Antigenic Properties of the Second Loop of Transforming Growth Factor α by Synthetic Peptides

Josephine Bahr and Björn Lüning

Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-10691 Stockholm, Sweden

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We describe synthesis and immunological properties of peptides constructed based on the second loop of TGF-α. The peptides were synthesized manually using a standard solid-phase Boc protocol. Sulfhydryl groups were protected as acetamidomethyl derivatives during the synthesis and the disulfide bridges were formed at high dilution by oxidation with iodine in the presence of glutathione. In ELISA assays antibodies to the peptides were inhibited by the native hormone to various extents. In addition, two peptides reacted well with a polyclonal antibody to TGF-α, but this reaction was only slightly inhibited by TGF-α in solution.

Transforming growth factor α (TGF-α) is a 50 amino acid polypeptide that exhibits 35 % sequence homology with epidermal growth factor (EGF), including the conservation of three disulfide bonds.[1,2] Both polypeptides compete for binding to the EGF receptor in a nearly indistinguishable manner,[3] inducing a receptor-associated tyrosine kinase activity in the cytoplasm[4] leading to mitosis.

Soluble TGF-α is found in a wide variety of tumor cell lines and virally transferred cells[1,5,6] where it interacts with the EGF-receptor via an autocrine mechanism,[7] thereby sustaining the transformed character of the cell population. TGF-α has also been isolated in the urine of cancer patients.[8]

Two-dimensional nuclear magnetic resonance (NMR) studies which have appeared during the course of this work show that EGF and TGF-α have very similar structures in solution.[9–11] (Fig. 1). The second loop of EGF has the same antiparallel β-sheet structure, including a 'hairpin-loop', as TGF-α.[12] This structural feature, combined with a clear 'hydrophilic propensity'[13] near the middle of the loop, indicates high immunogenicity in this area. TGF-α exhibits least homology to EGF in the second loop[14] with only four out of 17 residues conserved.

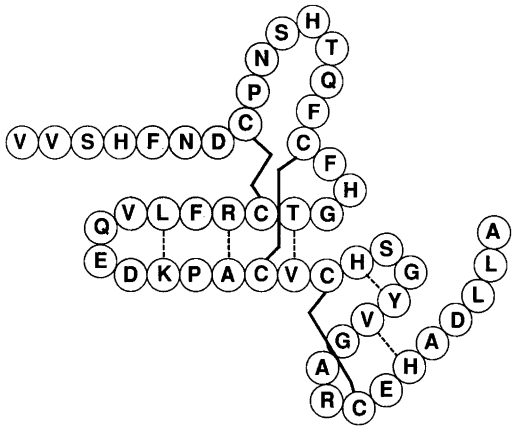

Fig. 1. Mature human TGF-α, with three disulfide bridges. The broken lines depict peptide backbone hydrogen bonds according to NMR studies (see the text). Predicted hydrophilic regions are shaded.

Due to the established association of TGF-α with human malignancies, this hormone is of potential therapeutic value for diagnostic purposes as well as for the rational design of antagonists. Antibodies capable of discriminating

Table 1. Sequences of hTGF-α and synthetic peptides.

	1	5	10	15	20	25	30	35	40	45	50
hTGF-α	V V S H F N D C P D S H T Q F C F H G T C R F L V Q E D K P A C V C H S G Y V G A R C E H A D L L A										
pep. **1**						R F L V Q E D K P A					
pep. **2**				K F C F H G T S R F L V Q E D K P A C V							
pep. **3**					K G T C R F L V Q E D K P A C V						
pep. **4**	V V S H F N D	V	A	N	P	G	C T S R F L V Q E D K P A S V C A				

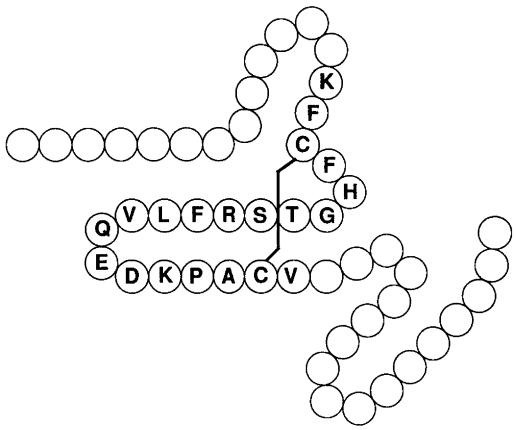

Fig. 2. Synthetic peptide **2**, with a disulfide bridge between the cysteines.

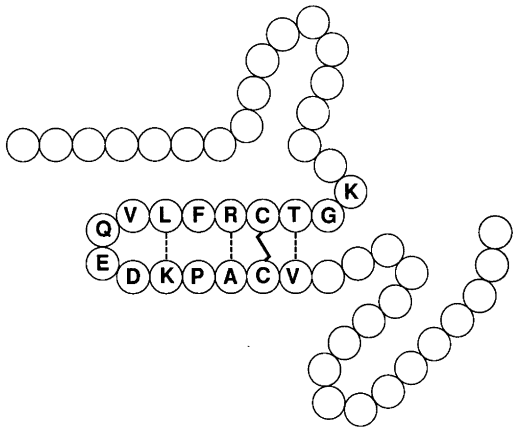

Fig. 3. Synthetic peptide **3** with an 'unnatural' disulfide bond.

between TGF-α and EGF should be preferentially produced against peptides matching the sequence of the second loop.

In an attempt to establish a method of measuring TGF-α in body fluids, we synthesized peptides **1**, **2** and **3** (Table 1) covering the sequence of the second loop of TGF-α, initially covering the whole loop but later, using the growing knowledge of the conformation of the hormone (Fig. 1), including unnatural disulfide bridges and attaching the *N*-terminal part of the molecule (Figs. 3 and 4). Antibodies were raised against the peptides and were tested for their ability to recognize native TGF-α in ELISA assays.

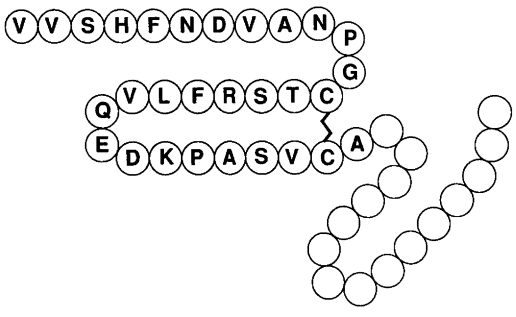

Fig. 4. Synthetic peptide **4** with an 'unnatural' disulfide bond.

Experimental

Materials. Protected amino acid derivatives and synthetic biologically active human TGF-α were purchased from Bachem, Switzerland. Rabbit anti-EGF antibodies were obtained from Collaborative Research, Inc. (USA). Goat anti-hTGF-α polyclonal antibodies were from AMS Biotechnology (Europe) Ltd. Horseradish-peroxidase-labeled antibodies to rabbit or goat immunoglobulins were from Dakopatts, Denmark.

Peptide synthesis. In this paper the one letter code for amino acids is used. The peptides **1–4** (Table 1) were synthesized manually using Merrifield's solid-phase technique with *t*-butyloxycarbonyl (Boc) protected amino acids.[15] The first Boc-amino acid was covalently linked to a Biorad® chloromethylated polystyrene resin (0.3 mequiv. g^{-1} substitution) according to the KF method[16] in DMF. The following *N*-α-Boc amino acids were side-chain protected: D, E, S and T by Bzl, C by acetamidomethyl (Acm) and K by 2-Cl-Bzl. Boc-R was N^{G}-tosylated and Boc-H was protected with an N^{Im}-2,4-dinitrophenyl (DNP) group.

The Boc group was removed in each step by double treatment with a solution of 45 % trifluoroacetic acid (TFA) and 5 % anisole in CH_2Cl_2 for 1 min and 20 min, respectively. The resin was neutralized with 5 % diisopropylethylamine in CH_2Cl_2. Coupling of the Boc-amino acids to the *N*-terminal of the growing peptide was carried out with symmetrical anhydrides preformed in CH_2Cl_2 using diisopropylcarbodiimide as the activator. G, N, Q, V, H and R were coupled as active esters in a three- to four-fold excess in the presence of 1-hydroxybenzotriazole with CH_2Cl_2–*N*,*N*-dimethylformamide (DMF) 10:1 as the solvent. 4-Dimethylaminopyridine (10 mM) was added to he couplings of L, T and V. The coupling reaction was monitored for completion with a ninhydrin test.[17] Double couplings (2 h each) were routinely performed in order to achieve full substitution, but in a few difficult cases the remaining free *N*-terminals were acetylated (acetic anhydride–pyridine 1:1).

The completed resin-bound peptide containing histidine was subjected to thiolysis in 50 % (v/v) thiophenol in DMF overnight to remove the N^{Im}-DNP groups.[18,19]

Deprotection and simultaneous cleavage from the resin was carried out according to the 'low–high' HF procedure.[20] The peptides were then purified on Sephadex® gel filtration columns (G15, G25 and G50) in 1 M acetic acid. Further purification was achieved by reversed-phase liquid chromatography. We used a Pharmacia FPLC Gradient Programmer equipped with a Waters Bondapak 10 μ C18 column in a Z-module and a water–100 % acetonitrile gradient, both solvents containing 0.05 % TFA. Components were detected by UV spectroscopy at 214 nm.

The removal of the Cys-Acm groups and subsequent formation of disulfide bridges[21] was performed in 800 ml of MeOH–water (1:6) at room temperature with a peptide concentration of about 0.06 mM. Iodine in MeOH was

added dropwise to the stirred solution at 1 h intervals until a the iodine color persisted. Addition of 1 M sodium thiosulfate removed the excess of iodine and the pH was adjusted to 7–8. In order to favor the formation of the thermodynamically most favorable disulfide bonds, ca. 1 mM glutathione and the corresponding disulfide[22] were added to the solution which was allowed to equilibrate at 4 °C for 5 days with constant stirring. The solution was subsequently concentrated on a rotary evaporator and the cyclic peptide was desalted on a Sephadex® – G15 gel filtration column in 1 M acetic acid. After cyclization and desalting, the peptide material was run through a Sephadex® – G50 column. A small polymeric fraction was collected first, which was then followed by the bulk of the material.

Amino acid analysis was performed at the Center for Amino Acid Analysis, Department of Biochemistry, Uppsala University Biomedical Center. Positive-ion fast-atom bombardment mass spectrometry (FAB-MS) was performed at Biocarb, Lund, and in our Department.

Polyclonal antibodies. Peptides **1**, **2** and **3** (Table 1) were conjugated to bovine serum albumin (BSA) by means of glutardialdehyde according to a standard procedure.

The immunizations for obtaining the polyclonal antibodies were carried out as follows. New Zealand White female rabbits were used in all experiments. In the first immunization four rabbits were immunized subcutaneously with 100 μg peptide in Freund's complete adjuvant. In the following biweekly immunizations the same amount of peptide was mixed with Freund's incomplete adjuvant and injected subcutaneously. Blood was always drawn prior to the first immunization. Serum for further studies was collected two weeks after the third immunization. Titers were determined by ELISA technology as described below.

ELISA tests were performed in 96-well flat-bottomed NUNC® microtiter plates. The wells were coated with 100 μl of 2.5–10 μg ml^{-1} unconjugated peptide in 0.1 M $NaHCO_3$ solution (pH 8.5) overnight at 4 °C. After the wells had been washed three times with phosphate-buffered saline containing 0.05 % Tween-20 (PBS-T), pH 7.5, 100 μl antiserum dilution were added and incubated at room temperature for at least 3 h. The wells were then washed again with PBS-T, and 200 μl horseradish peroxidase-conjugated antibody (diluted 1/2000 in PBS-T) were added. After 2 h at room temperature, the wells were again washed with PBS-T and once with 0.1 M sodium citrate buffer (pH 5.0). Addition of 100 μl per well of substrate solution (0.4 mg ml^{-1} *o*-phenylenediamine in citrate buffer containing 0.01 % H_2O_2) induced a colorimetric reaction, which was stopped after 20 min by the addition of 50 μl of 1 M H_2SO_4. Finally, the absorbance was measured automatically at 490 nm. In the case of inhibition-ELISA, the primary antibody was preincubated with the peptide overnight at 4 °C. Controls experiments were performed which ruled out any effects due to unspecific binding of primary and secondary antibodies to uncoated wells, as well as to secondary antibodies to the coated peptides.

Results and discussion

Synthesis of the peptides was relatively straightforward, but the cyclization and purification steps proved to be problematic. As has been observed by others in the synthesis of EGF, TGF-α and other cysteine-containing polypeptides, a substantial amount of insoluble polymeric material is obtained upon attempted cyclization. To overcome this, peptides **2**, **3** and **4** were synthesized using the Acm-group for protection of the SH-group and then purified by HPLC before removal of the Acm group. The Acm group was then detached with simultaneous cyclization with iodine at extreme dilution. Equilibration in the presence of glutathione buffer enabled reshuffling of the disulfides. With this method no insoluble material was formed and in Sephadex® G-50 gel filtration the main fraction was obtained at the elution volume expected for the monomeric cyclized form. FAB-MS analysis of peptides **2** (M^+/z = 2311), **3** (M^+/z = 1791) and **4** (M^+/z = 3087) confirmed that the completely deprotected and cyclized peptides had been obtained.

Amino acid analysis of all four peptides showed the composition to be in agreement with the theoretically required values (data not shown). The peptides were chromatographically pure, although a small percentage of deletion peptides and unwanted disulfide conformers cannot be excluded.

Initially two peptides were synthesized: peptide **1** (Table 1) covering the amino acids between Cys21 and Cys32 of TGF-α, and peptide **2** comprising the whole of the second loop (B-loop) (Fig. 2). Cys 21 was substituted for serine to avoid the formation of incorrect disulfide bridges. An *N*-terminal lysine was added to peptide **2** for BSA-conjugation.

During the course of this work the three-dimensional structures based on ^1H NMR studies of EGF and TGF-α were published[9–11] (*cf.* Fig. 1), and it was established that the conformation of the second loop is governed not only by the sequence, but also by the disulfide bridge forming the first loop. This forces the second loop to form an extra β-bend of four residues between Cys16 and Cys21 before entering the area of two antiparallel β-sheets connected by a 'hairpin-loop' (Fig. 1). A cyclic peptide encompassing the second loop with no further conformational restraints (Cys16–Cys32, i.e. peptide **2**), might therefore not show the correct 'growth-factor-like' conformation (Fig. 2). This implies that antibodies raised against such a peptide would not necessarily react with native TGF-α. However, a linkage between Gly19 and Cys34 would induce a conformation to the intervening sequence that is similar to the native B-loop. In peptide **3** an 'unnatural' disulfide bond is introduced, which should convey stability to the antiparallel β-sheet (Fig. 3). The presence hydrogen bonds to the *N*-terminal sequence of the native growth factor has been shown by weak nuclear Overhauser (NOE) effects to the B-loop in NMR.[10,12] Thus, a triple-stranded β-sheet structure is highly probable.[23] The synthetic peptide **4**, encom-

Table 2. Results from ELISA inhibition assays.[a]

Peptide antiserum	Titer[b]	Inhibition peptide				
		1	**2**	**3**	**4**	TGF-α
1	1/30000	100 (25)	[c]	–	–	–
2	1/10000	[c]	100 (70)	–	–	40 (0.1)
3	1/3500	–	–	100 (50)	–	10 (2.5)
4	1/5000	–	–	–	100 (2.5)	0 (2.5)

[a]Values show percentage of total inhibition for partially inhibiting peptides. Values in parentheses express the ratio between the concentration of the inhibiting peptide and that of the coating peptide valid for the given inihibition percentage. [b]Dilution at 0.5 × maximum affinity. [c]Reciprocal coating between **1** and **2** gives antibody titers indicating cross-reactivity.

Table 3. Titers from ELISA cross-activity assays.[a]

Coating	α-**4**	α-TGF-α
2	–	1/9000[b]
3	–	1/9000[b]
4	1/5000	1/11000
TGF-α	1/5000	1/11000

[a]Dilution at 0.5 × maximum affinity. [b]These curves had a lower inclination, indicating a weaker avidity for the antibody towards this antigen.

passing these structural features, was constructed with the *N*-terminal strand attached to the B-loop via an intermediate sequence excluding the A-loop (Fig. 4). Here amino acids with β-turn propensity[24] were chosen in order to assist and induce the triple-stranded β-sheet secondary structure.

None of the peptides showed significant growth-promoting activity in [^{3}H]thymidine incorporation assay with human foreskin fibroblasts.[25]

The peptides **1**, **2** and **3** conjugated with BSA, and peptide **4** without conjugation all induced the production of rabbit antibodies which could be inhibited by the corresponding peptides (Table 2). Although antibodies to TGF-α bound well to coatings of peptides **2**, **3** and **4** (Table 3), TGF-α was not efficient in inhibiting antibodies to the peptides (Table 2). Even anti-**4** antibodies, which are able to recognize coated TGF-α in the same way as anti-TGF-α antibodies (Table 3), are not inhibited by solvated TGF-α. This implies that the synthetic peptides might not have the same conformation as the corresponding sequence in native TGF-α, that their antibodies are incapable of recognizing native TGF-α in solution, and that coated TGF-α differs from the solvated form.

A commercial antibody to the third loop of the hormone did not give cross-reaction with any of the peptides synthesized, and peptides **1** and **2** did not cross-react with anti-EGF antibodies, as expected (data not shown).

A further phenomenon observed when using the commercial goat polyclonal antibody against intact native TGF-α is worth mentioning. Peptides **2**, **3** and **4** as well as TGF-α inhibit this antibody in wells coated with peptide **4** (Table 4). However, when the wells were coated with TGF-α, the peptides **2**, **3** and **4** partially inhibit anti-TGF-α, but TGF-α itself was unable to inhibit its antibody (Table 4, row 2). This could be due to the denaturation of TGF-α by Tween-20 in the incubation buffer. Inhibition of anti-TGF-α was more extensive when BSA-coated disposable test tubes were used (Table 4, row 3). This change in the procedure was necessary in order to circumvent the denaturing effect of Tween and, at the same time, prevent the unspecific adhesion of anti-TGF-α antibodies to the disposable test tubes.

Table 4. ELISA inhibition of goat anti-TGF-α antibodies.[a]

Coating	Titer[b]	Inhibition peptide				
		1	**2**	**3**	**4**	TGF-α
4	1/11000	–	45 (5)	45 (5)	95 (5)	70 (2.5)
TGF-α[c]	1/11000	–	55 (200)	30 (200)	35 (200)	0 (10)
TGF-α[d]	1/11000	–	–	35 (200)	15 (200)	70 (20)

[a]Values show percentage of total inhibition for partially inhibiting peptides. Values in parentheses express the ratio between the concentration of the inhibiting peptide and that of the coating peptide valid for the given inhibition percentage. [b]Dilution of TGF-α antibodies at 0.5 × maximum affinity. [c]Incubation of primary antibody and peptide was performed in PBS buffer containing Tween. Uncoated disposable tubes. [d]Disposable test tubes were coated with BSA prior to incubation of primary antibody with the peptide. No Tween in the incubation buffer.

Polyclonal antibodies to a complex molecule like TGF-α always contain a spectrum of gamma-globulins directed towards different conformations of a number of epitopes and with different affinities. It is therefore not surprising that this antibody, although it binds fairly well to the synthetic peptides, is only weakly inhibited by solvated TGF-α (Table 4).

The 'growth-factor structural motif' with its three disulfide bonds (Fig. 1) has made difficult the task of producing antibodies to partial structures which can be inhibited by the native hormone in a direct ELISA system. We tried to surmount this problem and preserve the 'native' conformation by the use of unnatural disulfide bonds. With the evaluation methods at hand, we have thus shown, that antibodies to these peptides were recognized by TGF-α as a coating material, although the degree of inhibition in some cases was low.

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