction of Colicin A with Phospholipid Monolayers and Liposomes†

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ABSTRACT: The insertion of colicin A into monomolecular films and liposomes composed of different phospholipids was studied. Although colicin A was able to penetrate many phospholipid monolayers, it interacted preferentially with negatively charged phospholipids such as phosphatidylglycerol. These interactions are highly dependent on the physical state of the lipid, the ionic strength, and the pH. Amino acid residues with a pK of 5.5 probably govern the lipid–protein interaction. At acidic pH, colicin A was able to insert into phospholipid vesicles and was as strong a penetrating agent as the lytic peptides bee venom mellitin and snake venom cardiotoxins. Below pH 5, colicin A induced aggregation and partial fusion of liposomes. At neutral and basic pH, colicin A penetration ability is limited, and the protein was unable to bind to phospholipid vesicles. However, association of colicin A with lipid vesicles could be achieved at pH 7 by the detergent dialysis technique. The apparent molecular area of colicin A inserted into phosphatidylglycerol films (2000 Å^2/molecule) suggests that a substantial part of the colicin A molecule inserts into the lipid surface.

Colicins A, E1, Ia, Ib, and K have been purified (Konisky, 1973; Schwartz & Helinski, 1971; Goebel, 1973; Jesaitis, 1970; Cavard & Lazdunski, 1979). The primary structure of E1 and A has been deduced from the nucleotide sequence of the gene (Yamada et al., 1982; Morlon et al., 1983a,b). These colicins depolarize the bacterial inner membrane and rapidly cause inhibition of active transport [see Konisky (1978)]. They can form voltage-dependent ion channels in planar bilayers (Schein et al., 1978; Weaver et al., 1981; Pattus et al., 1982, 1983), and they also have been reconstituted with liposomes (Tokuda & Konisky, 1979; Uratini & Cramer, 1981). For

† From the Centre de Biochimie et de Biologie Moléculaire du CNRS, 31, Chemin Joseph-Aiguier, 13402 Marseille Cedex 9, France. *Received March 1, 1983.* Financial support has been received from the CNRS ATP "Conversion de l'Energie dans les Membranes Biologiques".

0006-2960/83/0422-5698$01.50/0