Human Transforming Growth Factor Type β2: Production by a Prostatic Adenocarcinoma Cell Line, Purification, and Initial Characterization

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ABSTRACT: Human type β2 transforming growth factor (hTGF-β2) was purified from tamoxifen-supplemented, serum-free medium conditioned by the human prostatic adenocarcinoma cell line PC-3. The purification of hTGF-β2 was monitored in a growth inhibition assay and was achieved by batch purification on methylsilyl-controlled pore glass, followed by gel permeation chromatography and reversed-phase high-performance liquid chromatography. The overall recovery of hTGF-β2 was 75% of the initial activity and yielded 22 μg of hTGF-β2/L of conditioned medium. The concentration of hTGF-β2 required for half-maximal inhibition of Mv 1 Lu mink lung epithelial cells (CCl-64) was approximately 5 pM when assayed in the presence of 10% fetal bovine serum. The purified hTGF-β2 has a molecular weight of 24 000 when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and consists of two disulfide-linked, apparently identical polypeptide chains, with a molecular weight of 13 000. The amino-terminal sequence of hTGF-β2 was determined. Alignment of the amino acid sequences of hTGF-β2 and hTGF-β reveals statistically significant sequence homology. On the basis of the extensive amino acid sequence homology, we propose the term TGF-β2 for this newly isolated polypeptide. The reported results suggest that TGF-β (TGF-β1) and TGF-β2 may have evolved from a common progenitor.

The growth of normal cells in culture is believed to be regulated in part by a balance between growth-stimulatory and growth-inhibitory polypeptides. Unrestrained proliferation of cancer cells may result from the abnormal production and release of polypeptide growth factors (Sporn & Todaro, 1980; Todaro et al., 1981) or from their failure to express or respond to growth-inhibitory polypeptides (Sporn & Roberts, 1985). African green monkey kidney cells (BSC-1) produce a polypeptide growth inhibitor when arrested at saturation density (Holley et al., 1978). This growth inhibitor has been purified from the conditioned medium of BSC-1 cells and shown to inhibit the proliferation of epithelial, but not fibroblast, cell lines (Holley et al., 1980). Evidence has been presented (Tucker et al., 1984) indicating that the growth-inhibitory polypeptide from BSC-1 cells and type β transforming growth factor (TGF-β)[1] from human platelets are functionally related. TGF-β refers to a family of structurally highly conserved growth-regulatory polypeptides that both stimulate and inhibit cell proliferation, depending largely on the cell type [for review, see Sporn et al. (1986)].

The isolation of cartilage-inducing factor B (CIF-B) from bovine demineralized bone has been reported (Seyedin et al., 1985), and the structural and functional relationship of CIF-B to TGF-β has been demonstrated (Seyedin et al., 1987). We describe in this paper the purification of a growth-inhibitory polypeptide that is produced by a human prostatic adenocarcinoma cell line and released into tamoxifen-supplemented, serum-free medium and report the amino-terminal amino acid sequence. The data clearly define the structural relationship to bovine CIF-B. On the basis of the extensive sequence homology, we propose the term TGF-β2 for this new family of growth regulators. Human (Derynck et al., 1985) and mouse (Derynck et al., 1986) type β TGF will be termed TGF-β1 to emphasize the functional and structural relationship between the two families of growth regulators.

The findings described here, that a cloned line of human prostatic adenocarcinoma cells produces hTGF-β2, a close relative to hTGF-β1, should allow studies on its biosynthesis and how it might be hormonally regulated.

Experimental Procedures

Source of TGF-β2. TGF-β2 was purified from tamoxifen-supplemented medium conditioned by a line of human prostatic adenocarcinoma cells, PC-3 (Kaighn et al., 1979), derived from a bone metastasis. The PC-3 line was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown to confluence on 850-cm^2 plastic roller bottles (Corning 25140) in 50 mL of F-12 HAM (Sigma, St. Louis, MO) and Dulbecco's modified Eagle's medium (1:1), supplemented with 10% heat-inactivated fetal bovine serum. The monolayers were washed 3 times with phosphate-buffered saline. The medium was discarded and replaced with 50 mL of fresh serum-free medium supplemented with tamoxifen [1-[*p*-[β-(dimethylamino)ethoxy]phenyl]-*trans*-1,2-diphenylbut-1-ene; Sigma] and dissolved in dimethyl sulfoxide to reach a final concentration of 1.4×10^{-5} M. Serum-free conditioned medium was collected every 48 h for a 6-day period by decantation, clarified by low-speed centrifugation at 1000*g* for 15 min, and passed through a filter with a pore size of 0.45 μm (Nalgene, Rochester, NY). Aprotinin (3.6 mg/L) and phenylmethanesulfonyl fluoride (10 mg/L) were added to the filtrate.

Batch Adsorption on Methylsilyl-Controlled Pore Glass (MS-CPG). PC-3-conditioned medium was acidified to pH 4.0 with concentrated TFA. In the batch adsorption procedure, 30 g of MS-CPG (40-μm particle size, Sepralyte; Analytichem International, Harbor City, CA), previously washed with 100% acetonitrile containing 0.1% TFA, was suspended in 1.6 L of

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[1] Abbreviations: TGF, transforming growth factor; hTGF, human transforming growth factor; CIF, cartilage-inducing factor; TFA, trifluoroacetic acid; MS-CPG, methylsilyl-controlled pore glass; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Gdn, guanidine.

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acidified conditioned medium in a vessel equipped with a microcarrier magnetic stirrer (Bellco Glass, Vineland, NJ). The mixture was stirred for 1 h at 4 °C. The beads were resuspended in 10 volumes of 0.1% TFA and transferred to a column (5.0 × 3.2 cm) for elution. The column was washed with 200 mL of 0.1% TFA containing 30% acetonitrile and 0.2 M NaCl and batch eluted with 120 mL of 50% acetonitrile, 0.2 M NaCl, and 0.1% TFA. The eluate containing TGF-β2 was dialyzed for 60 h against 0.1 M acetic acid, concentrated by lyophilization, and reconstituted in 0.1% TFA containing 40% acetonitrile.

Gel Permeation Chromatography. The supernatant containing TGF-β2 activity was further purified by gel permeation chromatography on a column (21.5 × 600 mm) of Bio-Sil TSK-250 (Bio-Rad Laboratories, Richmond, CA). The column was equilibrated with 0.1% TFA containing 40% acetonitrile at 2 mL/min at 22 °C; 4-mL fractions were collected.

Reversed-Phase High-Performance Liquid Chromatography. The final purification of hTGF-β2 was achieved by reversed-phase HPLC. All separations were performed on a μBondapak C_{18} column (10-μm particle size, 3.9 × 300 mm; Waters Associates, Milford, MA). A linear acetonitrile gradient composed of 0.05% TFA in water as starting buffer and 0.045% TFA in acetonitrile as limiting buffer was used. The column was operated at a flow rate of 0.2 mL/min at 22 °C. The column effluent was collected in 1-mL portions. Pools of fractions comprising the major growth-inhibitory activity were diluted 2-fold with 0.05% TFA and rechromatographed on the same column, previously equilibrated with 0.05% TFA in water. The column was then eluted with a linear 1-propanol gradient containing 0.035% TFA at 0.2 mL/min at 22 °C. The column effluent was collected in 1-mL fractions.

Protein Determination. Total protein was determined (Bradford, 1976) by using bovine serum albumin as a standard. Protein was also determined by UV absorbance at 214 nm, comparing peak areas with the peak area of a known amount of bovine insulin.

Growth Inhibition Assay. Mv l Lu mink lung epithelial cells (CCl-64; American Type Culture Collection) were subcultured on flat-bottomed 96-well tissue culture plates (Costar 3596, Cambridge, MA) in 50 μL of complete Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum at 3.5×10^3 cells/well. Aliquots from column fractions to be assayed for growth inhibition were diluted in complete medium and assayed in triplicate with 50 μL of the diluted sample added to each well. The cells were incubated for 72 h at 37 °C in a humidified 5% CO_2–95% air atmosphere. At the end of this incubation period, each well was treated for 24 h with 100 μL of complete medium containing 5-[^{125}I]iodo-2′-deoxyuridine (IdU) (0.05 μCi/well; Amersham IM.355V). The monolayers were washed with phosphate-buffered saline, fixed in 95% methanol, and the [^{125}I]IdU incorporated by the cells was solubilized with 200 μL of 1 N NaOH. The amount of cell growth was measured by the amount of [^{125}I]IdU incorporated into the DNA of actively growing cells. One unit of activity was defined as the amount of TGF-β2 required to give 50% maximal response in the above assay.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). A 12–20% acrylamide gradient slab (140 × 120 × 0.75 mm) was prepared with a 5% stacking gel. Molecular weight standards were ovalbumin (M_r 43000), α-chymotrypsinogen (M_r 25700), β-lactoglobulin (M_r 18400), lysozyme (M_r 14300), and bovine trypsin inhibitor (M_r 6200). After electrophoresis, gels were fixed in 40% methanol and 10% acetic acid overnight, washed in 10% methanol and 5% acetic acid for 2 h, and stained with silver (Merril et al., 1981).

Amino Acid Sequence Determination. For amino-terminal sequence analysis, hTGF-β2 (8 μg) was reduced with dithiothreitol (20 mM) in 100 μL of 0.4 M Tris·HCl/6 M Gdn·HCl/0.1% Na$_2$EDTA, pH 8.5, for 2 h at 50 °C and subsequently S-pyridylethylated with vinylpyridine (100 mM) for 4 h at 22 °C. The reaction mixture was acidified to pH 2.0 with 20% TFA and desalted on an RP-300 column (2.1 × 30 mm; Applied Biosystems, Foster City, CA). The concentration of acetonitrile was increased linearly (1%/min) during 1 h, at a flow rate of 100 μL/min, at 35 °C. One symmetrical polypeptide peak was eluted with a gradient of aqueous acetonitrile containing 0.085% TFA.

Automated sequence analysis of S-pyridylethylated TGF-β2 was performed on a Model 470A amino acid sequencer (Applied Biosystems) with the 03RPTH program. A total of 3.0 mg of BioBrene Plus (Applied Biosystems) was applied and subjected to three precycles of Edman degradation prior to sample application. Conversion of the thiazolinone derivatives to phenylthiohydantoin amino acids was carried out with 25% TFA. Phenylthiohydantoin amino acid derivatives were separated by reversed-phase HPLC on a PTH C_{18} column (2.1 × 220 mm; Applied Biosystems) with a sodium acetate buffer/tetrahydrofuran/acetonitrile gradient (Hunkapiller & Hood, 1983), on-line, on a Model 120A PTH analyzer (Applied Biosystems).

RESULTS

Source and Initial Fractionation of hTGF-β2. hTGF-β2 was isolated from tamoxifen-supplemented, serum-free conditioned medium of the human prostatic adenocarcinoma cell line PC-3. The quantitation of hTGF-β2 was based on its ability to inhibit DNA synthesis in epithelial mink lung (CCl-64) cells. The amount of hTGF-β2 produced was increased by treatment of PC-3 cells with tamoxifen. The addition of tamoxifen to serum-free medium at 1.4×10^{-5} M increased the level of secretion of hTGF-β2 2–5-fold compared to tamoxifen-free controls after 6 days of treatment.

The supernatant fluids were collected every other day for a 6-day period. Culture conditions were such that at the end of the culture period more than 90% of the cells were still viable and attached as monolayers. A summary of the steps leading to the isolation of hTGF-β2 and its recovery is presented in Table I.

Batch adsorption on MS-CPG at 20 g/L acidified PC-3-conditioned medium removed all detectable growth-inhibitory activity present in the medium. After the adsorption step, the silica-based adsorbent was transferred to a column and washed with 30% acetonitrile containing 0.2 M NaCl and 0.1% TFA. Some adsorbed protein was removed, but the growth-inhibitory activity was still retained by the support. Elution with 50% acetonitrile containing 0.2 M NaCl and 0.1% TFA removed the bound hTGF-β2 from the support. Typically, about 50 mg of protein was recovered per preparation with a reduction in volume of about 8-fold and with a yield close to 90% of the initial growth-inhibitory activity.

Purification of hTGF-β2. Dialysis of the MS-CPG eluate against acetic acid, lyophilization, and subsequent gel permeation chromatography of the acid-soluble partially purified hTGF-β2 resulted in 83% recovery of the initial growth-inhibitory activity. A representative chromatogram is illustrated in Figure 1. One peak of activity was found with an M_r of

Table I: Purification of hTGF-β2 from Conditioned Medium of Human Prostatic Adenocarcinoma Cells, PC-3

purification step	volume (mL)	protein recovered (mg)	hTGF-β2 activity recovered (units)	relative specific activity (units/μg)	degree of purification (x-fold)	recovery (%)
(1) PC-3-conditioned medium	1600	686	3.5×10^6	5.1	1	100
(2) eluate from MS-CPG	220	50	3.1×10^6	62	12	89
(3) Bio-Sil TSK-250	12	2.3	2.9×10^6	1260	250	83
(4) μBondapak C_{18} (acetonitrile)	7	0.340	2.8×10^6	8240	1620	80
(5) μBondapak C_{18} (1-propanol)	6	0.035	2.6×10^6	74300	14600	74

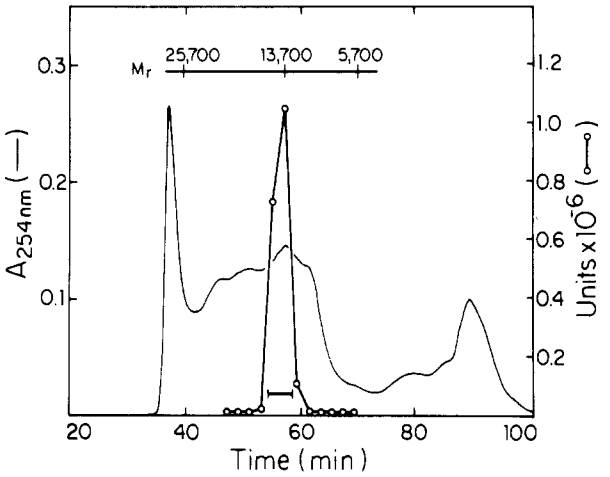

FIGURE 1: Gel permeation chromatography on a Bio-Sil TSK-250 column of 36 mg of protein eluted with 50% acetonitrile (0.2 M NaCl) from MS-CPG after batch adsorption of 1150 mL of PC-3-conditioned medium. Aliquots of the indicated fractions were assayed for growth-inhibitory activity on CCl-64 cells (–O–). The solid line gives the protein absorbance at 254 nm. The following proteins were used as markers: α-chymotrypsinogen (M_r 25 700), bovine pancreatic ribonuclease A (M_r 13 700), and insulin (M_r 5700).

14 000. hTGF-β2-containing fractions were pooled as indicated, diluted 2-fold with 0.05% TFA, and further purified by reversed-phase HPLC. A typical elution pattern is illustrated in Figure 2A. Growth-inhibitory activity of individual fractions was determined. hTGF-β2 was well separated from the bulk of contaminating protein, which eluted at lower and higher concentrations of organic solvent (not shown). Fractions indicated with a bar were pooled, diluted 2-fold with 0.05% TFA, and taken for rechromatography. A 7-fold purification of hTGF-β2 after gel permeation chromatography was obtained; 80% of the initial growth-inhibitory activity was recovered (Table I).

Rechromatography of hTGF-β2-containing fractions on the C_{18} support and elution with a linear 1-propanol gradient containing 0.035% TFA separated a well-defined peak of activity from the bulk of UV-absorbing material. The growth-inhibitory activity copurified with a distinct absorbance peak at 20.8% 1-propanol, as shown in Figure 2B. Fractions indicated with a bar were pooled and further analyzed. The purification of hTGF-β2 was approximately 15 000-fold with a yield of 74% of the initial total growth-inhibitory activity. The overall recovery of hTGF-β2 per step was 89–96%. The highest specific activity observed was 74×10^6 units/mg, which gives half-maximal activity in the range of 5×10^{-12} M.

Characterization of hTGF-β2. The purity of the final hTGF-β2 preparation was determined by analytical NaDodSO$_4$–polyacrylamide gel electrophoresis and compared with TGF-β1 isolated from bovine spleen.[2] One major polypeptide band, with an M_r of 24 000, was observed under nonreducing conditions, as shown in Figure 3A. When samples were electrophoresed under reducing conditions, the polypeptide

[2] M. N. Lioubin, T. Ikeda, and H. Marquardt, unpublished results.

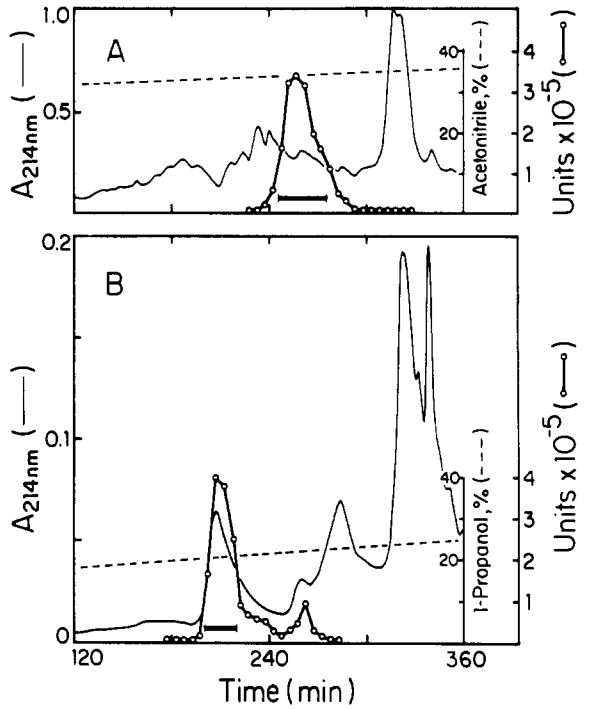

FIGURE 2: Final purification of hTGF-β2 by reversed-phase HPLC. (A) Elution pattern of 2.3 mg of protein from gel permeation chromatography purified hTGF-β2 (Figure 1) on a μBondapak C_{18} column in 0.05% TFA. Elution was achieved with a linear 10-min gradient of 0–30% acetonitrile in 0.045% TFA, followed by a linear 10-h gradient of 30–40% acetonitrile in 0.045% TFA and a 10-min gradient of 40–60% acetonitrile in 0.045% TFA. The conditions are described under Experimental Procedures. Aliquots of the indicated fractions were assayed for growth-inhibitory activity on CCl-64 cells (–O–). The horizontal bar indicates fractions of the column run pooled for rechromatography. (B) Rechromatography of pool hTGF-β2; elution pattern of 0.340 mg of protein on a μBondapak C_{18} column in 0.05% TFA. Elution was achieved with a linear 10-min gradient of 0–15% 1-propanol in 0.035% TFA, followed by a linear 6-h gradient of 15–25% 1-propanol in 0.035% TFA and a linear 20-min gradient of 25–40% 1-propanol in 0.035% TFA. Aliquots of the indicated fractions were assayed for growth-inhibitory activity on CCl-64 cells (–O–). The horizontal bar indicates pooled hTGF-β2. UV-absorbing material was measured at 214 nm (—); the dashed line (---) denotes the concentration of the organic modifier.

stained as a single band at M_r 13 000 (Figure 3B).

Automated Edman degradation of hTGF-β2 was performed with 10 pmol (based on the initial yield of identified Y-6) of the reduced and S-pyridylethylated TGF-β2 derivative. A single amino-terminal amino acid sequence was obtained. Unambiguous identification of phenylthiohydantoin derivatives of amino acids was possible up to residue 51. The amino-terminal amino acid sequence is given in Figure 4 and compared with the amino acid sequence of hTGF-β1.

Discussion

In this study, a growth inhibitor produced by the human prostatic adenocarcinoma cell line, PC-3, and released into tamoxifen-supplemented, serum-free medium was purified to apparent homogeneity and partially characterized. The polypeptide has been structurally defined as a member of the

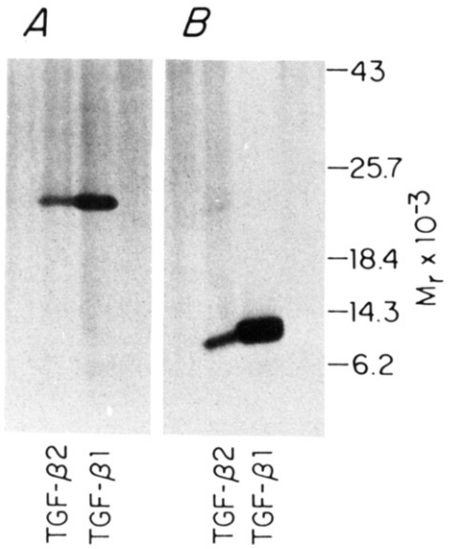

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of hTGF-β2 and bovine TGF-β1 on a 12–20% acrylamide gradient slab in the absence (panel A) or the presence (panel B) of β-mercaptoethanol. The position of marker proteins used to construct standard plots of log molecular weight vs. mobility are indicated: ovalbumin (M_r 43000), α-chymotrypsinogen (M_r 25700), β-lactoglobulin (M_r 18400), lysozyme (M_r 14300), and bovine trypsin inhibitor (M_r 6200).

	1				5				10				15				20
hTGF-β1	A	L	D	T	N	Y	C	F	S	S	T	E	K	N	C	C	V	R	Q	L
hTGF-β2	A	L	D	A	A	Y	C	F	R	N	V	Q	D	N	C	C	L	R	P	L

	25				30				35				40
hTGF-β1	Y	I	D	F	R	K	D	L	G	W	K	W	I	H	E	P	K	G	Y	H
hTGF-β2	Y	I	D	F	K	R	D	L	G	W	K	W	I	H	E	P	K	G	Y	N

	45					50		
hTGF-β1	A	N	F	C	L	G	P	C	P	Y	I
hTGF-β2	A	N	F	C	A	G	A	C	P	Y	L

FIGURE 4: Alignment of amino-terminal sequences of hTGF-β1 (Derynck et al., 1985) and hTGF-β2. Amino acid residues are given in single letter code. Residues in the amino acid sequence of hTGF-β2 that differ from those in the sequence of hTGF-β1 are underlined.

type β TGF family. hTGF-β2 was quantitated by utilizing one of its properties, to inhibit the proliferation of the epithelial mink lung cell line, CCl-64.

hTGF-β2 was isolated from the acid-soluble fraction of PC-3-conditioned medium and purified by batch adsorption on silica-based adsorbents (Henderson et al., 1983), gel permeation chromatography, and reversed-phase HPLC on the same column support with sequentially acetonitrile and 1-propanol as the mobile phase and TFA as the ionic modifier for elution (Marquardt & Todaro, 1982). High recoveries (89–96%) of growth-inhibitory activity were obtained at each purification step to yield purified hTGF-β2 with an approximate specific activity of 74×10^6 units/mg of protein. Half-maximal inhibition of CCl-64 cells was observed with approximately 0.1 ng of hTGF-β2/mL. These results were similar to those obtained with hTGF-β1 isolated from human platelets (Tucker et al., 1984).

The purity of the final hTGF-β2 preparation was established by $NaDodSO_4$–polyacrylamide gel electrophoresis. Only one major single band, with an M_r of 24000, was observed (Figure 3A). TGF-β2, like TGF-β1 isolated from bovine spleen,[2] consists of two apparently identical polypeptide chains, with an M_r of 13000, cross-linked by disulfide bonds, as shown by $NaDodSO_4$–polyacrylamide gel electrophoresis after reduction with β-mercaptoethanol (Figure 3B), reversed-phase HPLC after reduction with dithiothreitol and subsequent S-pyridylethylation (Experimental Procedures), and amino-terminal amino acid sequence analysis of the purified hTGF-β2 subunit (Figure 4). Reduction of the disulfide bridges completely abolished the ability of hTGF-β2 to inhibit the proliferation of mink lung cells.

Comparison of the amino-terminal sequences of hTGF-β2 and hTGF-β1 revealed a high degree of sequence homology. Alignment of the first 51 residues of hTGF-β2 with those of hTGF-β1 resulted in 36 common amino acid residues out of 51 possible comparisons. The complete amino acid sequences of human (Derynck et al., 1985) and mouse (Derynck et al., 1986) TGF-β1 are known from their cloned complementary DNAs and show remarkable conservation, differing only in one single amino acid residue. These studies define the biologically active TGF-β1 molecule as a homodimer of two disulfide-linked chains of 112 amino acids each. The amino-terminal amino acid sequences of bovine CIF-B (Seyedin et al., 1987) and human TGF-β2 are identical, emphasizing evolutionary relationship. The observed homology between TGF-β1 and TGF-β2 suggests that TGF-β1 and TGF-β2 may have evolved through a process of gene duplicaton from a common ancestor. Bovine CIF-B and human TGF-β2 are structurally closely related and are as well conserved as are human and mouse TGF-β1. Thus, the divergence of TGF-β2 from TGF-β1 occurred prior to the rodent–primate split, 70 million years ago. Since that time, it appears that TGF-β2 has been as well conserved as has TGF-β1.

Type β TGF is a multifunctional peptide that inhibits the proliferation of most epithelial cells and is mitogenic for a variety of fibroblastic cell types. The human prostatic adenocarcinoma cell, PC-3, a cloned line that produces TGF-β2, has ultrastructural characteristics of both epithelial (Kaighn et al., 1979) and neoplastic cells (Kaighn et al., 1980). However, PC-3 cells lost their inhibitory response in serum-free medium to TGF-β2. Thus, the endogenous production of TGF-β2 and its hormonal regulation by antiestrogens may play a role in an autocrine mechanism of growth control in the human prostatic adenocarcinoma cell line, PC-3. A physiological role for the abnormal expression of a functionally and structurally close relative of TGF-β1 in certain neoplastic cells or in tissues (Seyedin et al., 1985) characterized by excessive proliferation is at present unknown. Available structural data should allow studies of the regulation of TGF-β2 in normal and transformed cells.

Added in Proof

After this paper was submitted for publication, the amino-terminal sequence of porcine TGF-β2 was reported (Cheifetz et al., 1987). All of the identified residues in porcine TGF-β2 and human TGF-β2 were identical, confirming the predicted sequence conservation in this new family of growth regulators.

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Articles

Structure of the High-Affinity Binding Site for Noncompetitive Blockers of the Acetylcholine Receptor: [^{3}H]Chlorpromazine Labels Homologous Residues in the β and δ Chains†

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ABSTRACT: The membrane-bound acetylcholine receptor from *Torpedo marmorata* was photolabeled by the noncompetitive channel blocker [^{3}H]chlorpromazine under equilibrium conditions in the presence of the agonist carbamoylcholine. The amount of radioactivity incorporated into all subunits was reduced by addition of phencyclidine, a specific ligand for the high-affinity site for noncompetitive blockers. The labeled β chain was purified and digested with trypsin or CNBr, and the resulting fragments were fractionated by high-performance liquid chromatography. Sequence analysis resulted in the identification of Ser-254 and Leu-257 as residues labeled by [^{3}H]chlorpromazine in a phencyclidine-sensitive manner. These residues are located in the hydrophobic and potentially transmembrane segment M II of the β chain, a region homologous to that containing the chlorpromazine-labeled Ser-262 in the δ chain [Giraudat, J., Dennis, M., Heidmann, T., Chang, J. Y., & Changeux, J.-P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2719–2723]. These results show that homologous regions of different receptor subunits contribute to the unique high-affinity site for noncompetitive blockers, a finding consistent with the location of this site on the axis of symmetry of the receptor molecule.

The nicotinic acetylcholine receptor (AcChR)[1] from fish electric organ and vertebrate neuromuscular junction is a heterologous pentamer ($\alpha_2\beta\gamma\delta$) that both carries the acetylcholine binding sites at the level of the α chains and contains the agonist-gated ion channel [reviews in Changeux et al. (1984), Anholt et al. (1985) and Stroud and Finer-Moore

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[1] Abbreviations: AcChR, acetylcholine receptor; NCB, noncompetitive blocker; CPZ, chlorpromazine; $NaDodSO_4$, sodium dodecyl sulfate; PTH-amino acids, phenylthiohydantoin amino acids; HPLC, high-performance liquid chromatography; TPCK, N^{α}-tosylphenylalanine chloromethyl ketone; AUFS, absorbance units full scale.

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