Structure–function analysis of human transforming growth factor- α by site-directed mutagenesis

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Site-directed mutants of transforming growth factor- α (TGF- α) were expressed in an *Escherichia coli* outer membrane protein A (ompA) expression/secretion vector under the transcriptional control of the λ P_L promoter. TGF- α mutant proteins were isolated from cell pellets using alkaline extraction with 0.1 M-Tris (pH 10.5). The levels of protein expression of 23 TGF- α mutants were comparable with those of wild-type TGF- α , as determined by immunoblotting and radioimmunoassay. An analysis of biological activity using as assays radioreceptor binding competition and colony formation in soft agar showed that the following mutations destroy the activity of TGF- α : Gly-19 to Val, Val-33 to Pro and Gly-40 to Val. Mutations of Arg-42 to Lys, Leu-48 to Ala, Tyr-38 to Trp or Phe-17 to Tyr significantly decrease, but do not destroy, biological activity when compared with the wild-type. Mutations in 14 other residues did not significantly alter receptor binding or colony-forming activity. These studies suggest that two domains localized at the surface of TGF- α are important in receptor binding and colony-forming activity. Domain I involves amino acid residues which include Tyr-38 and Leu-48; domain II includes residues Phe-15, Phe-17 and Arg-42.

INTRODUCTION

Transforming growth factor- α (TGF- α) is a biologically active polypeptide with an M_r of 5616, and is derived from a 17000–19000- M_r glycosylated precursor (for reviews, see Burgess, 1989; Massague, 1990). TGF- α was first isolated from the culture media of oncogenically transformed cells (DeLarco & Todaro, 1978) and defined by its ability to induce proliferation of normal fibroblasts in soft agar, an effect which is now known to be dependent on the presence of another growth factor, TGF- β (Anzano *et al.*, 1983). Subsequently, TGF- α mRNA and/or protein has been found in a wide variety of cancer cell lines and tissues (Derynck *et al.*, 1987), including breast, colon and lung (Perroteau *et al.*, 1986; Dickson *et al.*, 1986; Hanuske *et al.*, 1987). Transfection of the TGF- α gene in fibroblasts induces phenotypic transformation and tumour formation in an animal model (Rosenthal *et al.*, 1986). This has led to TGF- α being implicated in the autocrine growth of many human cancers (Sporn & Roberts, 1985). In addition, normal cells and embryonic tissues synthesize TGF- α (Stromberg *et al.*, 1982; Samssoondar *et al.*, 1986; Coffey *et al.*, 1987), suggesting a physiological function and a key role in cellular proliferation. TGF- α belongs to a family of structurally related proteins characterized by their sequence similarity to epidermal growth factor (EGF). TGF- α has approx. 30% sequence similarity with EGF from various species (Marquardt *et al.*, 1984), and like EGF it binds to the EGF receptor (Todaro *et al.*, 1980) to elicit its biological effects. TGF- α also shows structural similarity with vaccinia (Brown *et al.*, 1985), Shope fibroma (Stroobant *et al.*, 1985), myxoma growth factor (Chang *et al.*, 1987) and amphiregulin (Shoyab *et al.*, 1989), as shown in Fig. 1.

In order to understand the role of TGF- α in neoplastic transformation, assess its physiological function and elucidate its various biological effects, it is necessary to investigate the structure–function relationship between TGF- α and EGF and

their receptor. The regions of TGF- α that are involved in receptor–ligand interactions are still unknown, despite the efforts of numerous investigators. Structural analysis of TGF- α has revealed that the six cysteine residues in the molecule define three looped regions required for biological activity. Any disruption of disulphide bridges renders the protein inactive, and attempts to evoke a response using individual synthetic loops or linear peptides have been unsuccessful (Komoriya *et al.*, 1984; Darlak *et al.*, 1988; DeFeo-Jones *et al.*, 1988). The structures of EGF and TGF- α are well documented (Montelione *et al.*, 1986; Campbell *et al.*, 1990) and we have found that the segment from Phe-15 to Asp-47 is conformationally well defined (Kline *et al.*, 1990). Using site-directed mutagenesis, studies from several laboratories have identified amino acid substitutions which result in the loss of biological activity; the most notable among these are L48A and R42K (Lazar *et al.*, 1988; DeFeo-Jones *et al.*, 1989). To date, no one has been able to separate binding activity from biological effect. Based on surface structures calculated from n.m.r. data, conserved amino acid sequence similarity and consensus sequences of the EGF family, we utilized site-directed mutagenesis to synthesize mutants of the TGF- α protein. This paper explores regions of the TGF- α molecule involved in receptor recognition and compares the biological activity of the wild-type to that of TGF- α mutants. We have recently developed a method for expressing high levels of biologically active human TGF- α using an outer membrane protein A (ompA) expression/secretion system in *Escherichia coli*, and have isolated and purified to homogeneity two forms of TGF- α : des-Val¹-Val²-TGF- α and Gly-Met-Asp-Pro-Met-TGF- α (G. Sathe, K. Johanson, J. Feild & M. Anzano, unpublished work). Both forms of TGF- α have biological activity equivalent to that of synthetic TGF- α and natural mouse EGF in various biological systems, including receptor binding competition, colony formation of NRK-49F cells in soft agar, thymidine incorporation in cell monolayers of A431 cells, tyrosine phosphorylation of the isolated membrane

Abbreviations used: TGF- α (β), transforming growth factor- α (β); EGF, epidermal growth factor; ompA, outer membrane protein A; LB, Luria broth medium; DMEM, Dulbecco's modified Eagle's medium; PEG 600, poly(ethylene glycol); IL-2, interleukin-2; r.i.a., radioimmunoassay; mutants are designated using the one-letter codes for amino acids, e.g. S11A defines a mutant in which alanine has been substituted for serine at position 11.

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		10			20			30			40			50							
Human TGF- α	VVSHFND	C	PDSHTQF	C	FH-	G	T	C	RFLVQEDKPA	C	V	C	HSG	Y	V	G	A	RC	EHAD	L	LA
Rat TGF- α	VVSHFNK	C	PDSHTQY	C	FH-	G	T	C	RFLVQEEKPA	C	V	C	HSG	Y	V	G	V	RC	EHAD	L	LA
Human EGF	NSDSE-	C	PLSHDGY	C	LHD	G	V	C	MYIEALDKYA	C	N	C	VVG	Y	I	G	E	RC	QYRD	L	KWWELR
Mouse EGF	NSYPG-	C	PSSYDGY	C	LNG	G	V	C	MHIESLDSYT	C	N	C	VIG	Y	S	G	D	RC	QTRD	L	RWWELR
Vaccinia GF	DIPAIRL	C	GPEGDGY	C	LH-	G	D	C	IHARDIDGMY	C	R	C	SHG	Y	T	G	I	RC	QHVY	L	VDYOR
Fibroma GF	VKHVKV	C	NHDYENY	C	LNN	G	T	C	FTI-ALDTPF	C	V	C	RIN	Y	E	G	S	RC	QFIN	L	VTY
Myxoma GF		C	NDDYKNY	C	LNN	G	T	C	FTV-ALDNPF	C	A	C	HIN	Y	V	G	S	RC	QFIN	L	ITIK
Amphiregulin	NP	C	NAEFQNF	C	IH-	G	E	C	KYIEHLEAVT	C	K	C	QQE	Y	F	G	E	RC	GEK		

Fig. 1. Comparison of amino acid sequences of TGF- α and related proteins

Sequence alignment of human TGF- α , rat TGF- α , human EGF, mouse EGF, vaccinia growth factor, fibroma growth factor (GF), myxoma GF and amphiregulin is shown (see the text for references). Amino acids are represented by one-letter symbols. Conserved residues are indicated with a box, and hyphens indicate gaps for maximum sequence alignment.

EGF receptor from A431 cells, and the ability to mobilize intracellular Ca^{2+} . We have expressed various site-directed and deletion mutants of TGF- α and have identified regions that are important for receptor binding and mitogenic activity. Our data and that of others (Campbell *et al.*, 1990) support the hypothesis that two domains of the TGF- α molecule are involved in maintaining biological activity: domain I, which consists of amino acids around Tyr-38 and Leu-48, and domain II, which consists of amino acids around Phe-15, Phe-17 and Arg-42. Either these regions are important in maintaining the structure of TGF- α , or they interact with the EGF receptor and elicit the biological action of the growth factor.

MATERIALS AND METHODS

Construction of the TGF- α plasmid

The sequence of mature human TGF- α , based on a human TGF- α cDNA (Derynck *et al.*, 1984), was assembled from single-stranded deoxyoligonucleotides. The synthetic TGF- α gene was subcloned between the *Bam*HI and *Sal*I-sites of M13mp18 (Messing & Vieira, 1982). Following confirmation of the correct TGF- α sequence, CJ236 *dut⁻ung⁻* cells (Kunkel, 1985) were infected with the M13mp18-TGF- α vector. Uracil-containing template DNA for mutagenesis was isolated from infected cultures using poly(ethylene glycol) (PEG 600) precipitation. Oligonucleotides (21-mers) containing single or multiple base-pair mismatches were synthesized using phosphoramidite chemistry (Sinha *et al.*, 1984) in an Applied Biosystems Model 380A DNA synthesizer (Foster City, CA, U.S.A.).

Mutagenesis of TGF- α

Mutagenesis was performed using the method of Kunkel (1985) with minor modifications (Geisseloder *et al.*, 1987). Briefly, 3 μM of the phosphorylated oligonucleotide primer was annealed to 0.1 μM of uracil-containing template DNA in a final volume of 10 μl of 20 mM-Tris (pH 7.4), 2 mM-MgCl₂ and 50 mM-NaCl for 5 min at 70 °C, and then cooled to less than 30 °C over a period of 40 min. Following annealing, 3 units of T₄ DNA ligase, 1 unit of T₄ DNA polymerase, 0.4 mM each of dATP, dCTP, dTTP and dGTP, 0.75 mM-ATP and 20 mM-dithiothreitol were combined in a final volume of 15 μl . The mixture was incubated on ice for 5 min, at room temperature for 5 min and then at 37 °C for 90 min. The reaction was brought to 100 μl with 10 mM-Tris/HCl, pH 7.4, and 10 μl of the mixture was added to 300 μl of competent JM109 cells for 1 h on ice. To initiate plaque formation, the reaction mixture was added to 300 μl of exponential-phase host

cells and plated on Luria broth (LB) plates containing 0.8% agar. Three plaques were selected at random and purified. Miniprep phage DNA was isolated and sequenced using the dideoxy chain termination method (Sanger *et al.* 1977). We selected a single plaque for each mutant whose sequence was confirmed by dideoxy sequencing, and stored it as a phage stock. Mutagenic efficiency was approx. 60%.

Construction of TGF- α mutant expression vector

Following mutagenesis, double-stranded replicative-form DNA was isolated from 250 ml cultures of infected JM109 cells using the alkaline lysis method (Birnboim & Doly, 1979). The 150 bp *Nco*I-*Sal*I fragment containing the mutated TGF- α was isolated by restriction enzyme digestion of DNA and preparative agarose gel electrophoresis. This fragment was subcloned between the *Nco*I and *Sal*I sites of an ompA expression plasmid, pGS-ompA (G. Sathe, K. Johanson, I. Feild & M. Anzano, unpublished work), such that the TGF- α mutant sequence was adjacent to and in frame with the ompA signal sequence. These constructs were transformed into competent host *E. coli* AR-120 cells.

Expression and isolation of mutant TGF- α

An overnight culture of *E. coli* AR-120 cells harbouring the pGS-ompA-TGF- α mutant plasmid was grown in 1 ml of LB medium containing 25 μg of ampicillin/ml, and was then diluted 100-fold with LB medium and grown at 37 °C in a 250 rev./min rotary shaker. When the A_{600} of the culture reached 0.6–0.8, expression of mutant TGF- α was induced by adding nalidixic acid to a final concentration of 60 μg /ml. Growth was continued for an additional 5 h. The cell culture was centrifuged for 10 min at 3000 *g*. The supernatant was saved for determination of TGF- α activity. The cell pellet was frozen quickly, thawed and resuspended in 2 ml of 50 mM-Tris (pH 7.4), 100 mM-NaCl, 5 mM-EDTA, 1 μg of pepstatin A/ml, 1 mM-phenylmethanesulphonyl fluoride and 0.5 mg of lysozyme/ml. The cell suspension was disrupted by vortex-mixing for 15 min at 4 °C, followed by homogenization for 30 s using a Polytron homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.). After centrifugation at 10000 *g* for 15 min, the supernatant (S_1) was saved and the pellet was resuspended in 2 ml of 0.1 M-Tris (pH 10.5). Brief sonication was used to assist in resuspension. The resuspended pellet was centrifuged at 15000 *g* for 20 min and the resulting supernatant (S_2) was used for assays. More than 90% of extractable TGF- α was localized in this fraction. A variety of agents including high-salt buffers, chaotropic salts, detergents and buffers at pH values

ranging from 4 to 11 were compared for their ability to liberate biologically active TGF- α . Of the agents tested, only alkaline Tris buffer proved effective (results not shown).

Partial purification of TGF- α

S₂ supernatant fraction (1 ml) from wild-type and mutant TGF- α cultures was made to 0.1% trifluoroacetic acid and passed through a Sep-Pak C₁₈ cartridge (Waters, Milford, MA, U.S.A.) pre-equilibrated in 0.1% trifluoroacetic acid. Samples were eluted with 2 ml of 60% acetonitrile in 0.1% trifluoroacetic acid. The eluate was lyophilized, dissolved in 1 ml of phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl, pH 7.2) and assayed for ¹²⁵I-TGF- α radioreceptor binding and colony formation in soft agar, as described below.

Western blot analysis

Wild-type and mutant TGF- α culture S₂ supernatants were run in 15% acrylamide SDS/PAGE gels under reducing conditions according to Laemmli (1970). Samples were transferred to nitrocellulose filters as described by Towbin *et al.* (1979). The primary antibody was a rabbit polyclonal antibody which recognizes a peptide representing residues 1–15 of human TGF- α . ¹²⁵I-Protein A and autoradiography were used to assess the level of TGF- α expression.

Radioimmunoassay

Quantification of immunoreactive TGF- α was determined by radioimmunoassay (r.i.a.) with IgG fractions of polyclonal goat antibody against synthetic human TGF- α (Biotope, Bellevue, WA, U.S.A.). Samples of diluted synthetic TGF- α standards (100 μ l) were added with 100 μ l of a 1:500 dilution of goat polyclonal antibody and ¹²⁵I-TGF- α (50 000 c.p.m./100 μ l) in a final volume of 0.5 ml of 10 mM-potassium phosphate buffer, pH 7.2, containing 0.01% Triton X-100. Following overnight incubation at 4 °C, immune complexes were precipitated by adding 50 μ l of bovine γ -globulin (50 mg/ml) and 500 μ l of 30% PEG 600, and incubating on ice for 30 min. Samples were centrifuged at 3000 *g* for 10 min, and precipitates were washed with 15% PEG and counted for radioactivity in a Beckman Gamma 5500 γ -radiation counter.

Receptor binding competition

Synthetic human TGF- α for receptor binding and r.i.a. was iodinated using the chloramine-T procedure (Hunter & Greenwood, 1962). The radioreceptor binding assay was performed in fixed A431 cells plated in 96-well plates as described by DeLarco & Todaro (1980).

Colony formation in soft agar

Wild-type and mutant TGF- α species that had been partially purified using Sep-Pak cartridges (Millipore, Bedford, MA, U.S.A.) were assayed for their ability to induce colony formation in soft agar as described by Anzano *et al.* (1983). Lyophilized samples were dissolved in 200 μ l of 4 mM-HCl containing 1 mg of BSA/ml as carrier. Activity was measured by counting the colonies of NRK-49F indicator cells formed in 0.4% agar in the presence of 80 pM-TGF- β using an Omnicon image analysis system (Dynatech Lab., Chantilly, VA, U.S.A.).

RESULTS

Expression of TGF- α mutants

The choice of TGF- α mutants was based on similarities between the TGF- α /EGF polypeptide family (Fig. 1) and information provided by the protein structure calculated from

two-dimensional n.m.r. data (Kline *et al.*, 1990). Several amino acid residues located at the surface of TGF- α are potential candidates for involvement in the interaction with the EGF receptor.

The TGF- α gene in the expression vector pGS-ompA-TGF- α was replaced with the TGF- α mutant gene and the mutated protein was expressed in *E. coli* lysogen AR-120 cells. Expression was induced by treatment with nalidixic acid (Shatzman & Rosenberg, 1987). When the absorbance at 600 nm reached 0.6–0.8, cells were centrifuged and fractionated using a modified osmotic lysis procedure. As with wild-type TGF- α , the majority of the immunoreactive material (> 70%) was localized in the S₂ supernatant fraction (result not shown). The size and identification of TGF- α mutants, as well as the level of expression, was initially analysed by SDS/PAGE and Western blotting using A-loop (residues 1–15)-specific rabbit primary antibody and detected using ¹²⁵I-Protein A and autoradiography. Our results show that an immunoreactive band with an *M_r* of 5000–6000, comparable with synthetic TGF- α , was observed for wild-type and mutant TGF- α , but was not seen in an ompA control vector without the TGF- α gene. The level of expression of 23 TGF- α mutants was comparable with expression of the wild-type (results not shown). To confirm this initial assessment of protein expression, the amount of immunoreactive TGF- α in each extract was quantified by r.i.a. A goat polyclonal antibody against synthetic human TGF- α -(1–50) was used to detect TGF- α by r.i.a. Results similar to those obtained by immunoblotting were seen, i.e. comparable levels of expression of both wild-type and mutant proteins. Fig. 2(a) depicts a representative r.i.a. showing approximately equivalent levels of expression of the wild-type and five mutant proteins. Thus these two antibodies are unable to distinguish between wild-type and mutant TGF- α forms.

Fig. 2. R.i.a. and radioreceptor competition of wild-type and mutant TGF- α

Various concentrations of wild-type and mutant TGF- α were assayed in an r.i.a. (a) and by radioreceptor binding competition (b). Mutants used: V1V (○), H18E (△), P17Y (▲), L48A (●), Y38W (■) and G19V (□).

Table 1. Levels of extractable protein, immunoreactive TGF- α and active TGF- α isolated from wild-type and mutant clones

Immunoreactive TGF- α , quantified by r.i.a., detects total TGF- α (active plus inactive), and radioreceptor binding detects active TGF- α only. Values are means $\pm$ S.E.M. of at least three separate experiments. ND, not detected; NA, not active. In some experiments, values > 1000 could not be extrapolated from the dose-response curve. V1V is the TGF- α mutant control.

Mutation	Protein concn. (μ g/ml)	Immunoreactive TGF- α (μ M)	Active TGF- α (μ M)	Radioreceptor binding ED ₅₀ values (nM)	Active TGF- α (%)
Wild-type	1.1 $\pm$ 0.2	7.7 $\pm$ 2.1	2.3 $\pm$ 0.4	7.6 $\pm$ 3.2	30
V1V	2.2 $\pm$ 0.4	7.6 $\pm$ 3.1	1.9 $\pm$ 1.4	5.1 $\pm$ 5.1	25
S11A	2.2 $\pm$ 0.7	3.2 $\pm$ 0.3	0.5 $\pm$ 0.5	24 $\pm$ 20	15
H12P	1.4 $\pm$ 0.3	8.6 $\pm$ 3.2	0.7 $\pm$ 0.1	11.4 $\pm$ 7.0	8
F17Y	0.8 $\pm$ 0.1	4.7 $\pm$ 4.2	0.6 $\pm$ 0.2	93 $\pm$ 22	13
H18A	2.0 $\pm$ 0.3	4.2 $\pm$ 3.2	0.6 $\pm$ 0.7	25 $\pm$ 17	13
H18E	1.6 $\pm$ 0.3	5.7 $\pm$ 2.8	1.1 $\pm$ 0.9	9.1 $\pm$ 6.5	19
G19V	1.5 $\pm$ 0.5	5.9 $\pm$ 2.2	ND	> 1000	NA
L24N	2.5 $\pm$ 0.6	7.5 $\pm$ 1.4	0.7 $\pm$ 0.6	35 $\pm$ 13	9
E27A	1.3 $\pm$ 0.2	2.0 $\pm$ 0.5	0.6 $\pm$ 0.4	4.5 $\pm$ 2.5	29
K29A	1.3 $\pm$ 0.1	7.9 $\pm$ 2.3	1.3 $\pm$ 1.4	9.5 $\pm$ 6.3	17
K29Q	1.7 $\pm$ 0.2	9.4 $\pm$ 0.9	2.1 $\pm$ 0.7	1.4 $\pm$ 0.5	22
V33P	1.6 $\pm$ 0.2	9.8 $\pm$ 2.4	ND	> 1000	NA
Y38W	2.9 $\pm$ 0.9	3.6 $\pm$ 1.0	0.016 $\pm$ 0.006	160 $\pm$ 46	0.4
G40V	2.1 $\pm$ 0.8	12.0 $\pm$ 3.2	ND	> 1000	NA
A41S	1.0 $\pm$ 0.1	3.0 $\pm$ 1.8	1.5 $\pm$ 0.5	16.0 $\pm$ 2.0	48
R42K	2.5 $\pm$ 0.6	3.2 $\pm$ 1.3	0.007 $\pm$ 0.001	1680 $\pm$ 722	0.2
E44A	0.8 $\pm$ 0.1	3.9 $\pm$ 0.7	0.29 $\pm$ 0.01	15.3 $\pm$ 4.5	7
H45A	1.6 $\pm$ 0.6	7.9 $\pm$ 0.9	1.2 $\pm$ 0.2	9.6 $\pm$ 2.4	15
A46S	0.7 $\pm$ 0.2	3.8 $\pm$ 3.7	0.63 $\pm$ 0.09	25 $\pm$ 21	16
D47A	1.0 $\pm$ 0.3	5.1 $\pm$ 2.2	1.4 $\pm$ 0.2	11.6 $\pm$ 3.3	28
L48A	2.4 $\pm$ 0.4	1.7 $\pm$ 1.0	0.005 $\pm$ 0.004	4667 $\pm$ 1604	0.3
E27Q/D28Q/K29Q	1.6 $\pm$ 0.1	9.0 $\pm$ 1.0	2.4 $\pm$ 1.6	7.0 $\pm$ 4.4	27
L24N/A31N/V33N	1.2 $\pm$ 0.2	3.3 $\pm$ 3.0	ND	> 1000	NA

Fig. 3. Colony-forming activity of wild-type and mutant TGF- α samples partially purified using Sep-Pak cartridges

Wild-type ($\square$) and mutant TGF- α forms were assayed for their ability to induce colony formation in soft agar using NRK-49F indicator cells in the presence of 80 pM-TGF- β . Mutants used: V1V ($\circ$), Y38W ($\blacksquare$), H18E ($\triangle$), P17Y ($\blacktriangle$), L48A ($\bullet$) and R42K ($+$).

Biological activity

In order to quantify the level of expression and calculate the amount of biologically active TGF- α , various concentrations of S₂ supernatant from wild-type and mutant extracts were analysed by r.i.a., which measures total TGF- α , and radioreceptor binding, which detects active and refolded TGF- α . Fig. 2 shows the r.i.a. and radioreceptor binding competition profiles of wild-type TGF- α and of several representative mutants. The ED₅₀ value in this r.i.a. was 2.1 nM for the wild-type control and 0.7–6.2 nM for five TGF- α mutants (0.3–3 times the wild-type value). In the radioreceptor binding competition assay, the ED₅₀ value for the

wild-type was 2.0 nM and for four mutant TGF- α proteins it ranged from 8 nM to > 1000 nM (4–500 times the wild-type value). No detectable activity was found associated with the G19V mutant. The control plasmid vector without the wild-type or mutant TGF- α gene insert did not secrete any detectable TGF- α -like protein, as determined by r.i.a. and radioreceptor competition. We have observed that the expression of a given TGF- α mutant from various extracts isolated at different times can vary up to 5-fold; it is therefore difficult to characterize mutants with partial activity. However, significant differences of two to three orders of magnitude are indicative of an inactive mutant protein.

The biological activity of our TGF- α mutant proteins can be classified into three groups: (1) wild-type-like, (2) significantly lower than wild-type by 1–3 orders of magnitude, and (3) completely inactive. Table 1 summarizes the protein, immunoreactive TGF- α and active TGF- α concentrations, based on the radioreceptor binding assay and ED₅₀ values in the radioreceptor binding competition assay with S₂ supernatants of 23 mutants. Values are averages of at least three separate experiments. Protein concentrations of various samples ranged from 0.77 to 2.9 μ g/ml. The mean immunoreactive TGF- α concentration for the wild-type was 7.7 μ M and for mutants it ranged from 1.7 μ M for L48A to 12.0 μ M for G40V. Active TGF- α concentrations based on the radioreceptor assay were as follows: 2.3 μ M for wild-type (30% active), undetectable levels for G19V, V33P and G40V, 0.005–0.028 μ M for L48A, R42K and Y38W, and 0.29–2.4 μ M for 16 other mutants. The ED₅₀ values in the radioreceptor binding assay were: 7.6 nM for wild-type, > 1000 nM for G19V, V33P, G40V, L48A, R42K and the triple mutant L24N/A31N/V33N, 160 nM for Y38W, 93 nM for F17Y, and 1.4–35 nM for 15 other mutants.

In order to correlate receptor binding data with mitogenesis,

Table 2. Analysis of partially purified wild-type and mutant TGF- α forms

TGF- α extracts were partially purified on a Waters Sep-Pak C₁₈ cartridge, eluted using 60 % acetonitrile in 0.1 % trifluoroacetic acid, lyophilized and analysed in receptor binding and mitogenesis assays, as discussed in the Materials and methods section. Immunoreactive TGF- α was determined by r.i.a. Values are means of duplicate samples; values > 1000 could not be extrapolated from the dose-response curve.

Mutation	Immunoreactive TGF- α (μ M)	ED ₅₀ values (nm)	
		Radioreceptor binding	Soft agar assay
Wild-type	7.3	4.0	1.4
V1V	2.0	9.0	0.3
S11A	11.7	18.0	3.2
H12P	7.5	11.0	3.0
F17Y	4.5	90.0	1.8
H18E	6.3	5.5	0.6
H18A	7.6	9.0	1.4
G19V	10.8	> 1000	> 1000
L24N	9.8	16.0	5.3
E27A	8.2	4.5	2.0
K29A	7.8	3.0	2.5
K29Q	7.7	1.8	0.1
V33P	12.4	> 1000	300
Y38W	1.3	> 1000	> 1000
G40V	11.8	> 1000	> 1000
A41S	2.9	12.0	0.2
R42K	2.2	> 1000	> 1000
E44A	1.1	14.0	0.8
H45A	5.4	8.0	1.2
A46S	0.8	6.0	2.5
D47A	12.5	25.0	2.5
L48A	1.9	> 1000	> 1000
L24N/A31N/V33N	0.3	> 1000	> 1000

S₂ supernatants were assayed for colony-forming activity in soft agar. The ability to induce colony formation in soft agar of NRK-49F indicator cells assayed in the presence of an optimum TGF- β concentration (80 pM) and 10 % fetal bovine serum is a response specific to the TGF- α /EGF family of polypeptide growth factors. Crude S₂ supernatants are toxic to NRK-49F indicator cells; thus it was necessary to partially purify S₂ fractions using adsorption of TGF- α on to Sep-Pak cartridges, followed by elution with 60 % acetonitrile containing 0.1 % trifluoroacetic acid. Fig. 3 shows the dose-response curves of representative mutants and of wild-type TGF- α . The ED₅₀ value for the wild-type and the V1V (control), H18E and F17Y mutants were 2.8, 0.3, 0.5 and 2 nm respectively; however, the two mutants R42K and L48A had ED₅₀ values > 1000 nm. Table 2 summarizes the immunoreactive TGF- α concentrations and the ED₅₀ values for radioreceptor binding and colony-forming activity in soft agar of partially purified wild-type and mutant TGF- α . The immunoreactive TGF- α concentrations following Sep-Pak cartridge purification ranged from 0.3 to 12.5 μ M. The ED₅₀ values in a radioreceptor binding assay were as follows: 4 nm for wild-type, > 1000 nm for Y38W, L48A, R42K, G19V, V33P, G40V and the triple mutant L24N/A31N/V33N, 90 nm for F17Y, and 1.8–25 nm for 15 other mutants. These values agree with the ED₅₀ values obtained with the crude S₂ supernatants (Table 1). The ED₅₀ values for colony-forming activity in soft agar were: 1.4 nm for wild-type, > 1000 nm for Y38W, L48A, R42K, G19V, G40V and the triple mutant L24N/A31N/V33N, 300 nm for V33P, and 0.1–5.3 nm for 15 other mutants. In general, mutants with high ED₅₀ values in the radioreceptor binding competition assay also had correspond-

ingly high ED₅₀ values in the colony-forming assay in soft agar, suggesting that mitogenic effects are coupled to receptor binding. Only the F17Y mutant showed a notable difference in activity between these two assays, with a receptor binding ED₅₀ of 90 nm and an ED₅₀ in the mitogenic assay of 1.8 nm. The significance, if any, of this difference remains to be determined.

DISCUSSION

We have reported the structure-activity relationship of the TGF- α molecule using site-directed mutagenesis. TGF- α mutants were expressed in *E. coli* using an ompA secretion system under the transcriptional control of the λ P_L promoter. Although two forms of TGF- α , des-Val¹-Val²-TGF- α and Gly-Met-Asp-Pro-Met-TGF- α , are produced in approximately equal amounts from wild-type constructs, biological characterization in five assays (receptor binding competition, colony-forming activity in soft agar, thymidine incorporation, intracellular Ca²⁺ mobilization and phosphorylation of the membrane EGF receptor) show that these recombinant TGF- α forms are equipotent to synthetic human TGF- α and natural mouse EGF (G. Sathe, J. Feild, D. Rieman & M. Anzano, unpublished work). Unlike a yeast expression system, which secretes low levels of mutant TGF- α compared with the wild-type (Lazar *et al.*, 1988), our expression system showed comparable levels of immunoreactive wild-type and mutant forms (Fig. 2 and Table 1). In addition, our alkaline extraction procedure with 0.1 M-Tris at pH 10.5 allows the refolding of TGF- α , as shown by its ability to bind to the EGF receptor (Fig. 2b; Tables 1 and 2) and to induce colony formation in soft agar (Fig. 3 and Table 2), with the degree of refolding ranging from 7 to 48 % (Table 1), which is comparable with the yeast expression system. Although wild-type and mutant TGF- α forms have been isolated and purified from inclusion bodies (DeFeo-Jones *et al.*, 1988), this method requires extraction and solubilization of TGF- α using denaturing agents and requires a refolding step, often resulting in low yields.

The localization of the majority of the immunoreactive TGF- α in the insoluble cellular/membrane fraction was unexpected, since our construct contains the ompA signal sequence which should facilitate transport and secretion of TGF- α into the periplasmic space. Using this type of expression system it has been demonstrated that β -lactamase and staphylococcal nuclease A are efficiently expressed and transported to the periplasmic space (Ghrayeb *et al.*, 1984; Takahara *et al.*, 1985). Our result on the localization of TGF- α in the cellular insoluble fraction resembles the data of Libby *et al.* (1987), who reported that granulocyte/macrophage colony-stimulating factor expressed from an ompA *E. coli* secretion vector was found associated exclusively with the membrane fraction. van Kimmenade *et al.* (1989) reported on the expression of mouse and human interleukin-2 (IL-2) using an ompA expression system. Even though both polypeptide sequences were fused to the same ompA bacterial signal peptide, only mouse IL-2 was translocated to the periplasm, whereas human IL-2 was localized mainly in the cytoplasm in the uncleaved form. Thus, although mouse and human IL-2 have very similar sequences, structural features unique to mouse IL-2 may contribute to its translocation to the periplasm. Another factor which may contribute to the insolubility of TGF- α in our system is the possibility of aggregation, which often occurs with high-level expression of recombinant proteins. In our expression system, protein analysis revealed cleavage of TGF- α from the signal peptide, yet attempts to release TGF- α from the periplasmic space by conventional osmotic shock techniques were not successful. Following osmotic shock, extraction of insoluble material with 0.1 M-NaCl did not release any TGF- α . However, after screening a variety of agents,

Fig. 4. Schematic representation of the structure of TGF- α and effects of mutation on biological activity

Conserved residues in the TGF- α /EGF polypeptide family are indicated by $\square$ and partially conserved residues by $\blacksquare$. Solid lines indicate disulphide bridges. Mutants represented by circle symbols behave like the wild-type, and those designated with triangles have lost biological activity in both the receptor-binding and colony-forming assays by more than a factor of 10 compared with the wild-type.

we found that immunoreactive TGF- α could be released from the insoluble fraction with 0.1 M-Tris, pH 10.5. Typically, one-third of this material was biologically active, as demonstrated by a radioreceptor binding assay. Our data suggest that the environment of the cellular insoluble compartment at pH 10.5 allows for refolding of TGF- α . This study supports the concept that the cellular localization of a protein fused to the ompA signal sequence depends on the nature of the protein being expressed.

Our studies show that mutants G19V, V33P, Y38W, G40V, R42K, L48A and the triple mutant L24N/A31N/V33N had little or no activity in the receptor binding assay. These results are summarized in Fig. 4. The colony-forming activity of the same mutants in soft agar correlated quite well with results from the receptor binding assay, indicating that the domain(s) involved in ligand-receptor interaction are probably coupled with the mitogenic response. We have observed that site-directed mutagenesis primarily detects mutations that destroy activity, but it has difficulty detecting mutants with partial activity, because of experimental variations inherent to the system. For example, the amount of active protein obtained from three different inductions of the same isolate of a given TGF- α mutant can vary up to 5-fold, probably due to variations in refolding, which range from 7 to 48%, and in efficiency of extraction. In spite of these limitations we have identified several mutants in which ED_{50} values can differ by two to three orders of magnitude compared with the wild-type activity (Table 1). Whether the loss of biological activity is the result of an alteration of the tertiary structure and the conformation of the TGF- α molecule, or whether it is due to the involvement of a particular residue in interacting with its receptor, requires further study. Since we were able to isolate to homogeneity 70 mg of des-Val-Val-TGF- α and 70 mg of Gly-Met-Asp-Pro-Met-TGF- α from 200 g of cells, a structural analysis of TGF- α mutants is feasible.

We have shown for the first time that the G19V, V33P and G40V mutations result in a loss of biological activity. Gly-19 and Gly-40 are well conserved in the TGF- α /EGF polypeptide family, and Val-33 is in the hinge region separating the B-loop/ β -sheet from the C-loop and C-tail region of TGF- α (Kline *et al.*, 1990). Consistent with previous reports, we have confirmed that the L48A and R42K mutations result in a decrease of activity (Lazar *et al.*, 1988; DeFeo-Jones *et al.*, 1989). However, our results with the Y38W mutation differ from those of Lazar *et al.* (1988), which indicated superagonist activity for this mutant. Whether the difference is due to the expression system remains to be investigated. We have found up to 5-fold differences in activity in the same mutant isolated at different times; therefore 2-fold stimulation, as reported for the Y38W mutant, is difficult to interpret.

Earlier studies on the structure-function activity of TGF- α and EGF strongly suggested that discontinuous regions of TGF- α are involved in receptor binding. These observations include: (1) alteration of any of the three disulphide bonds by mutagenesis or treatment with reducing agents (DeFeo-Jones *et al.*, 1988); (2) synthesis of various fragments of TGF- α representing the hydrophilic regions of the molecule (Heath & Merrifield, 1986) or various loops of TGF- α (DeFeo-Jones *et al.*, 1988) failed to inhibit binding of TGF- α to its receptor; and (3) site-directed mutants in specific regions of TGF- α , such as Tyr-38 to Ala (Lazar *et al.*, 1988), Arg-42 to Lys (DeFeo-Jones *et al.*, 1989) and Leu-48 to Ile (Lazar *et al.*, 1988) in the C-loop and C-tail abolished receptor binding and mitogenic activity. Some of these studies suggest that gross alteration of the TGF- α structure to study active domains is not productive, since often there may be no simple way to determine if structural integrity is maintained, and that further investigation on structure-function analysis of TGF- α requires more subtle changes to the molecule.

Correlation of our data and that of others, using site-directed mutants with structural information available for TGF- α and EGF obtained from two-dimensional n.m.r. methods, supports the prevailing idea that discontinuous regions of the TGF- α molecule are required for receptor binding and signal transduction, and that tertiary conformation is necessary for a productive interaction. Assuming that some single mutation does not grossly affect the conformation of TGF- α , it is possible that a mutation which results in a loss of biological activity may affect the interaction of the receptor or may modulate the binding process. We propose that at least two domains (I and II) of TGF- α are critical in binding to the EGF receptor. The first region (domain I) involves the C-loop and C-terminal end of the protein, particularly residues Leu-48 and to some extent Tyr-38. As suggested by Campbell *et al.* (1990) and the calculated structure of TGF- α (Kline *et al.*, 1990), Tyr-38 is situated at a crucial position between two turns, indicating a structural role. If Tyr-38 is changed to Trp (Table 1), Ala (DeFeo *et al.*, 1988; Lazar *et al.*, 1988) or Ser (Lazar *et al.*, 1988), or is deleted (Lazar *et al.*, 1988), activity is lost or decreased. Although Lazar *et al.* (1988) reported superagonist activity of the Y38W mutant (280% of wild-type activity), differences in the expression system might explain this discrepancy. A slight alteration in the side chain of TGF- α Leu-48, e.g. to Ala (Table 1), Ile or Met (Lazar *et al.*, 1988), or of the corresponding Leu-47 residue in EGF to His (Engler *et al.*, 1988) results in loss of activity. Structural analysis by two-dimensional n.m.r. of TGF- α L48A indicates that this substitution does not have any effect on protein structure (T. P. Kline & K. Kopple, unpublished work), suggesting that Leu-48 is a functional residue in the ligand-receptor interaction. Although the location of residue Leu-48 is ill-defined in solution, it is possible that, upon receptor binding, residues Leu-48 and Tyr-38 are positioned so as to interact to produce a binding site(s).

Fig. 5. Stereoview of the structure of TGF- α

Shown is the backbone as well as the positions of the following residues: Phe-15, Phe-17, Arg-42, Tyr-38 and Leu-48 [adapted from Kline *et al.* (1990)].

We propose that the second stage (domain II) that is critical for biological activity is composed of surface amino acid residues Phe-15, Phe-17 and Arg-42. Although these residues are not sequential, the calculated structure of TGF- α shows them to be grouped together. A conservative change of Arg-42 to Lys (Table 1; DeFeo-Jones *et al.*, 1989), or mutation to Ala or Leu, decrease activity. This particular residue is conserved throughout the TGF- α /EGF family (Fig. 1). Since the Arg-42 side chain is not important in the overall structure (Kline *et al.*, 1990) its role could be functional. Mutation of Phe-15 to Ala results in loss of activity (DeFeo-Jones *et al.*, 1988). This residue is partially conserved in the EGF family, existing as tyrosine in most of the proteins. A Phe residue at position 17 is unique to TGF- α ; a semi-conservative substitution to Leu occurs in most of the EGF family. Mutation of Phe-17 to Tyr resulted in a 10-fold decrease in receptor binding activity, but did not significantly alter mitogenic activity. This is the only mutant that we have identified where receptor binding activity did not correlate with mitogenic activity. The significance of this observation is unknown. The decrease in binding activity does indicate a possible role in receptor interaction. Although the mutants in domains I and II are separated in the primary sequence, two-dimensional n.m.r. data indicate that these amino acids define a patch of exposed surface residues of folded TGF- α (Fig. 5). The importance of the C-loop region for receptor binding is further supported by the observation that various deletions of C-loop Gly-37, Tyr-38 and Ala-41 result in a loss of activity, and by the fact that neutralizing antibodies to TGF- α specifically recognize the C-loop/C-tail peptide (K. Esser, personal communication).

Other mutations that destroy TGF- α activity are Gly-19 to Val, Gly-40 to Val and Val-33 to Pro. Glycine residues usually play a structural role in proteins, and these three residues most likely perform structural roles in the TGF- α molecule. Gly-19 is located at the end of the major β -sheet and is well defined in the calculated structure (Kline *et al.*, 1990). If an R group is substituted for the appropriate glycine C_{α} proton, the backbone must twist in order to accommodate the side chain, possibly disrupting the structure of the active site. A similar distortion is possible following mutation of Gly-40 to Ala, which is part of the turn involving residues Arg-42 and Glu-44. Additionally, the

structural importance of glycine residues 19 and 40 is further indicated in that these residues are well conserved in the TGF- α /EGF family of polypeptides (Fig. 1). The loss of activity due to a Val-83 to Pro mutation is probably a result of structural disruption. Values for ϕ (backbone torsional angle $C_{i-1}-N_i-C_i-C_{i+1}$) of residue 33 in the calculated structures range from -95° to -135° , and are consistent with a Pro ϕ value of -60° .

We have observed that a Ser-11 to Ala mutation in the flexible N-terminal region, as well as mutations in the β -sheet B-loop (Leu-24 $\rightarrow$ Asn, Glu-27 $\rightarrow$ Ala, Lys-29 $\rightarrow$ Gln and Lys-29 $\rightarrow$ Ala), do not affect activity (Table 1 and Fig. 4), suggesting that the N-terminus and β -sheet region are not critical in receptor binding. Antibodies recognizing epitopes in β -sheet/B-loop regions do not alter activity (Hoeprich *et al.*, 1989). Although Komoriya *et al.* (1984) reported that the linear and cyclic forms of B-loop in EGF (residues 20–31) compete with ^{125}I -EGF in receptor binding and stimulate cell proliferation in cell culture, the activity was only 0.003% of that of EGF.

In summary, we believe that residues Phe-15, Phe-17, Arg-42, Leu-48 and possibly Tyr-38 are involved in binding of TGF- α to its receptor. We recognize that it is possible in any mutagenesis study that binding of a mutated ligand may be altered because of a global effect on protein conformation rather than a direct effect on the binding of ligand to its receptor. Mutations G19V, G40V and V33P probably represent alterations in protein structure leading to disruption of binding. Further experiments are needed to demonstrate that inactive mutants do not disrupt the conformation, tertiary structure and folding of the molecule. Since the ompA TGF- α expression system is capable of producing large quantities of TGF- α mutants, structural analyses such as two-dimensional n.m.r. and X-ray crystallography studies are possible. Moreover, the interaction of antibodies of TGF- α with various mutants, as well as epitope mapping, will provide additional information to validate our two-site hypothesis of the interaction of TGF- α with the EGF receptor.

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