$(H3{\cdot}H4)_2H2A{\cdot}H2B$ hexamers and $(H3{\cdot}H4)_2$ tetramers would also bind as extra histones, the relative amounts of the three particles depending on the degree of modification and the salt concentration.

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Synthesis of a Biological Active Tumor Growth Factor from the Predicted DNA Sequence of Shope Fibroma Virus†

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ABSTRACT: A 55-residue peptide comprising the carboxyl portion (residues 26–80) of the Shope fibroma virus growth factor (SFGF), a predicted 80-residue DNA virus gene product that encoded a homologous sequence with the epidermal growth factor transforming growth factor α family, was synthesized by a stepwise solid-phase method. The synthetic SFGF (26–80) purified to homogeneity by reverse-phase HPLC was characterized by fission ionization mass spectrometry and amino acid analysis. The disulfide pairings were established by enzymatic digestion and mass spectrometry and were found to be similar to those of EGF and TGFα. Synthetic SFGF (26–80) was found to share about 10% of the activities as EGF in the radioreceptor binding to A431 cells, stimulation of [^{3}H]thymidine uptake in NRK cells, and induction of colony formation in soft-agar assay. Our results therefore confirmed that SFGF contained the putative biological activities of the EGF–TGFα family and that production of SFGF by Shope fibroma virus infected cells may account for the proliferative diseases associated with this particular virus.

Poxvirus comprises a large group of eukaryotic DNA virus whose exceptionally large double-stranded DNA genomes replicate within virosomes in the cytoplasm of the infected host cells. Several poxviruses are known to be responsible for proliferative tumorigenic diseases. Three notable examples of tumorigenic poxviruses are the following: (1) Shope fibroma virus (SFV)[1] (Shope, 1932; Chang et al., 1987), which induces benign fibromas in adult rabbit; (2) Yaba tumor virus, which causes subcutaneous histiocytomas in monkeys and man (Bearcroft & Jamieson, 1958); and (3) Molluscum contagiosum, which produces tumor-like epidermal lesions in man

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMS, dimethyl sulfide; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; SFGF, Shope fibroma virus growth factor; SFV, Shope fibroma virus; TFA, trifluoroacetic acid; TGFα, transforming growth factor α; VGF, vaccinia virus growth factor.

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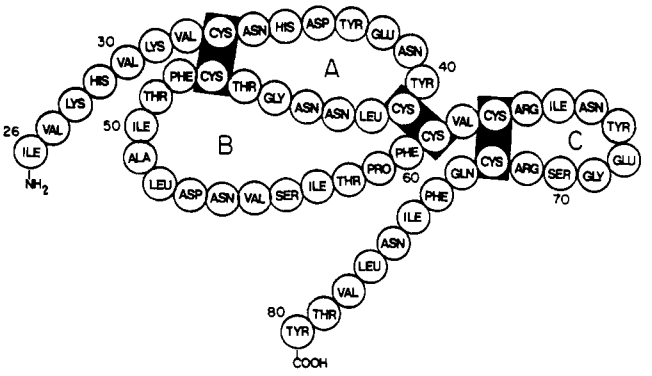

FIGURE 1: Structure of SFGF (26–80).

(Brown, et al., 1981). Since the poxvirus genome encodes the enzymes required for its own replication, it is believed that poxvirus may produce its own regulatory mitogenic agents such as growth factor to sustain its own growth. Recently, it has been shown that vaccinia virus, a cytolytic poxvirus of the genus *Orthopoxvirus*, encodes a gene product, vaccinia virus growth factor (VGF), which shares 45% amino acid homology with mouse epidermal growth factor (EGF) and 37% with the transforming growth factor α (TGFα) (Blomquist et al., 1984; Brown et al., 1985; Reisner, 1985; Upton et al., 1987). Similarly, it has been demonstrated that SFV also encodes a related gene product that possesses 33–45% sequence homology with the EGF–TGFα family of growth factors (Chang et al., 1987).

The amino acid sequence of a homologous Shope fibroma virus growth factor (SFGF) has been deduced from the gene sequence (Chang et al., 1987). SFGF is a protein consisting of 80 amino acid residues and is very similar in size to the secreted form of the VGF, a 77-residue protein. The six cysteinyl residues that form the three disulfide linkages to give the compact tricyclic structure of the EGF–TGFα family are all conserved in SFGF. Of the 13 conserved Tyr, Cys, Gly, and Arg residues found in EGF, TGFα, and VGF, 12 are retained in the SFGF sequence (Chang et al., 1987; Upton et al., 1987). Interestingly, the first and third disulfide loops are conserved in both amino acid sequence and size and have been shown to be important in biological activities (Tam et al., 1986a). The second disulfide loop, which is most diverse in amino acid sequence and is least important in biological activities, is three residues longer in SFGF than in EGF, TGFα, or VGF.

It has not been determined whether the SFGF is processed and secreted extracellularly like its vaccinia virus counterpart (Chang et al., 1987). The putative properties of SFGF as a growth factor are not known. To establish the biochemical properties of the novel SFGF, we have synthesized a 55-residue SFGF (Figure 1) comprising the homologous region of the EGF–TGFα family. Here, we demonstrate that the synthetic SFGF is mitogenic, binds to EGF receptor, activates tyrosine-specific protein kinases, and in general possesses biological activities similar to those of EGF and TGFα. However, unlike our previous synthesis of TGFα (Tam et al., 1986b; Tam, 1987), synthetic SFGF (26–80) in the reduced form was found to be insoluble even in 5 M urea and hence difficult to refold and oxidize to the expected tricyclic structure. We have therefore gone on to study an optimal condition for the oxidative refolding of SFGF.

Materials and Methods

Solid-Phase Peptide Synthesis. The synthesis of SFGF (26–80) was carried out in a Beckman 990 M synthesizer except for the final 12 residues, which were synthesized manually. The synthesis started on 2 g of N^{α}-*tert*-Boc-Tyr-(BzZ)-OCH_2-Pam resin (0.65 mmol/g of resin) (Mitchell et al., 1978). The butoxycarbonyl (Boc) group was used for the N^{α} terminus, and the side-chain protections were as follows: Arg(Tos), Asp(OBzl), Cys(4-MeBzl), Glu(OBzl), His(Dnp), Lys(2-ClZ), Ser(Bzl), Thr(Bzl), and Tyr(2-BrZ) (Bzl, benzyl; Dnp, dinitrophenyl; MeBzl, methylbenzyl; Tos, *p*-toluenesulfonyl; Z, benzoxycarbonyl). A 20-min deprotection by trifluoroacetic acid (CF_3COOH:CH_2Cl_2, 1:1 v/v) was preceded by two CF_3COOH prewashes containing 0.1% ethanedithiol for 2 min each. Neutralization was carried out by diisopropylethylamine (DIEA) after each deprotection and before each recoupling. A mixed mode of double-coupling protocol with symmetrical anhydride in CH_2Cl_2/DMF and then with dicyclohexylcarbodiimide (DCC) was used. Boc-Gly was coupled with DCC alone. In addition, double couplings of Boc-Asn, Boc-Gln, and Boc-Arg(Tos) were performed by the hydroxybenzotrizole (HOBt) active ester in DMF (Konig & Geiger, 1970). The yield in each coupling was monitored by a quantitative ninhydrin test (Sarin et al., 1981). Portions of synthetic peptide resin after cycles 17 and 32 were removed for analysis in order to monitor the progress of the synthesis. The cumulative yield was 95% on the basis of back-hydrolysis of synthetic SFGF-Pam resin.

HF Cleavage. Protected synthetic SFGF (26–80) peptide Pam resin (0.72 g) was treated 3 times with 15 mL of a 1 M solution of thiophenol in dimethylformamide for 8 h to remove the N^{im}-Dnp protecting group of His (Lin et al., 1972) and then with 50% (v/v) CF_3COOH/CH_2Cl_2 (15 mL) for 20 min to deblock the Boc group. The dried peptide resin was subsequently treated with the low–high HF method of cleavage (Tam et al., 1983).

In the low-HF procedure, the peptide resin was premixed with dimethyl sulfide (DMS), *p*-cresol, and *p*-thiocresol. To facilitate the evaporation of DMS during the time between low-HF and high-HF processes, 100 mg of cysteine was added. At −78 °C, the liquid HF was transferred into the deprotection mixture to reach a final volume of 10 mL (HF:DMS:*p*-cresol:*p*-thiocresol, 31:65:2:2 v/v). The reaction mixture was stirred at 0 °C for 2 h, and the HF and dimethyl sulfide were removed in vacuo. The high-HF procedure was initiated by recharging with liquid HF at −78 °C to reach a total volume of 15 mL of the mixtures (HF:*p*-cresol:*p*-thiocresol, 97.4:1.3:1.3 v/v). The reaction was performed at 0 °C for 1 h. After a complete evacuation of HF at 0 °C and two washes with prechilled ether/mercaptoethanol (99:1 v/v, 50 mL) to remove *p*-cresol and *p*-thiocresol, the crude reaction mixture was extracted with 100 mL of 8 M urea/0.2 M dithiothreitol (DTT)/0.1 M Tris buffer, pH 8.2. The cleavage yield was about 90% on the basis of back-hydrolysis of the HF-treated resin by propionic acid/12 N HCl (1:1 v/v).

Refolding and Purification. To refold the linear SFGF, the synthetic materials were subjected to a gradient refolding procedure in a decreasing concentration of urea solutions. The 8 M urea solution (100 mL) containing a crude mixture of SFGF (26–80) was dialyzed sequentially (spectrum 6, M_r cutoff 1000) against 4 L each of deoxygenated and N_2-purged 8, 6, and 4 M urea/0.1 M Tris-HCl buffer, pH 8.2, to exclude DTT and to allow refolding. However, synthetic SFGF (26–80) was found to precipitate at 5 M urea solution. The insoluble material was then redissolved in a 3 M Gdn-HCl/0.1 M Tris-HCl buffer, and the resulting solution was diluted very slowly with 0.1 M Tris buffer to 1.5 M at a concentration of 0.1 mg of peptide/mL, pH 8.2. Reduced and oxidized glu-

Table I: Synthesis of Shope Fibroma Growth Factor

starting materials	methods	yields (%)
(1) Boc-Tyr(BrZ)-OCH_2-Pam-Ⓡ	stepwise peptide synthesis for 54 cycles	95
(2) Boc-SFGF-OCH_2-Pam-Ⓡ	cleavage from the resin and final deprotection of side chain: (1) TFA; (2) thiophenol; (3) low and high HF	90
(3) crude SFGF	refolding and oxidation of disulfide: (1) DTT/dialysis; (2) oxidized and reduced glutathiones	38
(4) purified SFGF(S–S)$_3$	sequential purification by reverse-phase C_{18} HPLC	10

tathiones (1 mmol each) were added, and the reaction mixture was kept at ambient temperature with occasional stirring for 48 h (Saxena & Wetlaufer, 1970). A small amount of precipitate was observed during the refolding process. The supernatant containing the crude peptide mixture was purified by preparative reverse-phase C_{18} liquid chromatography (2.5 × 30 cm) eluted with 0.045% TFA/aqueous acetonitrile at a flow rate of 10 mL/min. The major fractions were lyophilized and further purified on a second C_{18} reverse-phase liquid chromatography (eluted with 0.045% TFA/acetonitrile at a flow rate of 1.5 mL/min and a shallower gradient system), and the major peak eluted at 39% acetonitrile. Lyophilization gave 18 mg of pure peptide that was identified as the expected SFGF (26–80) (Figures 2 and 3). The total synthetic yield, on the basis of the starting loading of tyrosine to the Pam resin, was 3% (Table I).

Digestion of SFGF (26–80) with Thermolysin. Purified synthetic SFGF (26–80) (50 μg) was digested with thermolysin (15 μg) in 0.1 mL of 0.1 M pyridine/acetic acid buffer, pH 6.5, at 45 °C for 24 h (Savage et al., 1973). The sample was filtered (Waters, 0.22 μm) and chromatographed on a C_{18} reverse-phase HPLC (Vydac, 5 μm, 0.4 × 25 cm) by use of a linear gradient of CH_3CN containing 0.045% of TFA. The peaks were collected and subjected to amino acid analysis (Table III).

Characterization of Synthetic SFGF (26–80). Analytical HPLC of a 20-μg sample was performed on a Vydac column (C_{18} reverse-phase) with a 30-min linear gradient of 15–85% buffer B at a flow rate of 1.5 mL/min (Figure 2). Buffer A contained 5% CH_3CN, 95% H_2O, and 0.0445% TFA. Buffer B contained 60% CH_3CN, 40% H_2O, and 0.039% TFA. Amino acid analysis was performed in 5.7 N HCl for 24 h at 110 °C (Table II). Synthetic SFGF (26–80) was analyzed by Cf-252 fission ionization mass spectrometry (Macfarlane & Torgerson, 1976; Chait et al., 1982) and found to exhibit an average molecular ion $(M + H)^+$ of 6344, corresponding closely to the calculated mean value m/z 6344 (Figure 3). Strong peaks corresponding to $(M + 2\ H)^{2+}$ and $(M + 3\ H)^{3+}$ were also detected in the expected range, 3173 and 2115 (Figure 3). No dimeric form of SFGF (26–80) of $(2\ M + 3\ H)^{3+}$ at 4230 was found. Furthermore, high mass ion at 2199 (Figure 3) that corresponded to the loss of the carboxyl-terminal 18 residues was also found. Such a fission fragment would only occur with the synthetic SFGF (26–80) containing a correct disulfide linkage at the carboxyl terminus (see C loop in Figure 1).

Biological Assays. Inhibition of [^{125}I]EGF binding to the EGF–receptor was examined by means of subconfluent monolayer of formalin-fixed A431 cells after a 1-h incubation at 22 °C with synthetic SFGF (26–80) (Table V) (De Larco & Todaro, 1978). A soft-agar assay was carried out on 49F fibroblast cells, and colonies were counted after 12 days by number and size (Table V) (De Larco & Todaro, 1978). Incorporation of [^{3}H]thymidine was measured in normal rat kidney fibroblasts, clone 49F (Table V) (De Larco et al., 1981).

Results

The chemical synthesis of a gene product encoded by a tumorigenic DNA virus (Shope fibroma virus) described in this work serves several purposes. The first and foremost is to confirm that SFV growth factor has the predicted biological activities putative to the family of EGF–TGFα. Second, the chemical synthesis of a fully biologically active growth factor from a gene product demonstrates the viability of the chemical approach in obtaining a biological active molecule from the predicted gene product. Furthermore, the synthetic linear product, which contained multi-cysteinyl residues, would refold in high yields to its native state.

Synthetic and Cleavage Strategies. The homologous sequence of SFGF with EGF or TGFα begins at residue 33 (Cys, the first of the six cysteinyl residues) and ends at residue 72 (Cys). Within this highly conserved stretch, SFGF shares respectively 45%, 35%, and 33% sequence homology with mEGF, TGFα, and VGF. Since EGF and TGFα are secreted as a soluble factor containing about 50 amino acids, the carboxyl two-thirds of SFGF, comprising residues 26–80 and containing all of the homologous growth factor like sequence, was chosen for the model of chemical synthesis to test for its growth factor activities.

The primary strategy for the synthesis of SFGF adopted the conventional stepwise solid-phase approach (Merrifield, 1963, 1986) using the differential acid-labile protecting group scheme with the *tert*-butyloxycarbonyl group for the N^{α}-amine terminus and the benzyl alcohol derived group for the side-chain protection. Recent improvements for the reduction of side reactions during the synthesis of a complex peptide were also implemented in the present synthesis (Mitchell et al., 1976; Tam et al., 1979). For example, a more acid-stable linkage to the resin support, Pam resin, was used to reduce the premature loss of peptide chain during the repetitively acidolytic deprotection cycles (Mitchell et al., 1976). In order to prevent the S-alkylation side reaction of the cysteinyl residues during the acidolytic deprotection cycles, ethanedithiol (0.1% by volume) was added only to the two 2-min TFA prewash solutions to scavenge the *tert*-butyl cation source as a result of the N^{α}-Boc removal reaction. Since the $t_{1/2}$ of Boc removal is less than 1 min, the thiol scavenger is therefore used for the initial 98% removal of the Boc group. A mixed mode of double-coupling protocol with symmetrical anhydride and then with DCC was used except in the cases of Boc-Gly, Boc-Arg(Tos), Boc-Asn, and Boc-Gln. In order to diminish side reactions that would lead to accumulation and amplification of deletion and termination products (Tam, 1987), repetitive quantitative coupling yield in each step is required. In most cases, the coupling reaction was complete or nearly complete within a few minutes. Since the coupling reaction rate in the solid phase is partly diffusion-controlled, incomplete couplings are usually caused by physical states such as aggregation of the peptide resin during the coupling. Improvement in such cases could be effected by using the appropriate solvent or cosolvent (Tam, 1987). In the present synthesis, coupling reactions were carried out in CH_2Cl_2 for 30 min and then in DMF/CH_2Cl_2 (1:1 v/v) for another 30 min, in both the DCC and symmetrical anhydride couplings. Satisfactory coupling yields averaging >99.8% were obtained in every coupling step. However, in the coupling steps of the Asn^{43}–Asn^{44} and

Gln^{73}–Phe^{74}, only 98% coupling yields were achieved. The remaining 2% of free amino groups were terminated by acetic anhydride.

The bulk of the synthesis was carried out in a Beckman 990 M automatic synthesizer. However, we found that the design of the Beckman automatic synthesizer would not allow base neutralization of TFA present in the nonwettable surface of the reaction cup and that which was trapped in the exhaust tubings. Extensive washing of the bulkhead above the reaction vessel and the side of the reaction cup did not remove the residual amount of TFA vapor. Since the presence of TFA in the coupling step will lead to both deletion and termination products (the salt formation of TFA with the free amine and the formation of an acylating agent with DCC), we found that coupling through performed symmetrical anhydride or active ester in the first coupling cycle with a small amount of tertiary amine base (DIEA) greatly reduced the danger of the termination side reaction. To completely avoid the danger of termination products that may complicate the purification of the desired SFGF, the final 12 residues containing the NH_2-terminal cysteinyl residue (Cys^{33}) were synthesized manually, with the assumption that terminated peptides would have contained an odd number of cysteinyl residues and would not be refolded correctly to give SFGF.

The deprotecting procedure for the side-chain protecting groups was the low–high HF method (Tam et al., 1983). Under such a condition, the oxygen-linked benzyl protecting groups were removed as sulfonium salts during the low-HF treatment by a S_N2 mechanism that minimized the danger of an alkylation side reaction (Tam, 1987; Tam et al., 1983). In addition, the S_N2 reaction also prevented acylium ion formation in glutamyl and aspartyl peptides. To remove Arg(Tos) and Cys(4-MeBzl), a high-HF procedure containing a concentrated HF was used. A small amount of cysteine was added in the reaction mixture as a scavenger to facilitate the evaporation of DMS during the transition from the low- to the high-HF treatment.

Refolding and Renaturation. A difficult point in the synthesis of SFGF (26–80) is the oxidative refolding of the six cysteinyl residues to the correct disulfide pairs. In the synthesis of TGFα (Tam et al., 1986b; Tam, 1987), we have successfully used a gradient dialysis method that uses a stepwise refolding procedure of sequentially lowering of denaturant in the presence of a combination of oxidized and reduced glutathiones. No aggregation that led to precipitation on these syntheses was observed. However, in the present refolding of SFGF, this strategy was unsuccessful because the reduced SFGF became insoluble at the intermediate stages of the gradient dialysis method. Unlike mouse EGF (Heath & Merrifield, 1986) and human TGFα (Tam et al., 1986b), both are acidic and contain about 26% hydrophobic amino acids in their sequences, SFGF (26–80) is rather hydrophobic (40% of the sequence is hydrophobic residues), especially at both termini (residues 73–80 and residues 26–32). In addition, SFGF (26–80) also contains an overall basic charge. These factors may lead to the precipitation of the unfolded synthetic SFGF in the neutral or basic condition. At 5 M urea, pH 8.2 solution, synthetic SFGF started to precipitate from the solution. Oxidation and regeneration of synthetic SFGF at 5 M urea, using air or a combination of an oxidized and reduced form of glutathione, resulted in an extremely poor yield. Since the precipitation in the gradient dialysis constituted a simple purification step that removed soluble byproducts, we took advantage of this observation. The precipitate from the urea solution was readily redissolved in 3 M Gdn-HCl. Dilution of the guanidine solution to 1.5 M did not result in precipitation, and the peptide was then subjected to oxidation and refolding.

Table II: Amino Acid Analysis of Synthetic SFGF (26–80)

amino acid (theory)	found	amino acid (theory)	found
Ala (1)	1.1	Leu (3)	3.0
Arg (2)	1.9	Lys (2)	2.0
Asp (9)	8.9	Phe (3)	3.2
Cys (6)	6.3[a]	Pro (1)	1.1
Glu (3)	3.5	Ser (2)	2.0
Gly (2)	2.4	Thr (4)	3.9
His (2)	1.8	Tyr (4)	3.8
Ile (5)	4.7[b]	Val (6)	5.8[b]

[a] As cysteic acid after performic acid oxidation. [b] Hydrolysis with propionic acid/12 N HCl, 1:1 v/v, for 10 h, 130 °C.

Table III: Amino Acid Composition of Peptide Obtained from Thermolysin Digestion

Glu-Asn-Tyr-Cys^{41}
|
Phe-Cys^{61}

amino acid	found (theory)	amino acid	found (theory)
Asn	0.8 (1)	Tyr	0.8 (1)
Glu	1.2 (1)	Cys	0.5[a]
Phe	1.2 (1)		

[a] No performic acid oxidation was carried out.

Table IV: Cf-252 Fission Fragment Mass Spectrometry of SFGF (26–80)

ion species	measd values	calcd values
SFGF (26–80) $(M + H)^+$	6344.2	6345.3
SFGF (26–80) $(M + 2\ H)^{2+}$	3173.2	3173.2
SFGF (26–80) $(M + 3\ H)^{3+}$	2115.2	2115.8
SFGF (63–80) $(M_c + Na)^+$	2198.7	2200.5

Two approaches to oxidation were compared for the formation of the disulfide linkages of SFGF (26–80): air oxidation and the mixed disulfide method using reduced and oxidized glutathione (Saxena & Wetlaufer, 1970). However, the duration for air oxidation as monitored by C_{18} reverse-phase HPLC was longer (>48 h) and provided a lower yield of the monomeric SFGF.

Because of the insolubility encountered in the synthesis of SFGF (26–80), a shorter SFGF analogue consisting of residues 31–80 and containing all 6 cysteinyl residues but lacking the basic terminal pentapeptide (26–30) was synthesized. SFGF (31–80) showed good solubility in all stages of the gradient dialytic refolding in urea similar to those observed for the synthesis of TGFα. Furthermore, SFGF (31–80) is acidic and becomes readily soluble in the basic refolding medium.

Purification and Characterizations. The reaction mixture after oxidation was easily purified to homogeneity by two successive C_{18} reverse-phase liquid chromatographies in 3% overall yield (Figure 2). The low yield could be attributed to the difficulties in the oxidative refolding process. Attempts to improve the yield by lowering the concentration of Gdn-HCl failed because of precipitation during further dilution.

Amino acid hydrolysis of the purified SFGF (26–80) with 5.7 N HCl was in good agreement with the expected composition (Table II). The values for Val and Ile were lower than expected and required a hydrolysis in propionic acid/12 N HCl at 130 °C (1:1 v/v) to provide the theoretical amount. Performic acid oxidation of the amino acid hydrolysate showed 6.3 cysteinyl residues, which agreed well with the expected value of 6. Analytical HPLC showed that synthetic SFGF (26–80) eluted as a single symmetrical peak (Figure 2).

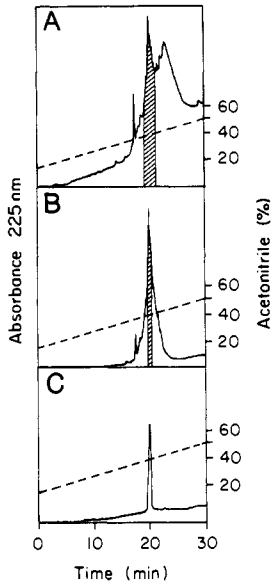

FIGURE 2: HPLC analysis on a C_{18} reversed-phase column of crude and purified Shope fibroma growth factor (26–80): (A) Crude peptide mixture after refolding disulfide pairings; (B) monomer fractions of SFGF; (C) analytical high-pressure liquid chromatography (HPLC) of synthetic SFGF (26–80) using C_{18} reverse-phase column.

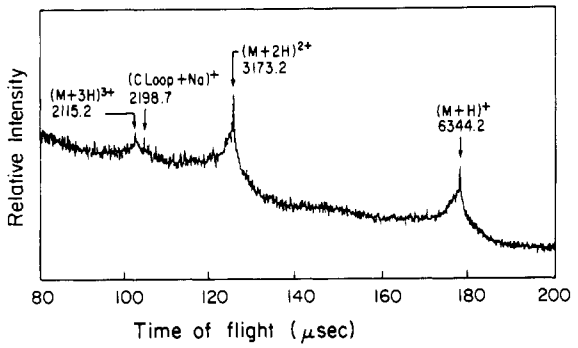

FIGURE 3: Cf-252 fission ionization mass spectrometry of synthetic SFGF (26–80).

Strong peaks shown by Cf-252 fission ionization mass spectrometry corresponding to $(M + H)^+$, $(M + 2H)^{2+}$, and $(M + 3H)^{3+}$ were detected at the expected range 6344, 3173, and 2115 (Figure 3 and Table IV). These correct values provided evidence that the synthetic SFGF (26–80) was not only monomeric but also refolded. In order to prove correct disulfide pairings, an enzyme digestion with thermolysin (Table III) was carried out and provided a fragment corresponding to the disulfide pairing of Cys^{41}–Cys^{61} (B loop in Figure 1). In addition, Cf-252 fission ionization mass spectrometry of SFGF exhibited a high mass ion at 2199 (Figure 3), corresponding to the fragment of the carboxyl-terminal region containing C loop paired by Cys^{63} and Cys^{72}. These results suggest that the disulfide pairings of synthetic SFGF (26–80) are similar to those of EGF–TGFα.

Biological Activities. The presence of a growth factor like sequence within the predicted genomic sequence of the unique Shope fibroma virus is of great interest and points to the possibility of this growth factor like sequence being a principal agent responsible for the tumorigenicity. The 55-residue synthetic Shope fibroma growth factor, SFGF(26–80), was found to compete with [^{125}I]EGF in EGF–receptor binding assay using a human epidermal carcinoma cell line, A431, with an IC_{50} value of 25 nM (Figure 4) and was about 10% as active as murine EGF (Table V). Synthetic SFGF (26–80) was also found to stimulate [^{3}H]thymidine uptake in normal

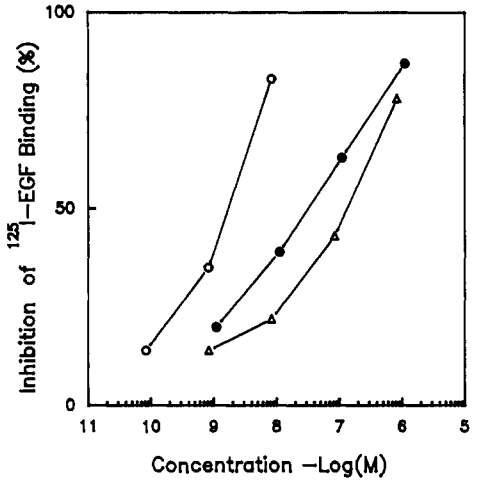

FIGURE 4: Radioreceptor assay on A431 cells. Binding curves are shown for EGF standard (○), synthetic SFGF (26–80) (●), and synthetic SFGF (31–80) (△).

Table V: Comparison of Synthetic SFGF (26–80), EGF, and TGFα by Different Assays

growth factors	binding A431[a] IC_{50} (nM)	soft-agar assay[b] EC_{50} (nM)	thymidine assay[c] EC_{50} (nM)
EGF	2	0.2	0.1
TGFα	6	0.1	0.7
SFGF (26–80)	25	1	2

[a] IC_{50} inhibition of binding of 0.7 nM [^{125}I]EGF to A431. IC = inhibitory concentration. [b] Performed in the presence of 10% calf serum. Colonies were quantified after 12 days. EC = effective concentration. [c] Incorporation of [^{3}H]thymidine was measured in NRK-49F after 8 h.

rat kidney fibroblast with EC_{50} at 2 nM; moreover, synthetic SFGF (26–80) induced colony formation of normal rat kidney fibroblast in soft-agar assay with EC_{50} at 1 nM. The latter assay has been found to correlate well with tumorigenicity (Table V).

Discussion

An 80-residue peptide encoded by a poxvirus, Shope fibroma growth factor, has been identified to be closely related to the EGF–TGFα family (Chang et al., 1987). Our results show that a 55-residue peptide comprising the homologous EGF–TGFα region and the carboxyl portion of this growth factor has the putative biological activities of the EGF–TGFα family. Furthermore, we have shown that the three disulfide alignments of this synthetic and biologically active Shope fibroma growth factor are similar to those found in EGF and TGFα.

Previously, we synthesized TGFα and its analogues rather efficiently with an overall yield of 10–30% (Tam et al., 1984, 1986; Tam, 1987). The chemical synthesis of this 55-residue Shope fibroma growth factor was found to be difficult, and we were successful at the second attempt with an overall yield of 3% (Table I). The difficulties arise during the refolding step due to extensive aggregation and precipitation of synthetic SFGF from the solution. This could be contributed by the basic character and the abundance of β-branched and hydrophobic amino acids in the SFGF molecule, which favor, at the basic pH of the refolding step, aggregation that ultimately leads to precipitation of the synthetic molecule. Thus, the refolding of the synthetic SFGF has to be conducted at a highly denaturing condition in order to disrupt the aggregation and to prevent precipitation. Moreover, the refolded and oxidized SFGF also reveals the tendency of aggregation

at concentrations >0.1 μmol, as evidenced in the EGF–receptor binding assay (Figure 4) that shows nonparallel dose–response curves between EGF and SFGF. To alleviate the aggregation problem, a shortened SFGF analogue consisting of 50 residues (31–80) but lacking the basic and hydrophobic amino-terminal residues was also synthesized. This 50-residue analogue was found to be soluble during the refolding steps, and the refolding molecule was nearly as active as the longer 55-residue analogue (Figure 4). Thus, it can be concluded that judicious choice of an appropriate sequence for chemical synthesis would minimize the hydrophobic aggregation and enhance solubility in the basic condition, which is important for the successful synthesis of a complex and cysteinyl-rich peptide.

The synthesis of SGF has made it possible for us to study this putative growth factor both in vitro and in vivo. In vitro assays, synthetic SFGF binds to the EGF–receptor, activates the receptor tyrosine-specific kinase (data not shown), and induces mitogenicity and colonies in soft agar. Furthermore, synthetic SFGF is found to be as active as mouse EGF or rat TGFα (Cohen, 1962; Tam, 1985) in inducing precocious eyelid opening and incisor eruption in newborn mouse (Ye et al., 1988). Taken together, these biological results show that synthetic SFGF comprising residues 26–80 of the deduced DNA sequence represents a fully biological active growth factor sequence. Thus, our synthesis of SFGF (26–80) and (31–80) presents an approach to confirm putative biological properties of a growth factor derived from the DNA gene products.

Two other members of the poxvirus family also encode the closely related EGF–TGFα sequences in their DNA sequences. Myxoma virus, an agent of myxomatosis, also encodes an 85-residue EGF-like growth factor (Upton et al., 1987). This myxoma virus EGF-like sequence shares 80% homology (within the EGF-like domain) with the 80-residue Shope fibroma EGF-like sequence. We have also synthesized this growth factor sequence from myxoma virus (MGF) and found that it contains the putative EGF growth factor like properties (Y.-Z. Lin and J. P. Tam, unpublished results). A 77-residue EGF-like peptide encoded by another poxvirus member, vaccinia virus, has been isolated and also found to possess EGF-like growth factor activities in vitro (Stroobant et al., 1985). Surprisingly, this EGF-like growth factor from vaccinia shares only limited sequence homology with either SFGF (37%) or MGF (34%). The presence of biological EGF-like sequences in the poxvirus family points to the possibility that this growth factor may play an important role in the life cycle of the poxvirus. It is possible that the binding of the EGF receptor by the EGF-like growth factor from the poxvirus provides the stimulation and proliferation of a specific class of host cell such as the epidermal cells, which might contribute to the disposition of the virus to infect epidermal cells. In turn, the production of EGF-like growth factor from this DNA virus might explain the distinct EGF-responsive cellular proliferation and benign tumors associated with the poxvirus family.

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