Synthesis of Biologically-active Human Transforming Growth Factor-α by Fluorenylmethoxycarbonyl Solid Phase Peptide Chemistry

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The synthesis of a biologically-active 50-amino acid peptide, human transforming growth factor-α (hTGF-α), by fluorenylmethoxycarbonyl (Fmoc) solid phase peptide chemistry is described.

Transforming growth factor-α (TGF-α or TGF-1) is a 50-amino acid residue polypeptide which can induce the transformation of non-transformed cell lines.[1] TGF-α is secreted by a number of cell lines[2,3] and shows marked sequence homology with the functionally related epidermal growth factor (EGF) family of polypeptides.[4,5] Both TGF-α and EGF interact with the EGF receptor activating a receptor-associated tyrosine kinase,[6] thus stimulating DNA synthesis and cell growth.

The refinement of peptide synthesis in recent years has been instrumental in successfully achieving the synthesis of several complex peptide hormones and growth factors. For example, biologically-active murine interleukin-3 (a 140-amino acid peptide containing two disulphide bridges), rat and human TGF-α (rTGF-α and hTGF-α, respectively, 50-amino acid peptides containing three disulphide bridges differing in only four amino acids), and murine EGF (a 53-amino acid peptide containing three disulphide bridges) have recently been synthesized by t-butoxycarbonyl (Boc)–polystyrene solid phase peptide chemistry.[7—10] Similarly, fluorenylmethoxycarbonyl (Fmoc)–polyamide solid phase peptide chemistry[11] has been utilised in the synthesis of conotoxin, a complex 15-amino acid peptide toxin from the sea snail *Conus geographis* containing two disulphide bridges.[12] To date, there are no examples of the synthesis of larger, more complex multi-disulphide-bridged molecules by the Fmoc method in the literature. We report here the production of biologically-active hTGF-α by Fmoc–polyamide solid phase peptide synthesis.

The synthetic strategy employed was to assemble the linear form of the 50-amino acid peptide with conventional Fmoc–polyamide side chain protecting groups [His(Fmoc), Ser(Bu^t), Asp(OBu^t), Glu(OBu^t), Thr(Bu^t), Tyr(Bu^t), Lys(Boc), and Arg(Mtr) (Mtr = 4-methoxy-2,3,6-trimethylbenzenesulphonyl)] except in the case of cysteine where the stable acetamidomethyl (Acm) group was employed. This strategy (outlined in Figure 1) allowed purification of the linear form of the molecule to homogeneity, as standard side chain deprotection conditions and cleavage from the resin support did not affect the six Acm-protected cysteine residues. The advantage

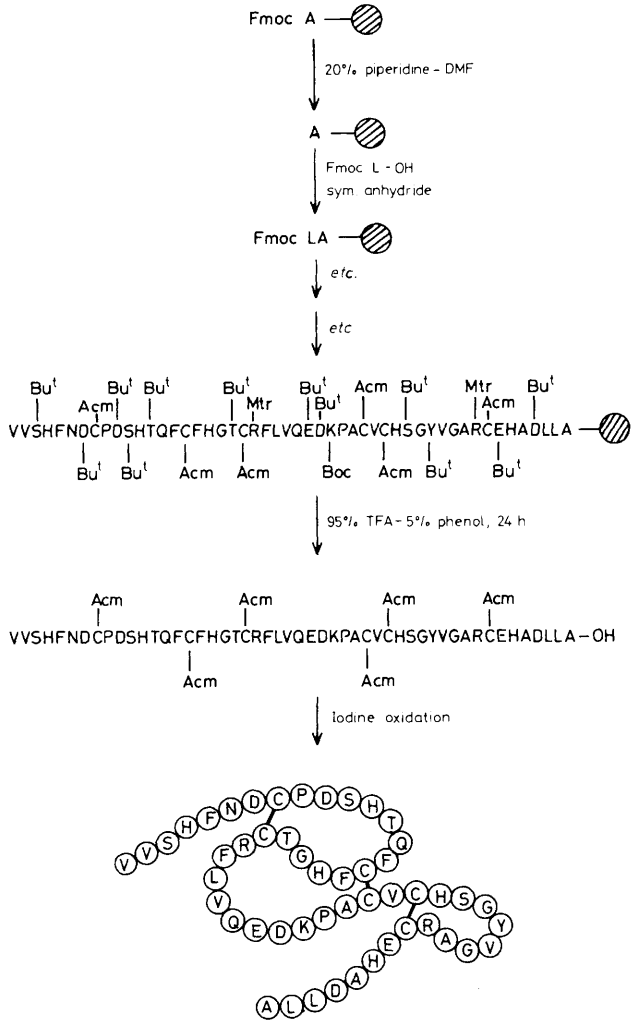

Figure 1. Flow diagram showing the strategy of synthesis and deprotection of hTGF-α. The 50-amino acid peptide was synthesised by the Fmoc–polyamide chemistry of Atherton *et al.*[11] Cleavage of the peptide from the polyamide resin using 95% TFA–5% phenol resulted in deprotection of all side chains except for the sulphydryl moiety of cysteine which was protected with the acid-stable Acm group. The hTGF-α was purified in this form prior to oxidative removal of the Acm groups and concomitant formation of disulphide bridges. (See text for details.)

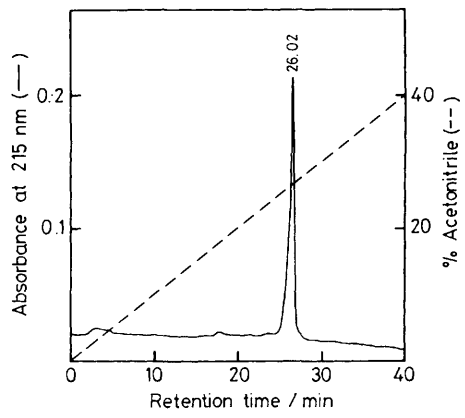

Figure 2. Analytical reversed-phase h.p.l.c. of purified synthetic hTGF-α. A sample of purified hTGF-α (reoxidized component B) was applied to a Brownlee RP300 column (2.1 × 30 mm) equilibrated with 0.15% (v/v) aqueous TFA. The flow rate was 100 μl/min and the column temperature was 45 °C. The column was developed with a linear 60-min gradient from 0 to 60% acetonitrile.

of this approach was that all termination and deletion peptide impurities were removed prior to disulphide bond formation. The final purification of disulphide-bridged TGF-α was, therefore, greatly simplified as it only involved separation of biologically-active TGF-α monomer from other inactive, incorrectly disulphide-bridged monomers or aggregates.

The synthesis was performed manually on a Kieselguhr–polydimethylacrylamide support by the procedure of Atherton *et al.*[11] The Kieselguhr support was designed as a flow resin, with coupling being performed by recirculating the activated amino acid through the non-swelling polymer support. Kieselguhr in this case, however, was used as a normal gel resin without a recirculating flow coupling procedure. The loading of the C-terminal amino acid, alanine, on the resin was 0.046 mmole g^{-1}. This value was considerably lower than the theoretical loading capacity of 0.1 mmole g^{-1} but this may be a reflection of forming the peptide–resin ester linkage under non-continuous flow conditions. Peptide bond formation, however, was found to proceed rapidly (generally complete within 45 min) using Fmoc amino acid symmetrical anhydrides in dimethylformamide (DMF), provided that the concentration of the anhydride was greater than 0.15 M and provided that 1 equiv. of 1-hydroxybenzotriazole (HOBt) was added to the coupling mixture in order to catalyse the reaction.[13]

Upon completion of the synthesis, the peptide was deprotected and cleaved from the resin by extended treatment with trifluoroacetic acid (TFA) (with 5% phenol as scavenger). The crude peptide was initially desalted by gel-permeation chromatography on TSK 40(F) and then further purified by high-pressure liquid chromatography (h.p.l.c.). The desalted crude product was resolved into two major components by ion-exchange chromatography on Mono Q (Pharmacia). Amino acid analysis of these two major components revealed that one was consistent with a termination product formed mid-way through the synthesis whilst the other component had an amino acid composition comparable with that of full-length hTGF-α. The latter product was further purified by h.p.l.c. on a Brownlee RP300 support. The pure synthetic hTGF-α(Acm) chromatographed as a single peak on h.p.l.c. and gave the expected amino acid ratios for full-length hTGF-α [found: Asx, 4.6(5); Thr, 1.7(2); Ser, 3.1(3); Glx, 3.9(4); Pro, 1.8(2); Gly, 3.8(3); Ala, 4.1(4); Cys, not determined (6); Val, 5.0(5); Leu, 3.0(3); Tyr, 1.2(1); Phe, 3.8(4); His, 3.4(5); Lys, 1.0(1); Arg, 1.7(2)].

The stable Acm group was removed by reaction with iodine according to the method of Kamber *et al.*[14] This treatment not only oxidatively removed the cysteine protecting groups but also randomly formed disulphide bonds within the molecule. The complex mixture of hTGF-α products from the iodine treatment was resolved by h.p.l.c. on a Brownlee RP300 support into two major components (designated components A and B). Since both components had identical amino acid compositions, presumably they represented differently disulphide-bridged forms of hTGF-α.

Both components were tested for biological activity in an ^{125}I-labelled murine EGF (^{125}I-mEGF) receptor binding assay using A431 cells.[15] ^{125}I-mEGF was bound to its receptor on the surface of the cells and hTGF-α components A and B were

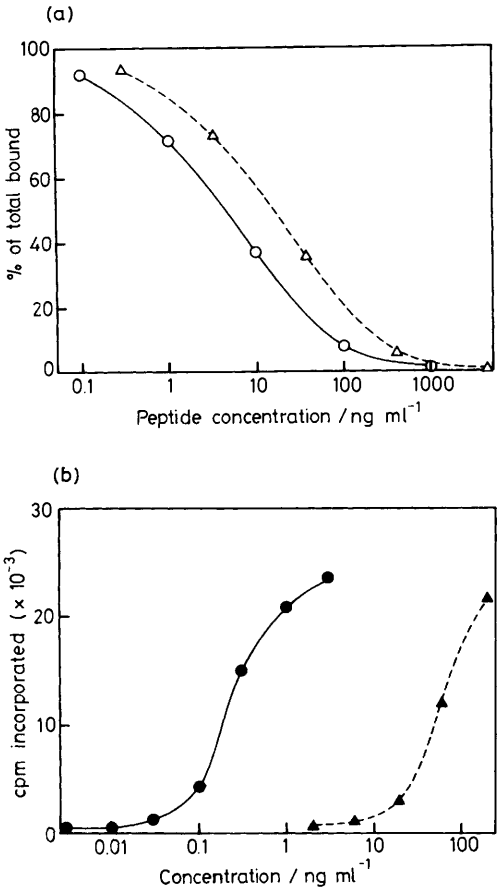

Figure 3. (a) Competition by synthetic hTGF-α for ^{125}I-labelled mEGF binding to A431 cells. Human epidermoid carcinoma cells (A431 cells) were incubated with ^{125}I-mEGF and unlabelled synthetic hTGF-α at increasing concentrations of hTGF-α (△). All assay concentrations were determined by amino acid analysis of companion aliquots and the assay results were directly compared with a standard companion assay of native mEGF (○). (b) Mitogenic activity of synthetic hTGF-α on murine 3T3 fibroblasts. [Methyl-^{3}H]thymidine incorporation into the DNA of quiescent 3T3 fibroblasts was measured at increasing synthetic hTGF-α concentrations (▲). All assay concentrations were determined by amino acid analysis of companion aliquots and the assay results were directly compared with a standard companion assay of native mEGF (●).

tested for their ability to displace the radiolabel at increasing concentrations of peptide. hTGF-α component B was found to be totally inactive in this assay whilst hTGF-α component A displaced 50% of the bound radiolabel at a peptide concentration of 2600 ng ml^{-1} (0.2% as active as native mEGF).

Both hTGF-α components were reduced with β-mercaptoethanol, and, after removal of the reducing agent by reversed-phase chromatography on a Pharmacia Pep-RPC column, were reoxidized using the glutathione procedure of Ahmed *et al.*[16] After this treatment hTGF-α components A and B were chromatographically indistinguishable on Pharmacia Pep-RPC and the reoxidized product was also shown to be homogeneous by reversed-phase h.p.l.c. on the same Brownlee RP300 support used in the purification and analysis of hTGF-α(Acm) (see Figure 2). The amino acid ratios [found: Asx, 4.8(5); Thr, 1.7(2); Ser 3.0(3); Glx, 4.3(4); Pro, 1.7(2); Gly, 3.5(3); Ala, 4.0(4); Cys, not determined (6); Val, 3.7(5); Leu, 2.8(3); Tyr, 1.0(1); Phe, 3.7(4); His, 4.0(5) Lys, 0.9(1); Arg, 1.7(2)] were consistent with full length hTGF-α. The final yield of isolated pure peptide was 0.1% compared with the initial loading of the first amino acid (alanine) at the commencement of synthesis.

In the radioreceptor assay both reoxidized hTGF-α components A and B displaced 50% of the bound ^{125}I-radiolabel at a concentration of 22 ng/ml [see Figure 3(a)]. This finding is comparable with the value of 4.1 nM (23 ng/ml) reported by Tam *et al.*[8] for rTGF-α synthesized by Boc chemistry and approximately 5.5 nM (33 ng ml^{-1}) reported by the same group[9] for hTGF-α synthesized by Boc chemistry. In the same assay native mEGF displaced 50% of the radiolabel at a concentration of 4 ng/mol [see Figure 3(a)]. Since mEGF and hTGF-α are reported to be equipotent in the A431 cell assay[17] the synthetic hTGF-α reported here is approximately 18% as active as native hTGF-α. Interestingly, the biological activity of hTGF-α was only approximately 1.6% that of native mEGF in a mitogenic assay using 3T3 fibroblast cells[15] [see Figure 3(b)]. Maximal mitogenic activity of synthetic hTGF-α was achieved at a peptide concentration of 34 nM; this value was similar to that reported by Tam *et al.*[8] (20 nM) for synthetic rTGF-α in the same mitogenic assay.

As the peptide chemist looks to synthesizing larger, more complex molecules, Fmoc–polyamide chemistry has great potential to achieve this aim. In contrast with Boc–polystyrene chemistry, however, there are few reports to date of large complex syntheses by the Fmoc method. The synthesis of biologically-active hTGF-α detailed here, therefore, is an indication of the extended capabilities of the Fmoc method.

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