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A SYNTHETIC FRAGMENT OF RAT TRANSFORMING GROWTH FACTOR α WITH RECEPTOR BINDING AND ANTIGENIC PROPERTIES*

John J. Nestor, Jr.[†], Sherry R. Newman[†], Barbara DeLustro[X], George J. Todaro[§] and Alain B. Schreiber[X]

Institutes of [†]Bio-Organic Chemistry and [X]Biological Sciences, Syntex Research, 3401 Hillview Ave., Palo Alto, CA 94304

[§]Oncogēn, 3005 First Ave., Seattle, WA 98121

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SUMMARY: A fragment of rat transforming growth factor α (TGFα) comprising the third disulfide loop (residues 34-43) was selected as a potential antigenic and receptor binding region. Immunization of rabbits with a peptide conjugate resulted in antibodies which were specific for both the peptide and rat TGFα, but not for the homologous epidermal growth factor (EGF). The synthetic decapeptide exhibited low affinity for EGF receptors on human cells. Affinity was increased 100x to 0.2% of EGF or TGFα binding by blocking the peptide ends. The blocked decapeptide had no mitogenic activity but prevented the mitogenic effect of EGF and TGFα on fibroblasts. This decapeptide is an antagonist and contains an important receptor binding region of TGFα.

Transforming growth factors (TGF) are mitogenic proteins which confer on normal cells a transformed phenotype (1). Two families of TGF's have been identified. TGFα's are structurally homologous to epidermal growth factor (EGF), bind to the EGF receptor and induce all EGF-mediated biological effects (2,3). TGFβ's do not bind to EGF receptors, but potentiate the biological activity of TGFα (4). Recently a 6 kD TGFα molecule was purified to homogeneity from transformed rat, mouse, and human cells (5), sequenced (3), and cloned (6,7). Rat, mouse, and human TGFα differ by only a few amino acid substitutions and bear clear homologies to mouse and human EGF. The similar positioning of three disulfide bridges is most striking. Although the degree of homology between TGFα and EGF permits binding to a common cellular receptor, it is not sufficient to allow either immunological cross-reactivity or binding of TGFα to EGF binding proteins (1).

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We synthesized fragments of TGFα in an attempt to find regions which may act as antigenic or receptor binding domains. During the course of such studies on several proteins we have focused on predicted β-turn regions (J. J. Nestor and J. G. Moffatt, unpublished results). Two such regions (Figure 1) from rTGFα were identified (residues 1-13; residues 34-43, third disulfide loop). Although an analog of region 1 (V-V-S-H-F-N-D-A-P-D-S-H-T) did not exhibit receptor binding or immunogenic properties, region 2 proved to be an important portion of the TGFα molecule. In this report we describe the generation of antibodies to the third disulfide loop which cross-react with native TGFα and binding studies which indicate that this peptide is a competitive antagonist of TGFα.

MATERIALS AND METHODS

Peptides

EGF was purified from mouse submaxillary glands by extraction with 1 M HCl containing 1% trifluoroacetic acid (TFA), 5% formic acid, and 1% NaCl, concentration on Sep-Pak columns (Waters), and reversed-phase high performance liquid chromatography (hplc) on μBondapak C-18 columns (Waters) as in (8). The complete synthesis of native rat TGFα has been described (9).

The synthetic peptide fragments were prepared on chloromethylpolystyrene-1% divinylbenzene resin (Bio-Rad) using N^{α}-t-butoxycarbonyl protection. Peptides were deprotected and cleaved from the resin using HF or, for the analogs with blocked C-termini, the peptide was first removed by ammonolysis (NH_3/MeOH). The crude deprotected peptides were diluted and cyclized to the disulfide form by oxidation with 0.01 M $K_3Fe(CN)_6$. The peptide solution was loaded on a Bio-Rex 70 cation exchange column, washed with 300 mL of H_2O, and eluted with a gradient to 50% AcOH. The peptide was purified by prep-hplc on a 2.5 x 100 cm column (Altex) of Vydac 218TP C-18 packing using ∿20% CH_3CN eluent, 0.03 M in NH_4OAc at pH 4.5 (10). Peptides 1 and 2 represent the third disulfide loop of rTGFα (Figure 1) with free or blocked (N-Ac; C-amide) ends, respectively. Peptide 3 is the methyl ester of the corresponding loop of hTGFα (Ac-C-H-S-G-Y-V-G-A-R-C-OMe) prepared by transesterification from the resin (base/MeOH).

Figure 1. Sequence of rTGFα. Disulfide bonds and probabilities (>0.75 predicts turn) of β-turn regions (calculated by Chou-Fasman method (20) using computer program written by U. Glatter, supplied by A. Wollmer, R.W.T.H.-Aachen) are shown. See Materials for identity of peptides synthesized.

Immunogen Preparation and Immunoassay

TGFα peptide 1 was coupled to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) by thiol/maleimide linkage as described (11). Carrier-peptide complexes were purified by gel filtration. An average of 60 moles peptide/mole KLH and 20 moles peptide/mole BSA was achieved. Rabbits were immunized with the KLH-peptide 1 conjugate by multiple s.c. and i.m. injections of 2 mg protein in complete Freund's adjuvant. Rabbits were boosted s.c. every two weeks with the immunogen in incomplete Freund's adjuvant. The IgG fraction of the antiserum after 5 boosts was used for immunoassays and radiolabeled using $Na^{125}I$ (NEN) and Enzymobeads (Bio-Rad) to 4 x 10^5 cpm/μg protein. Polyvinyl chloride 96-well plates (Costar) were coated with 200 μg/mL BSA-peptide 1 conjugate and countercoated with 10% normal rabbit serum in phosphate-buffered saline. Plates were incubated for 4 hours at 37°C with [^{125}I]anti-KLH-peptide 1 IgG (50,000 cpm/well) in the presence or absence of inhibitors, washed, and individual wells were counted.

Radioreceptor Assay

A-431 human epidermoid carcinoma cells or human foreskin fibroblasts (HFF), established from primary cultures, were grown to confluence in 24-well cluster dishes (Costar) in Dulbecco's minimal essential medium (DMEM, Gibco) containing 10% fetal calf serum (FCS, Hyclone). EGF was radio-iodinated as above to between 4 x 10^4 and 8 x 10^4 cpm/ng protein. Cells were incubated at 4°C for 60 minutes with 1 nM [^{125}I]EGF in the presence of inhibitor peptides in DMEM (pH 7.4; 20 mM Hepes) containing 0.1% BSA. Cells were washed 3x with cold buffer, lysed with 0.1 N NaOH, and cell-associated radioactivity was measured (γ-counter). Nonspecific binding assessed in the presence of a 100x excess of cold EGF was less than 5% and 10% of the specific binding for A-431 and HFF cells, respectively.

Cell Proliferation Assay

HFF cells were grown to confluence in 48-well cluster dishes in DMEM-10% FCS and brought to quiescence by starvation for 2 days in DMEM-0.5% FCS. Mitogens and peptides were incubated with cells at 37°C for 18 hours prior to a 4 hour pulse with 1 μCi [^{3}H]methyl-thymidine (NEN) and determination of the trichloroacetic acid precipitable radioactivity.

RESULTS

Rabbit antibodies against the KLH-peptide 1 conjugate reacted with BSA-TGFα peptide by solid phase RIA. This reaction was inhibited in a concentration-dependent fashion by peptide 1 and slightly less by native TGFα. In contrast, EGF did not cause significant inhibition (Figure 2).

Rat TGFα competitively displaces the binding of EGF to its receptors (9). We evaluated the ability of TGFα peptides to compete with [^{125}I]EGF binding to either A-431 cells or HFF. Peptide 1 partially inhibited [^{125}I]EGF binding to A-431 cells at >10^{-6} M (Figure 3). Peptides 2 and 3, however, exhibited an improved binding inhibition, with IC_{50}'s of 4 x 10^{-6} M and 4 x 10^{-7} M, respectively (i.e. ∿0.02 and 0.2% of the binding potency of EGF or TGFα).

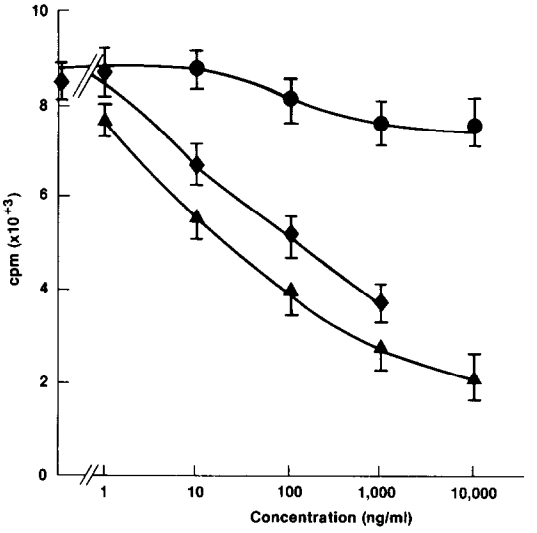

Figure 2. Specificity of anti-TGFα peptide 1-KLH antibodies. Plates were coated with BSA-peptide 1 conjugate, countercoated and incubated with [^{125}I]anti-peptide 1-KLH IgG (50,000 cpm/well) in the presence of EGF (●), TGFα (◆), or TGFα decapeptide 1 (▲).

Similar observations were made for the inhibition of EGF on HFF (Table 1). The receptor specificity of these interactions is illustrated by the inability of these peptides to inhibit either the binding or mitogenic effect of Endothelial Cell Growth Factor on HFF (not shown).

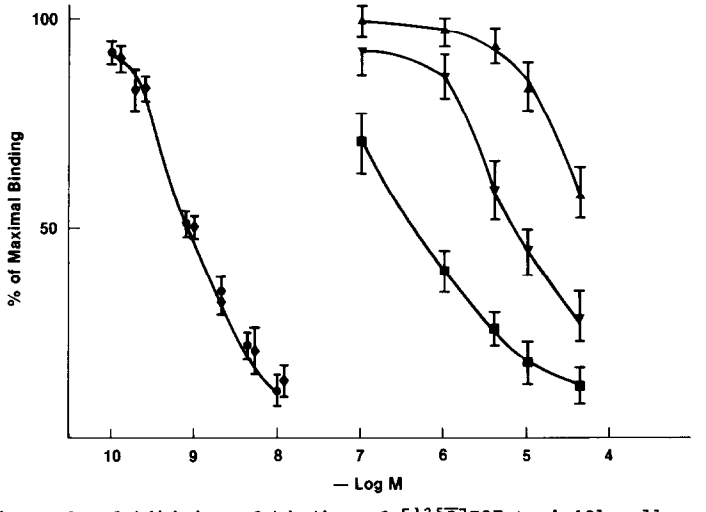

Figure 3. Inhibition of binding of [^{125}I]EGF to A-431 cells. A-431 cells were incubated with 1 nM [^{125}I]EGF in the presence of EGF (●), TGFα (◆), TGFα peptides 1 (▲), 2 (▼), or 3 (■), and the cell-associated radioactivity was determined.

TABLE 1

Biological Activities of Synthetic TGFα Fragments

	Binding[a] IC_{50} (M)		Inhibition of Mitogenesis[b] IC_{50} (M)	
	A-431	HFF	EGF	TGFα
Peptide 1	$8 \pm 2 \times 10^{-5}$	$6 \pm 2 \times 10^{-5}$	$>10^{-5}$	$>10^{-5}$
Peptide 2	$4 \pm 2 \times 10^{-6}$	$2 \pm 1 \times 10^{-6}$	$5 \pm 2 \times 10^{-6}$	$3 \pm 1 \times 10^{-6}$
Peptide 3	$4 \pm 1 \times 10^{-7}$	$3 \pm 1 \times 10^{-7}$	$6 \pm 2 \times 10^{-7}$	$5 \pm 3 \times 10^{-7}$

[a] IC_{50}'s derived from inhibition curves for the binding of 1 nM [^{125}I]EGF to cells. [b] IC_{50}'s are the concentrations that decrease by half the enhancement over control of [^{3}H]methyl-thymidine incorporation in quiescent HFF induced by 1 nM of EGF or TGFα.

None of the TGFα peptides possessed intrinsic mitogenic properties up to 10^{-5} M when incubated with quiescent HFF (data not shown). However, the TGFα peptides inhibited, in a concentration-dependent fashion, the induction of DNA synthesis in quiescent HFF by EGF (Figure 4). These antagonists were equally potent when TGFα was used as mitogen (Table 1). As seen for the binding potency, the antagonistic potency of the TGFα peptides was improved by capping of the amino- and carboxy-termini.

DISCUSSION

The binding of TGFα to EGF receptors on target cells results in the induction of all EGF-mediated biological effects studied. This interaction

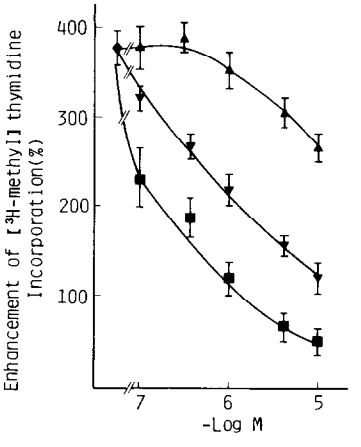

Figure 4. Inhibition of EGF induced DNA synthesis in HFF. Quiescent HFF were stimulated with 1 nM EGF in the presence of TGFα peptides 1 (▲), 2 (▼), or 3 (■) prior to a pulse with [^{3}H]methyl-thymidine and determination of the TCA precipitable radioactivity.

has been invoked as the basis for the autocrine growth control of tumor cells (12), wherein the secretion of TGFα would impair homeostatic growth regulation via the EGF receptor. Sequence (3) and gene cloning (6,7) data indicate a clear evolutionary relationship between TGFα and EGF in several species.

We have chosen predicted β-turn regions during our search for protein fragments with either immunogenic or active core properties (13). The β-turn is usually a prominent surface feature of proteins which frequently is implicated in receptor binding and enzyme recognition (14). The third disulfide loop of rTGFα (residues 34-43) was selected as a possible fragment with these properties because of its highly predicted β-turn and inherent rigidity as a small disulfide loop. Six of the ten residues in this loop region are common between TGFα and EGF.

Coupling of the cyclic decapeptide 1 to a KLH carrier resulted in the generation of rabbit antibodies which cross-reacted with native TGFα but not EGF. This lack of cross-reactivity is an important attribute in view of the high levels of EGF in normal serum. Lack of reactivity with EGF was also found in antibodies raised against the C-terminal 17-amino acid peptide (residues 34-53) of TGFα (15).

In addition, this decapeptide loop competes with EGF for binding to its receptors and is an antagonist of the induction of cellular proliferation by EGF. Capping of the N- and C-termini greatly improved both binding and antagonistic properties. This result may be due to protection of the molecule from enzymatic degradation or may be in accord with the hypothesis that ligand-receptor interaction is preceded by ligand-membrane interaction (16). This hypothesis would suggest that the more hydrophobic, capped analogs (2, 3) would have greater affinity for the plasma membrane and therefore greater receptor affinity. Further analog studies which support this hypothesis and which employ synthetic unnatural amino acids designed to favor phospholipid membrane interaction are under way.

The data reported here clearly demonstrate the importance of the third disulfide loop (residues 34-43) of TGFα for EGF receptor binding. Also demonstrated is a separation between the binding sequence and the region of TGFα necessary for the induction of mitogenesis, since these synthetic fragments are antagonists with no intrinsic agonistic activity.

Recent sequence searches revealed a homology between a vaccinia viral protein and both EGF and TGFα (17,18). It is noteworthy that the region of highest homology corresponds to the third disulfide loop of EGF and TGFα. Although an antigenic and receptor binding region of EGF was recently reported to be within residues 23-32 (19), it is quite likely that the homologous third disulfide loop of EGF contains an important binding region, by analogy to TGFα. Synthesis studies to confirm this point are under way.

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