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Assessment of Biological Activity of Synthetic Fragments of Transforming Growth Factor-Alpha

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Transforming growth factor-alpha (TGF-α) is a single chain polypeptide hormone of 50 amino acids that stimulates growth of some human cancer cells via an autocrine mechanism. The domain(s) of TGF-α that bind and activate its receptor have not been reported. Hydrophilicity plots of TGF-α indicate three discrete sequences that are theoretically exposed on the hormone's surface and thus potentially able to interact with the TGF-α receptor. Fragments of TGF-α encompassing these hydrophilic domains were prepared by using solid-phase peptide synthesis (SPPS) techniques and purified by use of high performance liquid chromotography (HPLC). Assessment of biological activity of the TGF-α fragments indicated that none of the fragments significantly inhibited binding of EGF to the receptor, stimulated DNA synthesis of cells, inhibited EGF-induced DNA synthesis of cells, stimualted growth of cells in soft agar, or induced phosphorylation of the receptor or p35 protein. These results indicate that the receptor binding domain of TGF-α is not totally encompassed by any of the separate fragments tested and probably is formed by multiple separate regions of TGF-α.

Key words: peptides, mitogens, solid-phase peptide synthesis

TGF-α is a mitogenic hormone that is thought to play important roles in normal fetal development and in tumor growth. Northern analysis of RNA isolated from rat embryos indicated that TGF-α transcripts were expressed at high levels during early development (8–10 days) then declined to undetectable levels at birth [1]. Analysis of normal adult human tissues for TGF-α transcripts found no mRNA for TGF-α but approximately half of 39 human tumor specimens contained mRNA for TGF-α [2]. Also, TGF-α was detected in urine of patients with various malignancies [3] and was secreted in vitro by a variety of malignant human cells [2]. Recently, TGF-α was shown to be the agent responsible for growth of MCF-7 human breast cancer cells in vivo by an autocrine mechanism [4]. Estrogen appears to stimulate growth of MCF-7 cells indirectly by inducing synthesis and secretion of TGF-α.

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TGF-α is synthesized as a large, single chain, transmembrane glycoprotein of 160 amino acids from which the smaller 50 amino acid polypeptide hormone of is cleaved by the action of an uncharacterized protease that cleaves between Ala-Val residues at both the N- and C-terminals [5]. TGF-α has 30% sequence homology with epidermal growth factor (EGF) and vaccinia virus growth factor (VGF) [6]. All three growth factors share similar placement of three intrachain disulfide loops and all three bind and activate the tyrosine kinase activity of a common membrane receptor. The domain(s) of TGF-α that bind and activate its receptor have not been reported. Hydrophilicity plots of TGF-α indicate three discrete sequences that are theoretically exposed on the hormone's surface and thus potentially able to interact with the TGF-α receptor. We synthesized and purified fragments of TGF-α encompassing these hydrophilic domains using solid-phase peptide synthesis (SPPS) techniques and high performance liquid chromatography (HPLC) and analyzed the fragments for biological activity in several assays. In some cases where disulfide bridging overlapped, selected cysteine residues were replaced by alanine to conserve structural and hydrophobic parameters.

MATERIALS AND METHODS

Synthesis of TGF-α Fragments

The primary amino acid sequence of rat TGF-α (1–50) is shown in Figure 1. Fragments of TGF-α are listed in Table I and peptides 1–7 were prepared by SPPS using the standard Merrifield method [7] with a Peptides International Synthor 2000 automated synthesizer (Louisville, KY) and peptides 8–13 were prepared by Peninsula Laboratories (San Carlos, CA). Alpha-amino groups were protected with t-butyloxycarbonyl (Boc) group and the following side chain protecting groups were used: Arg(Tos), Asp(Cxl), Cys(Acm) or Cys(Meb), Glu(Cxl), His(Bom), Lys(Cl-Z), Ser(Bzl), and Tyr(Dcb). Each synthetic cycle consisted of 1) 5-min and 25-min deprotection with 40% trifluoroacetic acid (TFA)/10% anisole/50% dichloromethane, 2) 5-min neutralization with 10% triethylamine/dichloromethane, and 3) double couplings (3-hr and 16-hr) with Boc-amino acid in the presence of N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC), all at 2.5-fold mole excess over amino acid on resin. Asn and Gln were coupled with their preformed p-nitrophenyl esters in the presence of HOBt, and Arg(Tos) and Gly were coupled with DCC omitting HOBt. All couplings were monitored with the Kaiser test. Triethylamine was distilled from ninhydrin; dichloromethane was distilled from potassium carbonate; dimethylformamide was distilled under reduced pressure. Other solvents and reagents were of analytical grade.

Peptides were deprotected and cleaved from resin with anhydrous HF (1 hr at 0°C) in the presence of 5% p-cresol (v/v) and 5% dimethyl sulfide (v/v). After evaporation of HF and scavengers, solid residues were washed with diethyl ether, extracted with 10% acetic acid and lyophilized. Residues were dissolved in 30% acetic acid and desalted on a Sephadex G-25 column eluted with 30% acetic acid. The major peak of each peptide was pooled, lyophilized, and purified by reversed phase preparative high performance liquid chromatography (HPLC) using a Vydac C-18 column (250 × 10 mm, 300-angstrom pore size, Separations Science, Hesperia, CA) with a gradient of 15% to 30% acetonitrile/water containing 0.05% TFA. HPLC columns had not been used previously to purify any growth factors.

Peptides containing cystine disulfide bonds were produced by washing the residues obtained after HF cleavage with diethyl ether containing 1% 2-mercaptoethanol, then extracted with 10% acetic acid under nitrogen. After lyophilization, residues were dissolved in 0.2% acetic acid saturated with nitrogen, and 2 N aqueous ammonia was added gradually to give a final pH of 7.0 to 7.5. Solutions of the peptides were treated with 20 μM $K_3Fe(CN)_6$ until a permanent yellow color was generated and then stirred for an additional 20 min. The pH was adjusted to 4–5 with acetic acid, and then the solutions were stirred with anion exchange resin (AG 3 × 4, acetate form) to remove excess ferri- and ferrocyanide ions and then lyophilized. Residues were desalted by chromatography on a Sephadex G-15 column eluted with 30% acetic acid and then purified by HPLC as described above.

Competition of EGF Binding

Peptides were tested for their ability to compete for ^{125}I-EGF binding using two receptor sources: placental cell membranes and A-431 cells. Normal term human placenta was homogenized at 4°C with a Brinkman Polytron in a solution of 250 mM sucrose, 1 mM calcium chloride buffered to pH 7 with 10 mM Tris, then filtered through four layers of cheese cloth [8]. Aliquots of placental membrane (50 μg) were incubated with a constant amount of ^{125}I-EGF and increasing amounts of unlabeled EGF or peptides for 2 hr at 37°C. Four milliliters of cold buffer was then added and reaction tubes were centrifuged at 7,000g for 20 min at 4°C and pellets counted with a Beckman gamma scintillation counter. Values represent the mean of triplicate determinations. Competition binding assays using A-431 cells were conducted as described and the values reported represent the mean of duplicate determinations [9].

Induction of DNA Synthesis

Peptides were tested for their ability to stimulate DNA synthesis using diploid human foreskin fibroblasts (HFF) or mouse 3T3 fibroblasts. Confluent, quiescent cultures of HFF, which had been seeded into 96-well plates and held in 0.2% calf serum for 2 days, received 10 ng/ml of TGF-α or 100 ng/ml of peptides. After 8 hr, cultures were labeled with 5-[^{125}I]iodo-2-deoxyuridine (Amersham, 10 μCi/μg) and the amount of isotope incorporated into TCA-insoluble material measured in triplicate wells [9]. Assays using mouse 3T3 fibroblasts were conducted as described below.

Inhibition of EGF-Induced DNA Synthesis

Confluent cultures of the J-2 clone of mouse 3T3 fibroblasts (H. Green, Harvard University) were washed with PBS and held in chemically defined medium (CDM) (equal parts of Dulbecco's modified Eagle medium, Medium 199, Ham's F-10, and buffered with 25 mM HEPES to pH 7.4) containing 0.5% calf serum for 24 hr and then harvested with trypsin. Twenty-four-well plates were seeded with 30,000 cells in 500 μl of CDM containing 0.5% calf serum and tritiated thymidine (1 μCi/ml, Amersham, [methyl,1′,2′-^{3}H]thymidine, final specific activity 100 μCi/mmol). Five hundred microliters of CDM containing 0.5% calf serum and the indicated levels of serum EGF or peptides was added and incubated for 72 hr. Culture wells were washed twice with PBS, 5% TCA, methanol, then dissolved in 1 N sodium hydroxide and radioactivity measured with a beta scintillation counter. Quadruplicate wells were measured and averaged.

Stimulation of Anchorage-Independent Cell Growth

Soft agar growth assay was performed using normal rat kidney cells (NRK) [9]. The number of colonies represents those colonies containing a minimun of 20 NRK cells per 6 random low power fields 10 days after seeding plates with TGF-α or peptides. Plates contained 2 ng/ml of TGF-β purified from human platelets [10] and the designated amounts of TGF-α or peptides. Plates of NRK cells treated with TGF-β alone did not form colonies.

Growth Factor-Induced Phosphorylation

Placental membranes (50 μg) were added to a reaction mixture at 4°C containing TGF-α or peptides at the designated amounts and ^{32}P-ATP and AMP-PNP to reduce phosphatase activity. After 5 min reactions were stopped by addition of SDS and processed by polyacrylamide gel electrophoresis and autoradiography [8].

RESULTS

Chemical Characterization of TGF-α Fragments

The primary amino acid sequence of rat TGF-α [11] is shown in Figure 1. The hydrophilicity plot [12] of TGF-α is shown in Figure 2. Three regions contain high indexes of hydrophilicity and encompass amino acids 8–13, 25–31, and 42–47. These sequences are located essentially within the three disulfide loops. Table I lists the fragments of TGF-α synthesized and Table II lists expected and measured amino acid compositions of the TGF-α fragments (1–6). Values for all the peptides are within experimental error of predicted values. Table III lists the physiochemical properties of the TGF-α fragments (1–6), and all the peptides were greater than 95% pure by HPLC analysis and gave a single spot in two TLC systems.

Competition of EGF Binding

As shown in Figure 3, EGF effectively competes for ^{125}I-EGF binding to human placental membranes with 50% displacement at approximately 2 nM and 90% of the

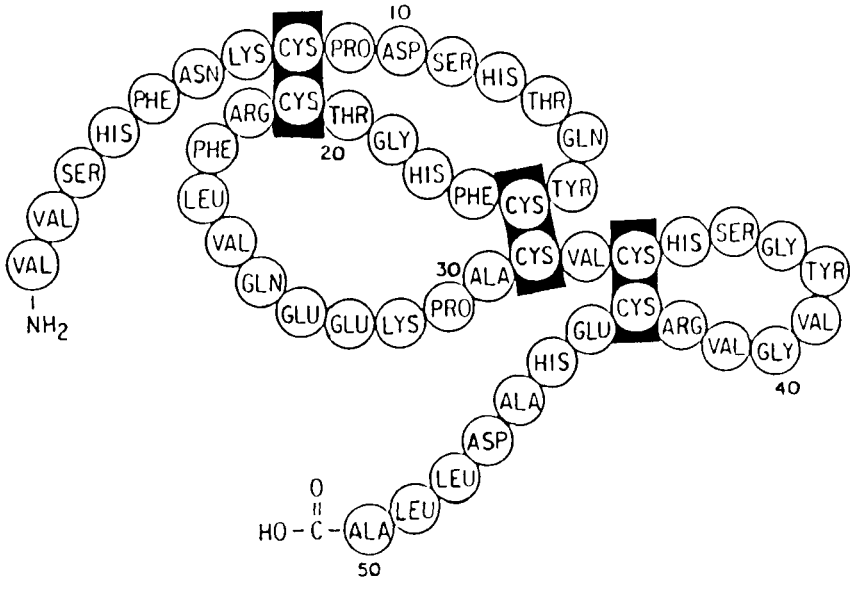

Fig. 1. Primary amino acid sequence of rat TGF-α. TGF-α has substantial sequence homology with EGF and VGF including the alignment of the three disulfide bonds.

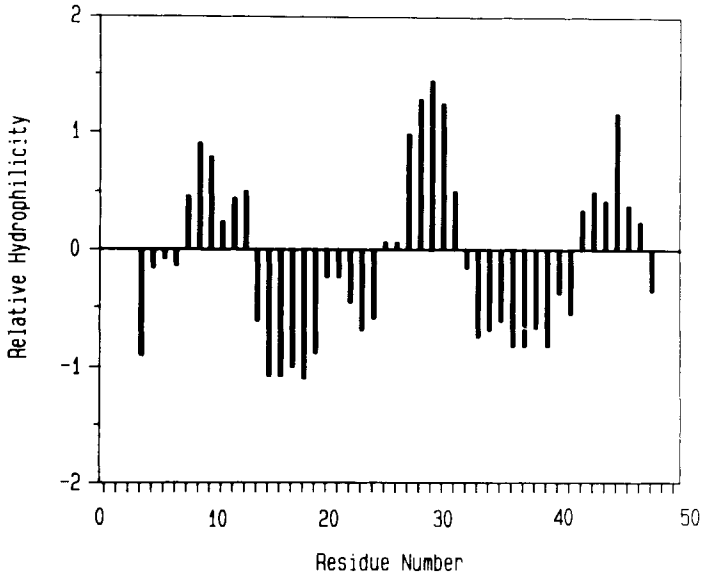

Fig. 2. Hydrophilicity plot of rat TGF-α. TGF-α contains three separate regions of hydrophilicity.

TABLE I. TGF-α Fragments*

Sequence	Peptide number
TGF-α (34–50)	1
Ac-TGF-α (34–43)-NH_2	2
[S-AcmCys^{21,32}]TGF-α (21–33)	3
[Ala^{32}]TGF-α (22–43)-NH_2	4
[Ala^{16}]TGF-α (1–21)-NH_2	5
[S-AcmCys^{16,32}Ala^{21}]TGF-α (15–33)	6
[Ala^{21}]TGF-α (16–33)	7
TGF-α (1–15)	8
[Tyr^{32}]TGF-α (22–32)	9
TGF-α (34–50)	10
TGF-α (34–43)	11
TGF-α (41–50)	12
TGF-α (26–36)	13

*Peptides 1 through 7 were prepared by Merrifield method of solid phase peptide synthesis, and peptides 8 through 13 were prepared by Peninsula Laboratories.

total binding displaced by 10 μM unlabeled EGF. In contrast, none of the TGF-α fragments (1–7) effectively competed for ^{125}I-EGF binding even at 100 μM concentrations. By the use of A-431 cells as the receptor source the same results were obtained: EGF (10 nM) displaced 92% of ^{125}I-EGF binding while none of the TGF-α fragments (1–5, 7) competed for ^{125}I-EGF binding even at 100 μM (Fig. 4).

Induction of DNA Synthesis

TGF-α (10 ng/ml) stimulated incorporation ^{125}IdU sevenfold over control cultures of human foreskin fibroblasts (Table IV). Fragments of TGF-α (1–5, 7) tested

TABLE II. Amino Acid Composition of Synthetic TGF-α Peptides*

Amino acid	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Peptide 6	Peptide 7
Ala	2.06 (2)	—	0.87 (1)	0.87 (1)	1.01 (1)	2.03 (2)	1.99 (2)
Asp	0.98 (1)	—	—	—	1.79 (2)	—	—
Arg	0.97 (1)	1.17 (1)	0.93 (1)	0.93 (1)	—	1.04 (1)	0.97 (1)
$(Cys)_2$	1.08 (1)	0.89 (1)	—	—	0.60 (1)	—	ND
Glu	1.05 (1)	—	3.01 (3)	3.02 (3)	1.00 (1)	3.07 (3)	3.23 (3)
Gly	2.00 (2)	2.07 (2)	—	—	1.00 (1)	1.00 (1)	1.00 (1)
His	1.92 (2)	1.08 (1)	—	—	3.06 (3)	1.01 (1)	0.86 (1)
Leu	2.09 (2)	—	1.00 (1)	1.00 (1)	—	0.98 (1)	1.01 (1)
Lys	—	—	0.98 (1)	0.97 (1)	0.89 (1)	1.06 (1)	1.00 (1)
Phe	—	—	0.98 (1)	0.98 (1)	1.98 (2)	1.96 (2)	1.89 (2)
Pro	—	—	1.22 (1)	1.42 (1)	1.25 (1)	1.46 (1)	1.29 (1)
Ser	0.87 (1)	0.83 (1)	—	—	1.59 (2)	—	—
Thr	—	—	—	—	1.76 (2)	0.89 (1)	0.91 (1)
Tyr	1.03 (1)	1.14 (1)	—	—	0.98 (1)	0.96 (1)	—
Val	2.10 (2)	2.00 (2)	1.95 (2)	1.95 (2)	1.52 (2)	1.82 (2)	2.11 (2)

*Peptides (0.5 mg) were hydrolyzed with constant boiling hydrochloric acid (0.4 ml) containing phenol (20 μl) in evacuated and sealed ampules for 24 hours at 110°C. Analyses were performed on a Dionex analyzer. Values in parentheses are predicted molar ratios. ND, not determined.

TABLE III. Physiocochemical Properties of TGF-α Fragments*

Peptide number	R_f		K′
	BAW	BAWP	HPLC
1	0.08	0.77	2.90
2	0.05	0.76	3.96
3	0.09	0.78	4.40
4	0.02	0.70	3.39
5	0.15	0.74	4.55
6	0.05	0.78	3.18
7	0.16	0.77	3.92

*Purified peptides shown in Table II gave the indicated relative migration in thin layer chromatography systems: BAW, 1-butanol-acetic acid-water (4:1:5, v/v, upper phase); BAWP, 1-butanol-acetic acid-water-pyridine (15:12:10:3, v/v). Analytical C-18 reversed phase HPLC was performed using 20-min linear gradient (flow rate 1 ml/min) from 15–40% acetonitrile/water v/v containing 0.05% trifluoroacetic acid.

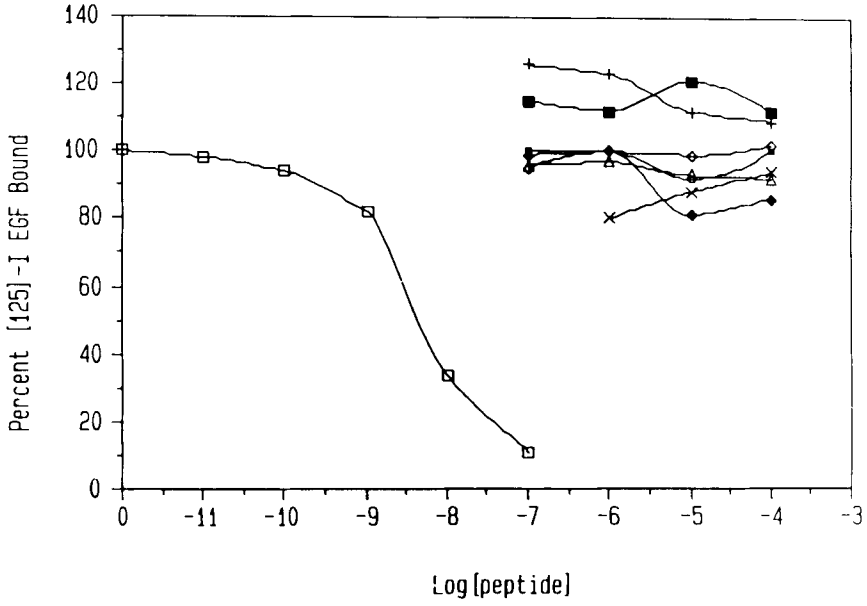

Fig. 3. Competition of EGF binding to human placental membranes. Aliquots of placental membranes (50 μg) were incubated with a constant amount of ^{125}I-EGF (100 pM) and unlabeled EGF or peptides at the indicated levels. After 2 hr at 37°C, tubes were centrifuged and pellets counted. Values are the mean of triplicate samples. Peptides are EGF (⊡), peptide 1 (◇), peptide 2 (△), peptide 3 (◆), peptide 4 (×), peptide 5 (■), peptide 6 (□), and peptide 7 (+).

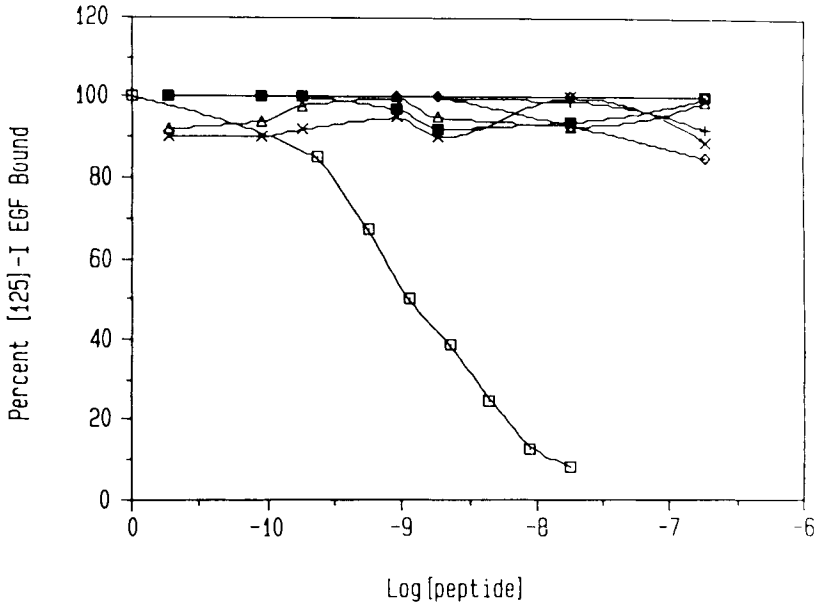

Fig. 4. Competition of EGF binding to A-431 cells. Cultures of formalin fixed A-431 cells were incubated with ^{125}I-EGF (300 pM) and increasing amounts of unlabeled peptides. After 1 hr at 37°C, wells (16 mm) were washed and radioactivity measured. Values are the mean of triplicate samples. Peptides are EGF (□), peptide 1 (◇), peptide 2 (△), peptide 3 (♦), peptide 4 (×), peptide 5 (■), and peptide 7 (+).

TABLE IV. Induction of DNA Synthesis*

Peptide	[^{125}I]IdU incorporated (cpm/dish)
TGF-α	7,824
1	856
2	1,012
3	956
4	979
5	1,005
7	804
No addition	1,075

*Quiescent cultures of diploid human foreskin fibroblasts were seeded into 96-well plates and cultured in medium containing 0.2% calf serum. After 2 days TGF-α (10 ng/ml) or peptides (100 ng/ml) were added and 8 hr later labeled with [^{125}I]IdU for 8 hr and TCA insoluble radioactivity measured. Values are mean of triplicate samples.

at 100 ng/ml all failed to stimulate DNA synthesis above control levels. When 3T3 fibroblasts were used for mitogenesis assay, EGF (1 nM) stimulated thymidine incorporation twofold over control cultures and peptides 1 to 4 (100 μM) again failed to stimulate DNA synthesis.

Inhibition of EGF-Induced DNA Synthesis

As shown in Figure 5, 3T3 cells incubated in CDM containing 0.5% calf serum incorporated small amounts of tritiated thymidine. Addition of 10% calf serum increased thymidine incorporation fivefold and addition of 1 nM EGF increased thymidine incorporation twofold over incorporation in the presence of 0.5% calf serum. Simultaneous addition of 1 nM EGF with each of TGF-α fragments 1 through 4 at 100 μM failed to reduce thymidine incorporation below the level of stimulation measured with EGF alone.

Stimulation of Anchorage-Independent Cell Growth

TGF-α stimulated extensive numbers of colonies of NRK cells in soft agar. In contrast, peptides 1–5 and 7 failed to stimulate colony formation even at tenfold higher concentration (50 ng/ml) (Table V).

Growth Factor-Induced Phosphorylation

Autoradiography of SDS polyacrylamide gels of placental membranes incubated with EGF or TGF-α (2 μM) and ^{32}P-ATP showed enhanced phosphorylation of the EGF/TGF-α receptor (170,000 daltons) and p35 (35,000 daltons) relative to membranes incubated without the growth factors (Fig. 6). Placental membranes incubated

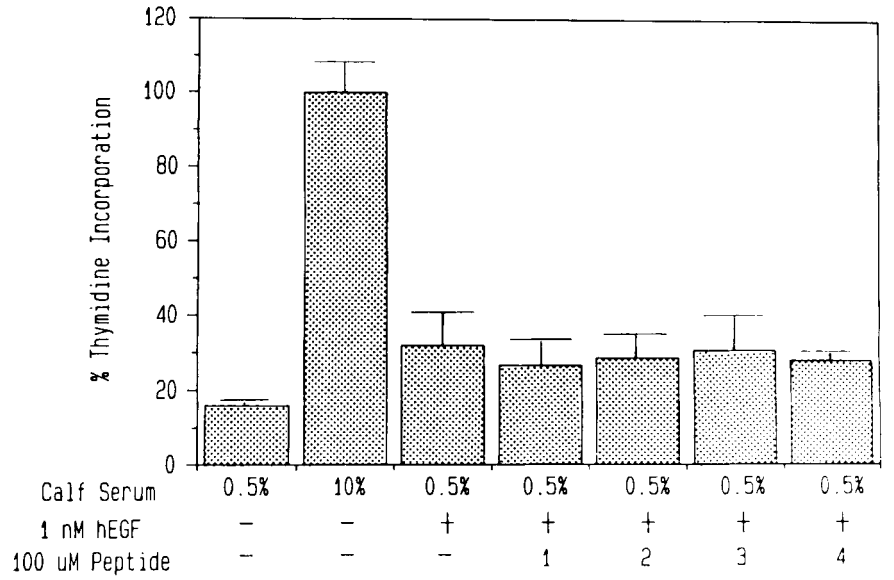

Fig. 5. Inhibition of EGF-induced DNA synthesis. Cultures of mouse 3T3 cells were maintained in low serum (0.5%) containing tritiated thymidine (1 μCi/ ml) and EGF alone or with the indicated peptides. After 72 hr, cultures were washed and radioactivity measured. Values are the mean and standard error of quadruplicate samples.

TABLE V. Stimulation of Anchorage-Independent Cell Growth*

Peptide	Concentration (ng/ml)	Soft agar colonies
TGF-α	5	253
1	5	<20
	50	<20
2	5	<20
	50	<20
3	5	<20
	50	<20
4	5	<20
	50	<20
5	5	<20
	50	<20
7	5	<20
	50	<20

*Normal rat kidney (NRK) cells were seeded in soft agar containing 2 ng/ml TGF-β purified from human platelets and TGF-α or peptides at the designated concentrations. The number of soft agar colonies represents the number of colonies containing a minimum of 20 NRK cells per 6 random low power fields 10 days after seeding (1.4×10^4 cells/ml) with TGF-α or peptides. Plates treated with TGF-β alone did not form colonies.

with TGF-α fragments (1–5, 7) at 200 μM showed no increased phosphorylation of the EGF/TGF-α receptor or p35 above control.

DISCUSSION

Hydrophilicity plots of TGF-α indicate three discrete sequences with high indexes of hydrophilicity separated by stretches of hydrophobic amino acids. Each of the three hydrophilic sequences is located nearly entirely within one of the three internal disulfide loops. It is highly probable that the hydrophilic sequences are located on the surface of TGF-α and, therefore, bind to the receptor. The three-dimensional structures of TGF-α or EGF have not been reported. We synthesized seven peptides encompassing the hydrophilic sequences of TGF-α using SPPS techniques and purified them to homogeneity by HPLC. Amino acid analysis confirmed the correct composition of the fragments.

Analysis of the peptides for activities characteristic of TGF-α gave uniformly negative results in five different assays. The peptides did not compete for ^{125}I-EGF binding to receptors of intact A-431 cells or human placental membranes even at 1,000 times the concentration of EGF that completely displaced the labeled EGF. Concentrations of the peptides up to 10 μM did not stimulate DNA synthesis of human foreskin fibroblasts or 3T3 cells nor did they stimulate anchorage-independent growth of NRK cells. Also, the peptides did not inhibit EGF-induced stimulation of DNA synthesis or stimulate phosphorylation of the EGF/TGF-α receptor or p35 even when added at 10 μM.

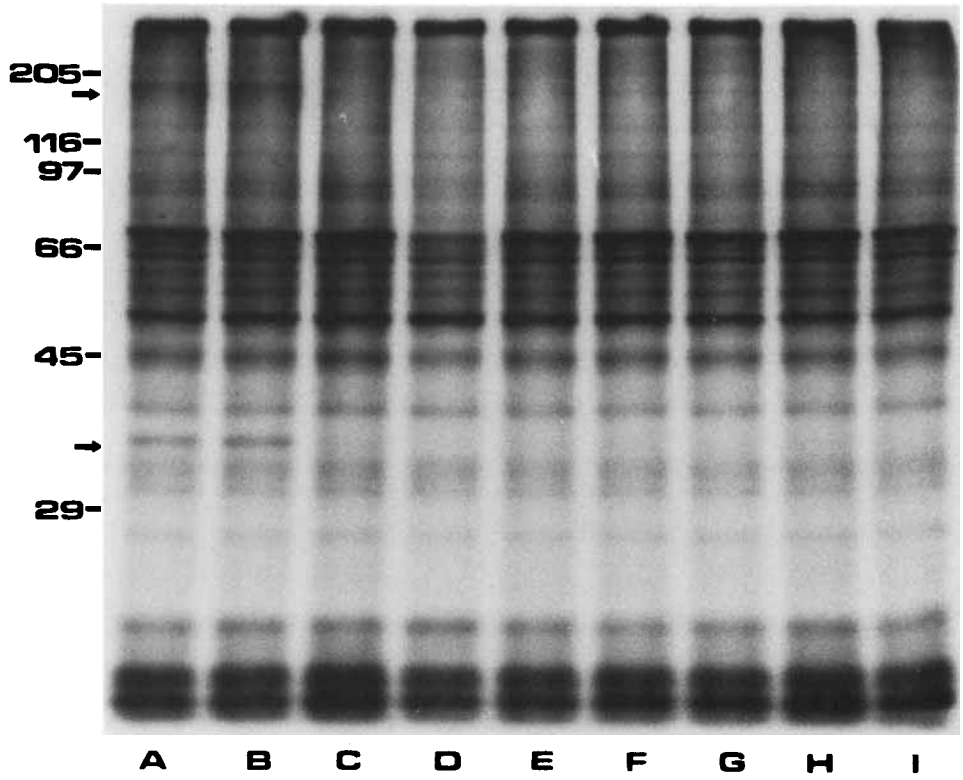

Fig. 6. Growth factor induced phosphorylation. Placental membranes (50 μg) were incubated with ^{32}P-ATP and AMP-PNP and peptides. After 5 min of reaction at 0°C, samples were chromatographed on SDS PAGE and autoradiography performed. Lanes contained 2 μM EGF (**A**), 2 μM TGF-α (**B**), buffer (**C**), or TGF-α peptide 5 (**D**), peptide 7 (**E**), peptide 2 (**F**), peptide 4 (**G**), peptide 3 (**H**), peptide 1 (**I**) all at 200 μM.

In addition to the seven peptides we synthesized, six peptides with sequences overlapping our peptides were synthesized and purified by Peninsula Laboratories. These peptides (8–13) also uniformly failed to produce any response characteristic of TGF-α in the five assays. Thus, results of assays testing the 13 peptides consistently indicate that the receptor binding domain of TGF-α is not contained within any of the individual sequences tested.

Since EGF, VGF, and TGF-α all compete for specific binding of ^{125}I-EGF to membrane receptors, it is likely that similar regions of the growth factors interact with the receptor. The region with the highest conservation of amino acid residues common to all three growth factors encompasses the third disulfide loop of the TGF-α. Nestor et al. [13] reported that a fragment of TGF-α comprising the third disulfide loop (residues 34–43) had low affinity (IC_{50} = 80 μM) for EGF receptors of human cells and that the affinity increased 10- to 100-fold when the N-terminal was blocked by acylation and the C-terminal was blocked by methylation or amidation. These decapeptides were antagonists of the mitogenic effect of EGF and TGF-α on fibroblasts. Also, Ac-TGF-α (34–43)-NHEt was reported to inhibit vaccinia virus infection purportedly by occupying the EGF receptor of target cells [14].

We also synthesized and evaluated the peptides encompassing the third disulfide loop including fragments with nonblocked N- and C-termini (peptides 1 and 10) and with acylated N-termini and/or amidated C-termini (peptides 2, 4, 10, and 11). All of these peptides failed to show inhibition of EGF-induced DNA synthesis in our assay systems. The reason(s) for the different results is not known.

Synthetic fragments of EGF have been reported to contain a major receptor binding domain. Komoriya et al. [15] reported that the cyclic second disulfide loop [Ala^{23}]EGF-(14–31) and the linear [S-Acm$Cys^{21,31}$]-EGF-(20–31) peptide induced all of the biological responses associated with intact EGF but were 10,000-fold less potent than intact EGF. Heath and Merrifield [16] reported that a fragment containing the cyclic third disulfide loop of EGF (residues 32–53) was 100,000-fold less potent than EGF in receptor binding and did not stimulate DNA synthesis. A fragment comprising 70% of EGF starting from the C-terminal (residues 15–53) was 10,000 times less potent than EGF in binding to the EGF receptor and stimulating DNA synthesis.

Based upon the failure of our 13 fragments of TGF-α to mimic or inhibit actions of intact TGF-α, we conclude the major receptor-binding domain is not contained entirely within any of the single peptide sequences reported here. This is supported by the reports that linear TGF-α and polymeric EGF molecules have 100- to 1,000-fold lower potency than the correctly folded hormones [16,17]. When the three dimensional structures of TGF-α or EGF become available, it may be possible to predict more accurately those sequences that form the receptor-binding domain. It is likely that the receptor binding domain is composed of separate regions of the TGF-α sequence that fold into the correct alignment when the hormone assumes its native conformation.

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