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Biochemistry

A synthetic approach to structure–function relationships in the murine epidermal growth factor molecule

(solid-phase peptide synthesis/epidermal growth factor fragments/acid deprotection)

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ABSTRACT **Murine epidermal growth factor, a 53-amino acid peptide that is mitogenic for a number of cell types, has been synthesized by the solid-phase method. The synthetic peptide is identical to the natural material in amino acid composition, chromatographic behavior, receptor binding, and stimulation of DNA synthesis. Fragments of the EGF molecule corresponding to residues 42–53, 32–53, and 15–53 were constructed as well as the methionine sulfoxide derivative of EGF, [Met(O)^{21}]EGF-(1–53), and a polymeric form of EGF. [Met(O)^{21}]EGF-(1–53) was slightly less active than EGF in receptor binding and stimulation of DNA synthesis. Polymeric EGF was 1/100th as active as EGF, while EGF-(15–53) was less potent than EGF-(1–53) by a factor of 10^4. EGF-(32–53) was even less active and EGF-(43–53) was inactive.**

Epidermal growth factor (EGF) is a 53-amino acid single-chain polypeptide isolated from the submaxillary gland of mice in 1962 (1). EGF is mitogenic for a number of cell types and demonstrates a variety of actions *in vitro* and *in vivo*, including stimulation of metabolite transport, activation of glycolysis, stimulation of production of RNA, protein, and DNA, enhancement of cell proliferation, alteration of cell morphology, and inhibition of gastric acid secretion (2). EGF contains three disulfide bridges of known orientation (Fig. 1) (3). Studies using circular dichroism and nuclear magnetic resonance (4–6) suggest that EGF forms a three-tiered antiparallel β-sheet structure formed with several chain reversals (β-turns). There have been limited studies of the activities of synthetic peptide segments of EGF. Komoriya *et al.* found that the linear and cyclic forms of the B-loop peptide (residues 20–31) were active in both receptor binding and biological stimulation in cell culture but to a very low extent (0.003% of EGF), while the other loops were inactive (7). On the basis of these data, they proposed that the B loop of EGF contains both the receptor-binding and biological-stimulation activities of the molecule. However, Nestor *et al.* have found that the cyclic C-loop peptide of the homologous transforming growth factor-α (residues 34–43) binds to the EGF receptor (0.2% of EGF binding) but does not stimulate mitogenesis (8).

Due to its relatively small size, the EGF molecule is a target for total synthesis. The mouse EGF molecule possesses a complex structure containing three disulfide bridges and a large number of trifunctional amino acids, presenting a considerable challenge to the peptide chemist. However, substantial improvements in the methodology of solid-phase peptide synthesis have occurred in recent years (9), and it was felt that such a synthesis of mouse EGF would be a good test of the method and would provide ready access to EGF derivatives. In this paper, we describe the synthesis of murine EGF and several analogs by the solid-phase method.

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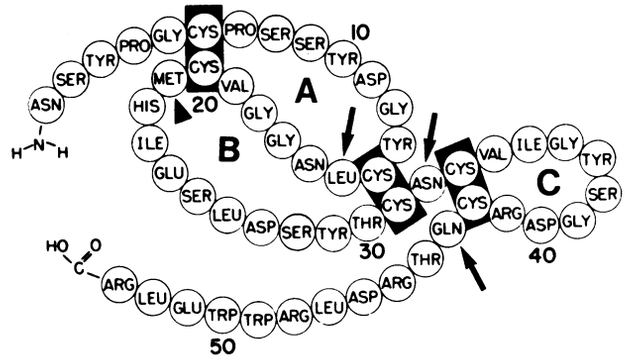

FIG. 1. Structure of mouse EGF. Arrows mark amino-terminal residues of synthetic EGF fragments, and the triangle denotes the position of the sulfoxide functional group in [Met(O)]EGF.

MATERIALS AND METHODS

Chemicals. Boc-amino acids were from Peninsula Laboratories (San Carlos, CA). The following side-chain-protected amino acids were used: Arg(Tos), Asp(OcHex), Cys(4-MeBzl), Glu(OBzl), His(Dnp), Met(O), Ser(Bzl), Thr(Bzl), Trp(For), and Tyr(Br-Z). All other chemicals and their purification have been described (10).

Synthetic Protocol. Aminomethyl-resin (0.40 mmol/g) prepared by the method of Mitchell *et al.* (11, 12) was allowed to react with Aoc-Arg(Tos)-4-(oxymethyl)phenylacetic acid (13) to give the starting Aoc-Arg(Tos)-Pam-resin (Aoc, *tert*-amyloxycarbonyl; Pam, phenylacetamidomethyl). [^{3}H]Leu was incorporated into position 52 at a specific activity of 1.72 mCi/mmol (1 Ci = 37 GBq). Synthesis of the peptide was performed manually in a 175-ml reaction vessel using the protocol previously described (10), with two exceptions: 0.1% dimethyl sulfide was added to the trifluoroacetic acid deprotection step to avoid alkylation of protected cysteine thioethers by *tert*-butyl cations (14), and the second coupling step involved the use of preformed symmetrical anhydrides of Boc-amino acids in dimethylformamide.

EGF-(1–53). The completed synthetic EGF-(1–53) protected peptide-resin was treated with 1 M thiophenol in dimethylformamide to deprotect His(Dnp) and 50% trifluoroacetic acid/CH_2Cl_2 to remove the amino-terminal Boc protecting group. The resin was then treated with HF/Me_2S/*p*-cresol/*p*-thiocresol (25:65:8:2, vol/vol, 0°C, 2 hr) followed by HF/*p*-cresol/*p*-thiocresol (90:8:2, vol/vol, 0°C, 1 hr) (15). After the HF was removed, the residue was stirred with diethyl ether/2-mercaptoethanol (49:1, vol/vol) to remove organic contaminants, and then the peptide was solubilized in 0.5 M

Abbreviations: Aoc, *tert*-amyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Br-Z, 2-bromobenzyloxycarbonyl; cHex, cyclohexyl; Dnp, 2,4-dinitrophenyl; EGF, epidermal growth factor; For, formyl; MeBzl, methylbenzyl; Met(O), methionine sulfoxide; Pam, phenylacetamidomethyl; Tos, *p*-toluenesulfonyl.

Tris·HCl/8 M urea/0.1 M dithiothreitol, pH 8.6. Quantitation of ^3H indicated a cleavage yield of 97%. The aqueous solution from the HF workup was transferred to a Spectra (Santa Clara, CA)/Por 6 dialysis bag (molecular weight cutoff, 1000) and dialyzed against 2 liters of N_2-saturated 0.1 M Tris·HCl/8 M urea, pH 8.8, for 16 hr. This was followed by dialysis against 2 liters of N_2-saturated 0.1 M Tris·HCl/4 M urea, pH 8.7 for 8 hr; 4 liters of N_2-saturated 0.05 M NH_4HCO_3, pH 7.8, for 8 hr; and 7 liters of N_2-saturated 0.01 M NH_4HCO_3, pH 7.8, for 18 hr. The slight precipitation that occurred during the dialysis was removed by centrifugation. The supernatant was diluted to 800 ml with H_2O and the peptide was allowed to fold and oxidize by gentle stirring for 7 days. The resulting solution was lyophilized.

Preparation of EGF Derivatives. During the course of the chain assembly of EGF-(1–53), portions of peptide-resin were removed and used to produce the EGF fragments, 43–53, 32–53, and 15–53 (see *Results*).

Biological Assays. EGF binding assays were performed on subconfluent layers of A-431 human carcinoma cells (8). Incorporation of [^3H]thymidine was measured in normal rat kidney fibroblasts, clone 49F (16).

RESULTS

The synthesis was initiated with 5 g of 0.40 mmol/g aminomethyl-resin, which was then derivatized to give Aoc-Arg(Tos)-Pam-resin in 99.9% yield. After incorporation of [^3H]Leu at the second step (position 52) to facilitate monitoring of the synthesis, the peptide chain was elongated with a double coupling protocol using CH_2Cl_2 and dimethylformamide as the solvents. This alternate solvent procedure is designed to ensure efficient coupling as the growing peptide chain alters the nature of the peptide-polystyrene polymer (17). If the ninhydrin monitoring (18) indicated that there was less than 99.9% incorporation of a particular residue after two couplings, a third coupling was done with the appropriate coupling conditions. The peptide-resin rapidly increased in weight and in its swelling properties as the peptide chain increased in length. After the addition of 10 residues, the product was already 50% peptide by weight, subsequently increasing to 80% by weight after the completion of the 53-amino acid sequence. The large increase in swelling of the peptide resin in both solvents necessitated a switch to a larger reaction vessel (350 ml), more thorough washing to counteract the greater solvent holdup by the polymer, and an increased number of base washes after the acid deprotection step to ensure complete deprotonation of the amino terminus of the peptide.

At the end of the synthesis and after removal of 800 mg (polystyrene weight basis) of resin samples for ninhydrin tests, resin hydrolyses, and EGF fragment production, 21 g of protected peptide resin was obtained. Hydrolysis of the peptide resin and subsequent amino acid analysis gave a substitution level of 0.40 mmol/g of polystyrene, indicating that the assembly of the peptide was essentially quantitative and that there was no apparent loss of peptide chains during the synthesis. Furthermore, quantitative ninhydrin analysis of the amino-terminal-deblocked resin gave a substitution value of 0.40 mmol/g, in agreement with the hydrolysis data and indicating that no appreciable termination of peptide chains had occurred during the synthesis. These data are expected, given the relative acid stability of the linkage to the Pam resin (11, 12) and improvements in the synthetic chemistry (9).

HF Deprotection. After removal of the Dnp group from histidine by thiolysis and the N^{α}-Boc group by 50% trifluoroacetic acid/CH_2Cl_2, the EGF-resin was treated with the HF/Me_2S/*p*-cresol/*p*-thiocresol procedure to simultaneously deprotect the side-chain protecting groups and cleave the peptide from the resin. HF cleavage yields were always >95%. Stirring the residue in Et_2O was used to remove the organic components of the reaction—i.e., cresols—mercaptoethanol being added to prevent oxidation due to any peroxide contaminants. The solubilization of the peptide after the Et_2O washes proved to be difficult, and initial efforts involving aqueous acetic acid and basic Tris buffers were less than successful. Finally, the use of basic buffers under denaturing conditions such as 8 M urea or 6 M guanidine hydrochloride proved satisfactory. Thiols were added to ensure total reduction of the EGF cysteine thiols. Later, it was found that the HF-deprotected EGF could be solubilized equally well in 1 M aqueous NH_3 alone. Dialysis was used to remove water-soluble contaminants such as benzyldimethylsulfonium salts from the peptide solution. Sequential dialysis against basic buffers containing decreasing amounts of urea was necessary to prevent precipitation of the protein. The buffers were kept as oxygen free as possible by degassing and subsequent N_2 saturation to prevent premature disulfide formation of the still denatured EGF. While slight precipitation of the peptide occurred on several occasions, very little (<5%) of the protein was lost during dialysis when the 1000 molecular weight cutoff dialysis tubing was used.

After the dialysis, two approaches to oxidation were compared for the formation of the disulfide bridges of EGF: air oxidation in dilute basic solution and the mixed disulfide method using reduced and oxidized glutathione (19). When the mixed disulfide method was used, an EGF fraction containing covalently bound glutathione could be isolated. After complete air oxidation of the crude EGF, HPLC analysis showed that the product consisted of a monomer fraction and polymeric fraction. The monomeric fraction contained 14% of the total protein and exhibited one major peak (>70%) on HPLC (Fig. 2) which was easily purified to homogeneity by using low-pressure liquid chromatography on C_{18}-silica in 10% overall yield (Table 1). The use of several less effective protocols gave lower but variable yields. The pure synthetic EGF coeluted with natural EGF on analytical HPLC (Fig. 2) and gave a good amino acid analysis (Table 2).

A number of acid deprotection conditions along with workup, dialysis, and oxidation conditions were examined to increase the ratio of monomer to polymer, with mixed results. The various schemes showed some difference in monomer yield but the result was always less than 15% of the total protein. When natural EGF was reduced, dialyzed against urea buffers, and then air oxidized, only low levels (<10%) of monomer could be recovered, with the remainder of the material behaving like polymer on HPLC.

Polymer. Investigation of the EGF polymer fraction revealed that it consisted predominantly (>90%) of dimers and

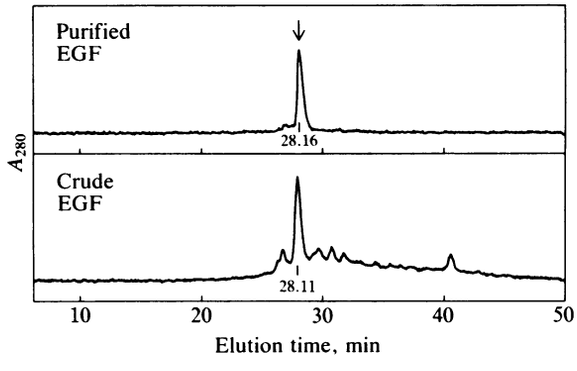

FIG. 2. HPLC of crude and pure synthetic EGF on Vydac (Hesperia, CA) C_{18} using a 45-min linear gradient of 26–38% (vol/vol) CH_3CN into H_2O/0.1% $CF_3CF_2CF_2CO_2H$, 1.5 ml/min. Arrow indicates the elution position of natural EGF.

Table 1. Yields of synthetic EGF-(1–53) and analogs

Compound	Disulfide bonds	Overall yield*
EGF-(1–53)	3	10†
[Met(O)]EGF-(1–53)	3	9
EGF-(43–53)	0	76
EGF-(32–53)	1	48
EGF-(15–53)	2	45

*Based on starting amino acid-resin.
†Recycling of polymer yielded an additional 1–5% monomer.

trimers on HPLC gel permeation chromatography and had an amino acid composition comparable to that of the monomer fraction (Table 2). Reduction and carboxymethylation of the polymer produced a material that was indistinguishable from the reduced and alkylated monomer on HPLC and by amino acid composition. *S*-Aminoethylation of the monomer and polymer fractions also suggested that the cysteine contents of the two were similar, ruling out loss of cysteine as the cause of polymer formation. Denaturation and reduction of the polymer followed by isolation of the reduced peptide and subsequent oxidation produced 1–5% more EGF monomer but was not fully satisfactory. Attempts to convert the polymer to monomer with treatment of the polymer in urea buffers by the mixed disulfide method were unsuccessful.

EGF Derivatives. To produce the methionine sulfoxide derivative of EGF-(1–53), the protected peptide-resin was deprotected and cleaved in high HF (HF/*p*-cresol, 9:1, vol/vol), since methionine sulfoxide is stable to high HF. All the synthetic EGF fragment peptide-resins were deprotected with low/high HF as for EGF-(1–53). The amino-terminal glutamine of EGF-(43–53) was cyclized to pyroglutamic acid in aqueous acetic acid (20). All fragments were purified to homogeneity by using low-pressure liquid chromatography (Table 1) and gave the expected amino acid ratios (Table 2).

Radioreceptor Assay on A-431 Cells. Binding of the growth factors to the EGF receptor was tested with A-431 cells. The

Table 2. Amino acid composition of synthetic EGF compounds

Amino acid	Molar ratio*					
	EGF-(1–53)		EGF-	EGF-	EGF-	[Met(O)]-EGF-
	Monomer	Polymer	(43–53)	(32–53)	(15–53)	(1–53)
Asp	7.0 (7)	6.9 (7)	1.0 (1)	3.0 (3)	4.9 (5)	7.0 (7)
Thr†	1.6 (2)	1.6 (2)	0.9 (1)	0.9 (1)	1.5 (2)	1.6 (2)
Ser†	5.2 (6)	5.2 (6)	—	1.0 (1)	2.6 (3)	5.2 (6)
Glu	3.1 (3)	3.2 (3)	1.9 (2)	2.2 (1)	2.9 (3)	3.2 (3)
Pro	2.0 (2)	2.0 (2)	—	—	—	2.0 (2)
Gly	6.2 (6)	6.1 (6)	—	2.1 (2)	3.9 (4)	6.2 (6)
Cys‡	6.0 (6)	5.2 (6)	—	1.9 (2)	3.8 (4)	6.0 (6)
Val	1.8 (2)	1.8 (2)	—	0.8 (1)	1.5 (2)	1.8 (2)
Met	0.9 (1)	0.8 (1)	—	—	0.8 (1)	0.9 (1)§
Ile	1.9 (2)	1.9 (2)	—	0.9 (1)	1.9 (2)	1.9 (2)
Leu	4.3 (4)	4.3 (4)	2.0 (2)	2.3 (2)	4.1 (4)	4.3 (4)
Tyr	5.1 (5)	5.0 (5)	—	1.2 (1)	2.1 (2)	5.1 (5)
His	1.1 (1)	1.0 (1)	—	—	1.3 (1)	1.1 (1)
Arg	4.1 (4)	4.1 (4)	3.2 (3)	4.6 (4)	3.9 (4)	4.1 (4)
Trp¶	2.0 (2)	1.9 (2)	2.0 (2)	2.0 (2)	1.7 (2)	2.0 (2)

Hydrolyses were performed in sealed, evacuated tubes with 5.7 M HCl, 110°C, 24 hr.
*One residue = (all residues but Thr and Ser)/*N*, in which *N* is the theoretical number of these residues present. Values in parentheses are theoretical.
†Thr and Ser were not corrected for loss during hydrolysis.
‡Cysteine was determined as cysteic acid after performic acid oxidation.
§Determined as Met(O) by using 3 M *p*-toluenesulfonic acid hydrolysis.
¶Trp was determined by using 4 M methanesulfonic acid hydrolysis.

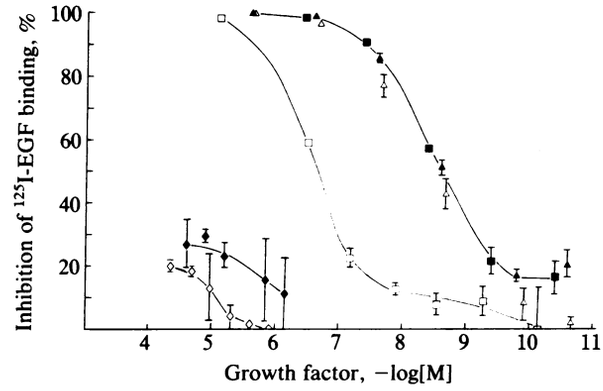

FIG. 3. Radioreceptor assay on A-431 cells. Binding curves are shown for synthetic (▲) and natural (■) EGF-(1–53); [Met(O)]EGF-(1–53) (△); polymer EGF (□); EGF-(15–53) (◆); and EGF-(32–53) (◇).

growth factor to be tested was mixed with 0.5 ng of ^{125}I-labeled EGF-(1–53) and allowed to bind for 1.5 hr at 4°C. Nonspecific binding was less than 5%. Binding was expressed as the ability to inhibit binding of the iodinated EGF to the receptor. Synthetic EGF-(1–53) and natural EGF-(1–53) [Collaborative Research (Waltham, MA), receptor grade] bound to the EGF receptor with similar affinities, as seen in Fig. 3. [Met(O)^{21}]EGF-(1–53) bound with a lower affinity (30% less) than EGF but still saturated the receptor at high doses (Fig. 3). Polymer EGF bound with 1/100 the affinity of EGF-(1–53), while EGF-(15–53) bound less well than EGF by a factor of 10^4. EGF-(32–53) showed even lower binding ($<1/10^5$ of EGF), and there was no detectable binding by EGF-(43–53).

Stimulation of DNA Synthesis. The ability of the growth factors to stimulate DNA synthesis was measured by the incorporation of [^{3}H]thymidine in normal rat kidney fibroblasts. As shown in Fig. 4, synthetic and natural EGF gave similar curves. [Met(O)^{21}]EGF-(1–53) was less potent than EGF, while polymer EGF was 1/100 as active. Of the fragments, only EGF-(15–53) was capable of stimulating DNA synthesis. EGF-(32–53) appeared to slightly inhibit DNA synthesis, while EGF-(43–53) was inactive in the assay.

DISCUSSION

The synthesis by the solid-phase method of a molecule as complex as EGF can be considered a rigorous test of the improvements in the chemistry of such a method. We emphasize that attention to detail—i.e., use of clean, well-

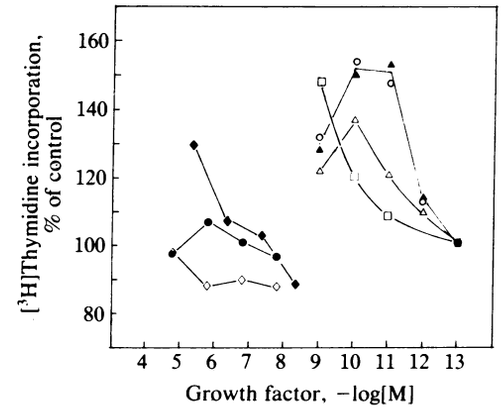

FIG. 4. Thymidine incorporation in NRK cells. Incorporation is shown for synthetic (▲) and natural (○) EGF-(1–53); [Met(O)]EGF-(1–53) (△); polymer EGF (□); EGF-(15–53) (◆); EGF-(32–53) (◇); and EGF-(43–53) (●).

characterized reagents, proper synthetic protocols, and monitoring of amino acid incorporation—is necessary to synthesize a peptide of high quality. As the experimental data show, the assembly of the 53-amino acid residue protected peptide on the resin proceeded smoothly with no indication of synthetic difficulties. The final peptide-resin was obtained in 99% yield based on weight gain of the resin, the ninhydrin test, and amino acid analysis. The incorporation of [^{3}H]Leu at position 52 greatly facilitated monitoring and quantitation of the synthetic peptides.

The acid deprotection step was a key step in the entire synthesis due to the fact that EGF contains a number of sensitive residues and sequences—i.e., Met, Trp, Cys, Tyr, Asp-Gly, and Asp-Ser. The low/high-HF procedure removed most of the precursors of harmful carbonium ions by an S_N2 mechanism before the final strong acid S_N1 step began. For EGF-(1–53) protected peptide resin, the low-HF step deprotected most benzyl type protecting groups, reduced Met(O) to Met, and converted Trp(For) to Trp. However, to fully cleave the linkage to the Pam-resin and to deprotect Asp(OcHex), Arg(Tos), and Cys(4-MeBzl), a second high-HF step was necessary. Using this method, we routinely obtained cleavage yields of 95%.

Subsequent results demonstrated that the most difficult part of the synthesis lay not in the area of chain assembly and acid deprotection but in the area of workup, renaturation of the molecule, and formation of the disulfide bonds. The reduced and denatured protein was difficult to handle, was very hydrophobic, and tended to aggregate. The yield of monomeric EGF-(1–53) after oxidation was always low (<15%) and did not increase in response to varied deprotection methods and the subsequent handling of the deprotected peptide. Experiments tend to rule out damage to the peptide during HF as a cause for low monomer yield and instead point to difficulty in refolding and oxidation of the molecule. Investigation of the polymer showed that it was mostly a mixture of dimers and trimers which could be reduced to form a material that behaved like reduced EGF monomer.

Earlier workers (21) reported that EGF activity could be recovered after reduction and denaturation of natural EGF followed by renaturation and air oxidation, but the EGF activity was never physically or chemically characterized. Results in this laboratory and others suggest that EGF recovery as monomeric EGF is low after reduction and oxidation and that the reduced EGF is unstable and tends to aggregate and polymerize. Lending some support to this theory is the fact that three syntheses of EGF (two classical solution and one solid-phase) using radically different synthetic approaches all suffered from low yields despite starting from pure or near-pure protected EGF-(1–53). Akaji *et al.* synthesized mouse EGF by using solution chemistry and CF_3SO_3H/CF_3COOH deprotection and obtained a 4.1% yield of pure EGF (22). This report also discusses the difficulty in converting the deprotected reduced molecule into the fully oxidized form. Human EGF (β-urogastrone) was synthesized by using solution chemistry and deprotected in two steps by HF and mercuric acetate in acetic acid to give pure human EGF in 2.6% yield (23). Mouse EGF synthesized by the solid-phase method (this report) and deprotected by using low/high-HF gave pure EGF in 10% yield.

Since EGF is synthesized *in vivo* as a 1200-amino acid precursor (24, 25) that is then processed to the 53-amino acid peptide, the disulfide bonds probably form before processing. Therefore, the folding of the precursor may be more favorable to the proper disulfide bridge formation. In the absence of the precursor sequences, the peptide may predominantly form the wrong intra- and intermolecular disulfide pairs and produce higher molecular weight products.

While the full 53-amino acid sequence was difficult to fold and oxidize to monomer, the smaller fragments were not. EGF-(32–53) and EGF-(15–53), containing two and four cysteines, respectively, refolded in high yield to monomers that could easily be purified to homogeneity (Table 1). Furthermore, the EGF-(15–53) polymer could be recycled in high yield. However, it should be noted that the disulfide pairs of the 15–53 fragment have not yet been established with certainty. The segment from positions 1–14 appears to be the cause of the folding difficulties. It does contain two extra cysteines and several predicted β-turns (26) that could complicate refolding.

A comparison of synthetic efficiency between solution and solid-phase methods in the synthesis of mouse EGF shows that to produce 0.108 mmol of protected EGF by the solution method, Akaji *et al.* used a total of 161.7 mmol of amino acids (22), and production of 0.108 mmol of protected EGF by the solid-phase method required 46.4 mmol of amino acids. By this criterion, the solid-phase method was somewhat more efficient than the solution method in producing EGF. We think the savings in time and labor are more important advantages.

Biological Activity of Growth Factors. *(i) Binding to the EGF receptor.* The receptor-binding assay using A-431 cells showed that synthetic and natural EGF bound with equal affinities (Fig. 3), confirming chemical evidence that synthetic EGF-(1–53) is identical to natural EGF-(1–53). [Met(O)^{21}]EGF-(1–53) bound slightly less well than natural EGF, indicating that the introduction of the sulfoxide functionality perturbs the binding (Fig. 3). This perturbation could be due to a change in the conformation or the polarity of the binding surface in the EGF molecule. The polymeric form of EGF, which consists of dimers and trimers, bound to the EGF receptor with 1/100 of the affinity of EGF. Either the polymer has a defined structure with very little affinity for the EGF receptor or it consists of a number of various structures with a limited subpopulation of structures having the ability to interact with the receptor.

EGF-(15–53) bound significantly to the EGF receptor, although very high concentrations were needed (10^4 more than EGF). EGF-(32–53) showed some binding at high concentration but was less potent than EGF-(15–53). EGF-(43–53) was totally unable to compete with ^{125}I-labeled EGF-(1–53) for the receptor. The results with EGF-(15–53) and -(32–53) appear to support the view that the B loop of EGF is important for receptor binding as opposed to the C loop. On the other hand, the greatly diminished affinity of all of the fragments points to a role of residues 1–14 in organizing the structure and presumably the binding surface of the EGF molecule.

(ii) Stimulation of DNA synthesis. The ability of the various growth factors to stimulate mitogenesis was tested with normal rat kidney fibroblasts by measuring the incorporation of [^{3}H]thymidine into DNA. This assay, although less accurate than the receptor binding assay, gave qualitatively similar results. As seen in Fig. 4, synthetic and natural EGF-(1–53) gave similar curves. The methionine sulfoxide derivative of EGF, [Met(O)^{21}]EGF-(1–53), gave less stimulation than EGF in this assay, while the polymeric form of EGF was 1/100 as active as EGF-(1–53). Of the fragments, only EGF-(15–53) was capable of inducing DNA synthesis, albeit only at very high concentrations. The activity of this derivative as well as that of [Met(O)^{21}]EGF-(1–53) lends further support to the role of the B loop in activity of the molecule. EGF-(43–53) was totally inactive in this assay. On the other hand, EGF-(32–53) appeared to be capable of inhibiting the mitogenic response to some extent. This supports the findings of Nestor *et al.* (8) that the C loop is capable of binding but not stimulation of mitogenesis. Occupation of the EGF receptor by EGF-(32–53) would inhibit the stimulation of mitogenesis by EGF in the calf serum in this assay.

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