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Articles

Murine Epidermal Growth Factor: Structure and Function

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ABSTRACT: Murine epidermal growth factor (EGF), a 53 amino acid protein, has been modified by enzymic digestion, site-specific chemical reactions, and recombinant DNA technology. After trypsin digestion the EGF derivatives EGF_{1-48} (called EGF-T) and EGF_{1-45} (called EGF-T_2) were separated from the residual EGF and the C-terminal pentapeptide by reversed-phase high-performance liquid chromatography. EGF-T competes for binding to EGF receptors with the same efficiency as EGF. The EGF-T_2 derivative had no detectable receptor binding activity even at 100 nM. The in vitro mitogenic potencies of EGF and EGF-T for Balb/c 3T3 cells were indistinguishable. Treatment of EGF-T with carboxypeptidase Y yielded two derivatives, EGF-T-(des-Arg_{48}) and EGF-T-des(Leu_{47}-Arg_{48}). There was only a 3–7-fold diminution in the binding efficiency and mitogenic potency for EGF-T-(des-Arg_{48}). However, there was more than a 100-fold decrease in the binding efficiency and mitogenic activity of EGF-T-des(Leu_{47}-Arg_{48}). These results indicated that Leu_{47} is intimately involved in the formation of the ligand–receptor complex. Studies with a number of proteases indicated that the C-terminus of EGF was susceptible to enzymic digestion; however, the N-terminus appears to be folded into a conformation which prevents access to proteolytic digestion. Consequently, the N-terminus was modified by preparing an analogue with recombinant DNA technology. Oligonucleotides corresponding to $EGF_{(3-48)}$.Met_3.Lys_{21} residues were ligated in frame to a β-galactosidase expression vector. The β-Gal–EGF fusion protein was cleaved with cyanogen bromide and $EGF_{(4-48)}$.Lys_{21} purified. This derivative was equipotent with EGF in the mitogenesis assay and bound to the EGF receptor with the same affinities as EGF. Disruption of the central antiparallel β-sheet structure of EGF at Met_{21} by treatment of EGF with cyanogen bromide reduced both the binding efficiency and the mitogenic activity of EGF more than 100-fold.

Murine epidermal growth factor (EGF) is a protein of 53 amino acids capable of stimulating the proliferation of both mesenchymal and epithelial cells (Savage et al., 1972; Taylor et al., 1972). Considerable progress is being made toward the determination of the three-dimensional structure of EGF (Mayo, 1985; Mayo et al., 1986; Montelione et al., 1986, 1987; Carver et al., 1986; Cooke et al., 1987), but there has been little progress toward an understanding of the function of particular residues or even specific regions of the EGF molecule. It is important to distinguish between the residues required for the folding and assembly of the three-dimensional structure, as well as those residues required for the binding to and activation of the EGF receptor. Preparation of EGF's and their analogues by complete chemical synthesis (Akaji et al., 1985; Heath & Merrifield, 1986; Tam et al., 1986; Scanlon et al., 1987) and by bacterial synthesis (Sumi et al., 1985) has shown that EGF mitogen can fold to its biologically active form. However, attempts to produce subdomains with full biological or potent antagonist activity have yielded derivatives with very low potency (Komoriya et al., 1984; Nestor et al., 1985; Heath & Merrifield, 1986). It is still not clear whether these analogues are missing specific residues important for the binding of EGF to its receptor or whether the short segments fail to fold into the conformation required for receptor binding. Disruption of the three-dimensional structure of EGF e.g., by reduction and carboxymethylation (Savage et al., 1972), destroys both its receptor binding and its biological activity. However, disruption of the polypeptide backbone by cyanogen

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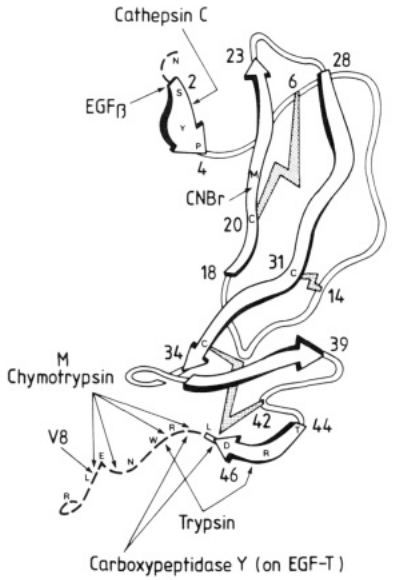

FIGURE 1: Cleavage sites for a number of proteolytic enzymes and cyanogen bromide are superimposed on the model for the three-dimensional structure of murine EGF (Montelione et al., 1987). EGF-β is a naturally occurring derivative (Burgess et al., 1982) and EGF-T (1–48) is the initial tryptic cleavage product. Leucine residue 47 (L) is conserved in all molecules known to bind to the EGF receptor (Simpson et al., 1985).

bromide cleavage at Met_{21} appears to alter the three-dimensional structure in a way which permits receptor binding but destroys its mitogenic activity (Yarden et al., 1982). The conversion of Met_{21} to the homoserine lactone is unlikely to be responsible for the altered mitogenic activity, as a substitution of Leu for Met at position 21 (Sumi et al., 1985) does not reduce its mitogenic activity.

EGF stimulates cellular proliferation by binding to a cell surface receptor kinase. This receptor also binds transforming growth factor α (TGF-α) which is related to EGF (Marquardt et al., 1984; Derynck et al., 1984); however, TGF-α is missing the final few residues of the EGF motif (Figure 1). Previous studies from our laboratories indicated that rat EGF (Simpson et al., 1985) was also lacking the final five residues present in the murine EGF. It had been reported previously that if the five C-terminal residues of EGF are removed by trypsin digestion (Savage et al., 1972), then the resultant molecule (EGF_{1-48}, also called EGF-T) is active in vivo but its in vitro activity appears to be impaired (Cohen et al., 1975). This result has always appeared anomolous, and we have investigated the binding and mitogenic activity of two EGF derivatives generated by partial tryptic digestion, namely, EGF_{1-48} (i.e., EGF-T), and EGF_{1-45} (i.e., EGF-T_2). The function of the C-terminal region of the EGF polypeptide chain was investigated further by sequential degradation of EGF-T with carboxypeptidase Y. The importance of residues at the N-terminus of EGF-T was investigated by preparing and purifying an amino-terminal truncated EGF-T analogue from *Escherichia coli* with the Müller-Hill expression system (Ruther & Müller-Hill, 1983).

As mentioned earlier, when EGF is split at Met_{21} by cyanogen bromide, the molecule can still bind to its receptor, but it is no longer mitogenic (Yarden et al., 1982). These properties of CNBr-cleaved EGF led to the suggestion that there are two distinct functional domains in the EGF molecule: one for receptor binding and another which mediates the mitogenic transduction. We have prepared CNBr-EGF and carefully separated the unmodified EGF from (CNBr)-cleaved EGF and compared quantitatively the binding and mitogenic activity of the native molecule with those of the CNBr-cleaved EGF derivative.

Materials and Methods

Reagents. Sodium chloride and hydrochloric acid (Aristar grade) and cyanogen bromide (Analar grade) were purchased from BDH (Poole, U.K.). Trifluoroacetic acid (99+% pure grade) was obained from Pierce (Rockford, IL) and [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) from Sigma (St. Louis, MO). All other chemicals were of the highest grade commercially available.

TPCK-treated trypsin and α-chymotrypsin were purchased from Worthington Biochemical Co. (Freehold, NJ) and carboxypeptidase Y (from yeast) from Boehringer Mannheim Biochemicals (Indianapolis, IN). *Staphylococcus aureus* protease V8 was purchased from Miles Laboratories Inc. (IL).

All organic solvents were of HPLC grade. Deionized water, obtained from a tandem Milli-RO and Milli-Q system (Millipore Inc., Bedford, MA), was used for all buffers.

High-Performance Liquid Chromatography. All separations were performed on a Hewlett-Packard Model 1090 M liquid chromatograph equipped with a Model 1040 diode array detector and a Model 79994A work station. Samples were injected with a Model 7126 rheodyne valve, equipped with a 2-mL loop, installed in the column oven compartment. Eluent fractions were collected manually (allowing for the delay due to the dead volume of the tubing between the detector flow cell and the collection port) or automatically with a Pharmacia FRAC 100 collector (Pharmacia, Uppsala, Sweden). Proteins were detected by their absorance at 215 and 280 nm. Tryptophan- and tyrosine-containing peptides were identified from their characteristic second-derivative absorption spectra (minima at 290 ± 2 and 280 ± 2 nm, respectively) as described previously (Grego et al., 1986).

Column Supports. Analytical and micropreparative reversed-phase (RP) separations (up to 50 μg) were performed on Brownlee RP-300 "guard" columns (30 × 2.1 mm i.d., Brownlee Labs, Santa Clara, CA) (Nice et al., 1985). Preparative separations (50–500 μg) were carried out on Brownlee RP-300 (100 × 4.6 mm i.d.) columns. Proteins were separated with a linear 60-min gradient between 0.15 (v/v) aqueous trifluoroacetic acid (TFA) as the primary solvent and 60% acetonitrile/40% H_2O/0.125% TFA as the secondary solvent. A flow rate of 0.1 mL/min was used for the 2.1 mm i.d. column and 1 mL/min for the 4.6 mm i.d. columns. Column temperature was maintained at 45 °C.

Anion-exchange (AEX) separations were performed on a Pharmacia Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) by use of linear gradients between a primary solvent of 20 mM Bis-Tris (pH 6.7) and a secondary solvent of 20 mM Bis-Tris (pH 6.7) containing 0.5 M sodium chloride as indicated in the figure legends. The flow rate was 0.5 mL/min, and the column temperature was maintained at 25 °C.

Preparation of Derivatives. Murine epidermal growth factor (form α1) was purified from acid extracts of male submaxillary glands according to the HPLC methods described previously (Burgess et al., 1982, 1983). These preparations were found to be greater than 99% homogeneous as indicated by both RP- and AEX-HPLC. Preparations were stored at 4 °C in a 0.15% aqueous TFA solution. Aliquots were dried in a Speedi-Vac concentrator prior to being dissolved in the appropriate buffers for chemical or enzymic modification.

Cyanogen Bromide Treatment. $EGF_{\alpha 1}$ (200 μg) was dissolved in 100 μL of 70% aqueous formic acid containing 10 mg of cyanogen bromide. The tube was flushed with nitrogen prior to incubation for 18 h at 25 °C. The sample was diluted

to 1 mL with water for injection onto the Brownlee RP-300 column. Material recovered from this column was diluted 5-fold with the 20 mM Bis-Tris (pH 6.7) buffer for further purification by AEX-HPLC as described in Figure 6.

Trypsin Digestion. $EGF_{\alpha 1}$ (500 μg) was dissolved in 100 μL of 0.1 M sodium phosphate (pH 7.0) containing 1 mM $CaCl_2$ and digested with TPCK-treated trypsin at an enzyme/substrate ratio of 1:50 (w/w) for 90 min at 37 °C. The peptides generated under these conditions were separated by RP-HPLC.

Carboxypeptidase Y Digestion. EGF-T (100 μg), produced by the tryptic digestion of $EGF_{\alpha 1}$ (see above) and purified by RP-HPLC, was dried in a Speedi-Vac concentrator and redissolved in 100 μL of 2 M urea in 0.1 M sodium phosphate (pH 6.2) before digestion with carboxypeptidase Y at an enzyme/substrate ratio of 1:10 (w/w) for 3 h at 37 °C. The reaction was stopped with 20 μL of 1.5% aqueous TFA. The generated peptides were purified to homogeneity by a combination of RP- and AEX-HPLC.

α-Chymotrypsin Digestion. $EGF_{\alpha 1}$ (54 μg) was dissolved in 1% (w/v) ammonium bicarbonate (pH 7.9) and digested with α-chymotrypsin at an enzyme/substrate ratio of 1:20 (w/w) for 18 h at 37 °C. The cleavage products were purified by RP-HPLC.

S. aureus Protease Digestion. $EGF_{\alpha 1}$ (54 μg) was dissolved in 1% (w/v) ammonium bicarbonate (pH 7.8) and digested with *S. aureus* at an enzyme/substrate ratio of 1:20 (w/w) for 18 h at 37 °C. The products were purified by AEX-HPLC.

Recombinant EGF Analogue. A β-galctosidase–EGF fusion analogue was prepared by annealing (Mullenbach et al., 1986) ten synthetic oligonucleotides encoding the sequence for met-$EGF_{(4-48)}$.Lys_{21} and flanked by *Bam*HI and *Hind*III sticky ends. The annealed oligonucleotides were ligated into the β-galactosidase expression plasmid pUR290 (Ruther & Müller-Hill, 1983) which had been digested previously with *Bam*HI and *Hind*III and subsequently phosphatased. The 5′–3′ oligonucleotides were GAT CCT ATG CCT GGT TGT, CCT TCT TCT TAT GAT GGT TAT TGT CTG AAT, GGT GGT GTT TGT AAG CAT ATT GAA, TCT CTG GAT TCT TAT ACT TGT AAT TGT GTT ATT GGT TAT TCT, GGT GAT CGT TGT CAA ACT CGT GAT CTG CGT TAA TAA T, CGA TTA TTA ACG CAG ATC ACG AGT, TTG ACA ACG ATG ACC AGA ATA ACC AAT AAC ACA AT, T ACA AGT ATA AGA ATG CAG AGA, TTC AAT ATG CTT ACA AAC ACC ACC ATT CAG ACA ATA ACC and ATC ATA AGA AGA AGG ACA ACC AGG AT AG. Each oligonucleotide (500 ng) excepting the ones corresponding to the N-terminus and C-terminus was phosphorylated, and all of the oligonucleotides were pooled. The pool was heated to 90 °C and subsequently cooled to 25 °C over a 1.5-h period. The pool was incubated in ligase buffer with 12 units of T4 DNA ligase overnight at room temperature. A further 10 units of T4 DNA lyase was added in the morning and the incubation at 37 °C continued for another 30 min. After the ligase was denatured at 65 °C for 15 min, 60 ng of this oligonucleotide mixture, 60 ng of the *Bam*HI, *Hin*dIII-digested PUR290 plasmid, and 10 units of T4 DNA ligase were incubated overnight at room temperature. A total of 3 μL of this mixture was used to transform *E. coli* NM522 (Maniatis et al., 1982). Sixty antibiotic-resistance colonies were obtained, 12 of which were used for small-scale plasmid preparations. Several plasmids with the appropriate insert were obtained [pEGF$_{(3-48)}$.M_3.K_{21}], and dideoxy sequencing confirmed that the insert corresponded to the sequence expected for β-galactosidase–$EGF_{(3-48)}$.Met_3.Lys_{21}. To produce the β-Gal–$EGF_{(3-48)}$.Met_3.Lys_{21} fusion protein, the recombinant bacteria were grown overnight in L broth and induced for 3 h with isopropyl thiogalactoside (20 μg/mL). After the induced bacteria were harvested, the soluble fusion protein was recovered by sonication, purified, and cleaved with cyanogen bromide. $EGF_{(4-48)}$.Lys_{21} was isolated from the cyanogen bromide digest as described under Results.

Preparation of Samples for Amino Acid and Sequence Analysis. Samples for amino acid analysis or N-terminal sequence analysis were concentrated and desalted by microbore RP-HPLC as described previously (Nice et al., 1984, 1985). Amino acid analysis was performed on a Beckman Model 6300 analyzer equipped with a SECA integrator. Samples were hydrolyzed in vacuo with gaseous HCl generated from 6 M HCl containing 0.1% (w/v) phenol. Norleucine was added as an internal standard to enable accurate quantitation.

Reduction of CNBr-EGF. An aliquot of CNBr-EGF from RP-HPLC (≃5 μg) was evaporated in a Speedi-Vac concentrator before reduction with 10 mM dithiothreitol (DTT) in 1% (w/v) NH_4HCO_3 (100 μL) for 30 min at 37 °C. The reduced peptides were separated by RP-HPLC.

Receptor Binding and Mitogenic Assays. The affinity of the EGF derivatives and peptides for the EGF receptor on murine 3T3 fibroblasts was followed by the inhibition of ^{125}I-EGF binding (Burgess et al., 1983) for 30 min at room temperature. The mitogenic activities of the EGF derivatives were measured with 3T3 fibroblasts (Burgess et al., 1983). The binding characteristics of the ^{125}I-labeled $EGF_{(4-48)}$.Lys_{21} analogue, prepared with iodogen (Burgess et al., 1983; Fraker & Speck, 1978), were determined by the method of Scatchard (1949).

Results and Discussion

EGF derivatives were prepared by selective proteolysis (Savage et al., 1972) and modification with cyanogen bromide (Gross, 1967). During previous studies EGF derivatives which were modified at the N-terminus or which were missing the first few amino acids (Koch et al., 1984; Burgess et al., 1983; Simpson et al., 1985) have been detected and found to be fully active in vitro. Thus, it is not surprising that modification of the α-amino group does not reduce the biological activity of EGF (Chatelier et al., 1986). However, modification of the EGF C-terminus has been reported to alter its biological activity (Cohen et al., 1975). Although the final two residues do not appear to be important, when the last five amino acids were removed (EGF-5, also called EGF-T), the molecule appeared to be active in vivo but was less than 5% as efficient as EGF for stimulating mitogenesis in vitro (Cohen et al., 1975). It was suggested that the Trp-Trp-Glu sequence was critical for the full in vitro activity of EGF. It has been puzzling why transforming growth factor α (which is missing the last four residues) and rat EGF (which is missing the last five species-specific amino acid residues) were as active as EGF in vitro. It is possible that early substitutions in residues 1–48 can compensate for the lack of the Trp-Trp-Glu sequence. We have investigated the importance of the residues 46–53 at the C-terminus of EGF.

Digestion of EGF with Trypsin. When native EGF (0.8 mM) was digested with TPCK-treated trypsin at an enzyme substrate ratio of 1:50 in phosphate buffer (pH 7.0), there was little or no reaction even after 2 h. However when $CaCl_2$ (1 mM) was added to reduce the rate of autodigestion of trypsin, it was possible to convert EGF to EGF-T within 90 min. The digestion mixture could be separated by reversed-phase chromatography on a Brownlee RP-300 column (Figure 2A). As expected (Savage et al., 1972), the two major products were

Table I: Amino Acid Composition of EGF Derivatives[a]

	amount (mol/mol)					
	tryptic fragments (Figure 2B)			carboxypeptidase Y fragments (Figure 5A)		
amino acid	EGF-T	EGF	EGF-T_2	EGF-T-(des-R,L)	EGF-T-(des-R)	EGF-T
Asx	7.0 (7)	7.0 (7)	6.1 (6)	6.5 (7)	7.0 (7)	7.0 (7)
Thr	2.0 (2)	2.1 (2)	2.1 (2)	2.4 (2)	2.0 (2)	2.0 (2)
Ser	5.9 (6)	6.2 (6)	6.0 (6)	6.0 (6)	6.2 (6)	6.0 (6)
Glx	2.0 (2)	3.3 (3)	2.2 (2)	2.2 (2)	2.2 (2)	2.0 (2)
Pro	1.7 (2)	1.8 (2)	2.4 (2)	2.2 (2)	2.0 (2)	1.9 (2)
Gly	5.9 (6)	6.2 (6)	6.0 (6)	6.4 (6)	6.1 (6)	6.0 (6)
Val	1.7 (2)	1.7 (2)	1.7 (2)	1.7 (2)	1.5 (2)	1.4 (2)
Met	0.7 (1)	0.7 (1)	0.8 (1)	0.9 (1)	0.8 (1)	0.7 (1)
Ile	1.7 (2)	1.7 (2)	1.7 (2)	1.6 (2)	1.5 (2)	1.6 (2)
Leu	3.0 (3)	4.2 (4)	2.1 (2)	2.1 (2)	2.9 (3)	2.9 (3)
Tyr	4.1 (5)	4.2 (5)	4.5 (5)	4.5 (5)	4.4 (5)	4.2 (5)
His	0.9 (1)	1.1 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.1 (1)
Arg	2.8 (3)	4.0 (4)	2.0 (2)	2.0 (2)	1.9 (2)	2.9 (3)

[a] Values given are for 24-h total acid hydrolysis. Figures in parentheses are theoretical values deduced from the sequence (Savage et al., 1972).

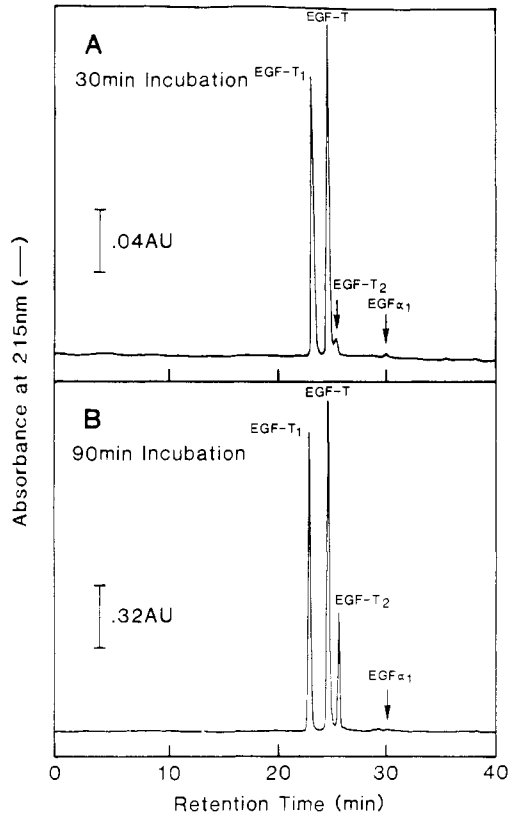

FIGURE 2: RP-HPLC separation of tryptic peptides of EGF. The column used for these separations was a Brownlee RP-300 (100 × 4.6 mm i.d.). A linear 60-min gradient between a primary solvent of 0.15% (v/v) aqueous TFA and a secondary solvent of 60% CH_3CN/40% water containing 0.12% (v/v) TFA was used to elute the peptides. The flow rate was 1 mL/min, and column temperature was maintained at 45 °C. (A) Analytical separation (≃12% of the sample) after 30-min incubation. (B) Preparative separation after 90-min incubation.

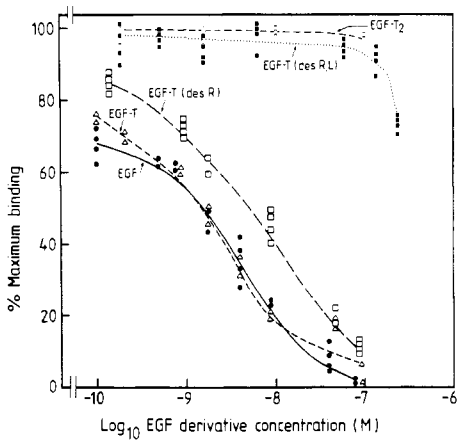

FIGURE 3: Competition between EGF derivatives and the binding of ^{125}I-labeled EGF (200 pM) to the EGF receptor: EGF (residues 1–53), EGF-T (residues 1–48), EGF-T-(des-R) (residues 1–47), EGF-T-(des-L,R) (residues 1–46), and EGF-T_2 (residues 1–45).

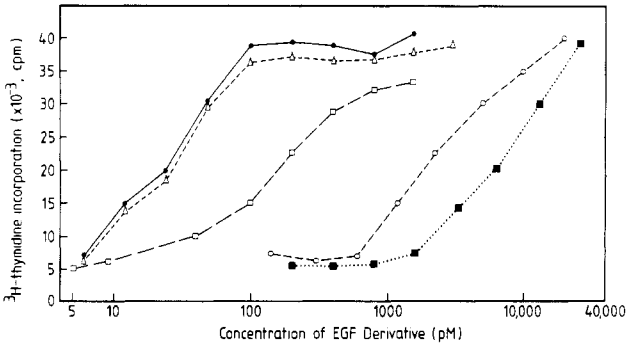

FIGURE 4: Stimulation of [^3H]thymidine uptake in Balb/c 3T3 cells by EGF derivatives: EGF (●), EGF-T (△), EGF-T-(des-R) (□), EGF-T-(des-L,R) (■), and EGF-T_2 (○).

the C-terminal five residues, EGF-T_1 (23 min) and EGF-T (24.7 min).

The identity of those products and of the small peak eluting at 25.7 min (EGF-T_2) was determined by quantitative amino acid analysis (Table I). The second-derivative spectra of these three products also allow the relationships between the products to be ascertained immediately. The distinct minima in the second-derivative spectrum at 291 nm for the first eluting peak (23 min) is typical of a tryptophan-containing peptide (Grego et al., 1986), and thus we can assign this peptide as residues 49–53. The troughs at 280 nm observed in the second-derivative spectra for the two later eluting peaks (at 24.7 and 25.7 min, respectively) are typical of tyrosine-containing peptides. Thus both the digestion kinetics and spectra indicate that EGF-T_2 is derived from EGF-T rather than EGF. These assignments are supported by the amino acid analysis presented in Table I. After 30-min digestion there was only a small peak of EGF remaining; however, by 90 min the yield of the second trypsin cleavage product EGF-T_2 (EGF_{1-45}) had increased significantly (Figure 2B). Further increases in the digestion time did not increase the relative proportion of EGF-T_2. The two trypsin cleavage products were resolved sufficiently for the analysis of their individual biological activities in both the receptor binding assay (Figure 3) and the 3T3 mitogenic assay (Figure 4).

Both EGF and EGF-T inhibited the binding of ^{125}I-labeled EGF (200 pM) with equal potency ($IC_{50} \sim 1$ nM). However, even at 10 nM, EGF-T_2 did not appear to compete for the binding of ^{125}I-EGF. At 100 nM, EGF-T_2 exhibited some weak competition (less than 10%) for binding, but at such high concentrations it is difficult to exclude nonspecific effects (e.g., the chromatography buffers). Thus the interaction between EGF-T_2 and the EGF receptor appears to be at least 500-fold weaker than that of either EGF or EGF-T. Similar results were obtained when the mitogenic activities of these derivatives were compared (Figure 4). Once again there was no detectable difference in the potency of EGF and EGF-T (both stimulated 50% of maximal [^{3}H]thymidine uptake at 25 pM). Almost 100 times more EGF-T_2 (2.4 nM) was required to achieve half-maximal mitogenic stimulation of the 3T3 fibroblasts (Figure 4).

It is clear that the last five residues of EGF do not influence either the receptor binding or in vitro mitogenic activity of the molecule. However, if the tryptic digest proceeds and EGF-T_2 is generated, both the mitogenic activity and receptor binding affinity are reduced 100–500-fold. These results need to be compared to the report of Cohen et al. (1975) in which the mitogenic potencies of EGF derivatives lacking the final two or five (equivalent to EGF-T) residues were compared to that of EGF. Although all of these derivatives were active in the in vivo assay (Savage et al., 1972), EGF-5 (i.e., EGF-T) appeared to be 20–100-fold less potent than EGF in the mitogenic assay using human fibroblasts. Although our experiments use murine 3T3 fibroblasts, it is unlikely that the mitogenic assays are at the basis for the difference between the in vitro results we have obtained with EGF-T and those of these earlier assays. Perhaps the generation of EGF-T_2 in our tryptic digests provides the key. If some batches of EGF-T in the earlier experiments had been overdigested, there may have been a considerable reduction in the in vitro mitogenicity because EGF-T_2 predominated. Thus the C-terminal residues missing from rat EGF do not appear to be required for the activity of the EGF, and compensatory changes in the body of the molecule would not be required to restore full biological activity.

The C-terminal five residues do not appear to be required to direct the folding of EGF. We have prepared fully reduced EGF-T, and in the presence of a mixture of glutathione (3 mM) and oxidized glutathione (0.3 mM) this derivative refolds to the biologically active form.

The considerable difference between the potency of EGF-T and EGF-T_2 suggests that the amino acids Asp_{46}-Leu_{47}-Arg_{48} are critical for receptor binding and subsequent activation of DNA synthesis. At this stage of our investigations it is not possible to distinguish between the importance of Asp_{46} to Arg_{48} for the conformational integrity of EGF and the importance of the interactions between Leu_{47} and the binding site of the EGF receptor. The NMR structure of the C-terminal domain for EGF (Montelione et al., 1987) reveals a complex pattern of folding. The cleavage after Arg_{45} may disrupt the juxtaposition of other side chains in this region, thus reducing the receptor binding affinity. This region of the EGF-T sequence was explored in more detail by use of carboxypeptidase Y digestion.

Carboxypeptidase Y Digestion of EGF-T. A comparison of the amino acid sequences for four species of EGF (human, rat, murine, and guinea pig), three transforming growth factors α (human, murine, and rat), and the vaccinia virus 19K EGF-like peptide revealed very few fully conserved residues (Simpson et al., 1985). One of these residues was in the region

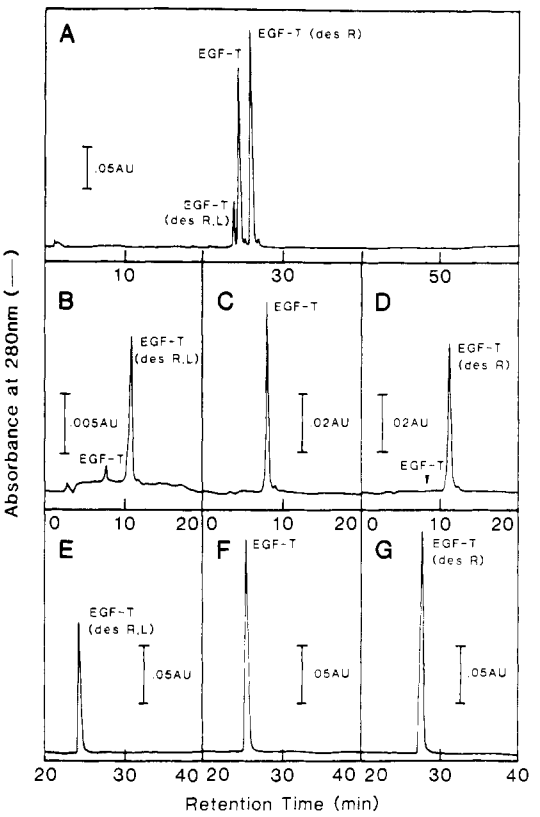

FIGURE 5: Isolation of peptides from a carboxypeptidase Y digest of EGF-T. (Panel A) Preparative RP-HPLC of a carboxypeptidase Y digest of EGF-T. Chromatographic conditions were as for Figure 2. (Panels B–D) Preparative anion-exchange chromatography of peak fractions from panel A. Column was Pharmacia Mono Q HR5/5. A linear 50-min gradient between a primary solvent of 20 mM Bis-Tris-HCl (pH 6.7) and a secondary solvent of 20 mM Bis-Tris-HCl (pH 6.7) containing 0.5 M NaCl was used to elute the peptides. Flow rate was 0.5 mL/min, and column temperature was 25 °C. An aliquot (150 μL) of each fraction from the RP-HPLC was diluted to 1.5 mL with primary solvent for injection. (B) EGF-T-(des-L,R); (C) EGF-T; (D) EGF-T-(des-R). (Panel E) Chromatographic concentration and desalting of material from the anion-exchange column by microbore RP-HPLC. The column was a Brownlee RP-300 (30 × 2.1 mm). Flow rate was 100 μL/min. Other chromatographic conditions were as for Figure 2. (E) EGT-T-(des-L,R) from panel B; (F) EGF-T from panel C; (G) EGT-T-(des-R) from panel D.

46–48, namely, Leu_{47}. We decided to digest EGF-T with carboxypeptidase Y. However, when EGF-T was incubated with carboxypeptidase Y at pH 6.2 (37 °C), no digestion was apparent even after 24 h. This problem can be overcome by adding urea (2 M) to the digestion mixture, whence both arginine and leucine are released from EGF-T by carboxypeptidase Y within 3 h. After this digestion three peptides were detected by reversed-phase chromatography on a Brownlee RP-300 column: the first peptide eluted at 24 min (i.e., 0.7 min before the EGF-T), and the third peptide eluted at 26.0 min (Figure 5A). Amino acid analysis indicated that these proteins were EGF-T-(des-L,R), residual EGF-T, and EGF-T-(des R) (Table 1). Before the biological properties of the two derivatives could be investigated in detail, it was necessary to ensure complete separation from the parent peptide EGF-T. Rechromatography of the proteins using a Pharmacia Mono Q ion-exchange column equilibrated with Bis-Tris-HCl (pH 6.7) achieved the required purification (Figure 5B–D). The individual peaks from the Mono Q column were concentrated for analysis and quantitation on a microbore Brownlee RP-300 reversed-phase column (Figure 5E–G).

EGF-T-(des-R) was 3 times less effective than EGF-T in

competing for ^{125}I-EGF receptor binding (Figure 3). EGF-T-(des-R) was also a less potent mitogen (7-fold) than EGF-T. The removal of Leu_{47} reduced both its receptor binding and mitogenicity at least 500-fold. This result confirms the prediction from homology of the importance of Leu_{47} for the function of EGF. Interestingly, the high-resolution NMR analysis of EGF indicated that the defined structure of EGF stops at Leu_{47} (Montelione et al., 1987). Proton NMR studies of EGF-T support the notion that removal of the last five residues has little effect on the overall conformation of EGF (De Marco et al., 1986). However, cleavage of the peptide bond between Arg_{45} and Asp_{46} starts to disturb its three-dimensional structure. In particular, the aromatic ring of Tyr_{37} becomes more exposed. It will be particularly interesting to examine the juxtaposition of the Leu_{47} and Tyr_{37} side chains in the models of EGF being developed by high-resolution two-dimensional NMR measurements (De Marco et al., 1986; Cooke et al., 1987; Montelione et al., 1987). Although it is still not clear whether Leu_{47} is important for determining the conformation of a critical binding locus on EGF or whether it is important for direct interactions with the EGF receptor, Leu_{47} is involved one way or the other in the formation of the ligand–receptor complex.

The small difference in the mitogenic potency of EGF-T_2 (i.e., residues 1–45) and EGF-T-(des-L,R) indicates that the structure of the polypeptide chain in the region of Leu_{47} is complex. The introduction of a C-terminal carboxyl at position 46, together with the aspartyl side chain, appears to reduce the mitogenicity even more than the presence of a C-terminal carboxyl at position 45 in the EGF-T_2. The relative binding affinities of these two derivatives are difficult to compare because of their low potencies. However, the fact that both are mitogenic between 1 and 10 nM indicated that both derivatives occupy the EGF receptor with low affinity, but still activate the high-affinity state of the EGF receptor. The lack of competition for EGF receptor binding even at 100 nM suggests that the binding of these derivatives to the low-affinity sites is almost abolished.

Chymotryptic Digest of EGF. After digestion of EGF with α-chymotrypsin for 18 h at 37 °C, four products could be resolved by RP-HPLC. Amino acid analysis combined with second-derivative spectroscopy (to identify tryptophan-containing peptides) revealed that these were EGF-T_{1-48}, EGF-T-(des-R), EGF_{1-51}, and EGF_{1-50}. The two later derivatives had full mitogenic and receptor binding activity. The activity of the other derivatives was identical with that of the corresponding peptides produced by the tryptic digestion of EGF or carboxypeptidase Y digestion of EGF-T described above.

S. aureus V8 Protease Digestion of EGF. Following *S. aureus* digestion of EGF (residues 1–53) for 18 h at 37 °C, no discernible product was revealed by RP-HPLC. However, anion-exchange HPLC on Pharmacia Mono Q (using the conditions described under Materials and Methods) yielded a single product (retention time 30.2 min) clearly distinguishable from the substrate molecule (retention time 24.0 min). The second-derivative absorption spectrum of this product clearly indicated the presence of tryptophan residues. Following buffer exchange and concentrations by microbore RP-HPLC, amino acid analysis indicated that this peptide was EGF_{1-52}. Receptor binding affinity and mitogenic activity of this peptide were identical with those of EGF.

Amino-Terminus Truncation of EGF-T. Whereas the C-terminal region of EGF was readily cleavable by a number of enzymes, the N-terminus proved remarkably resistant to enzymic digestion. It has been reported (Taylor et al., 1972) that the dipeptidase cathepsin C removes the two terminal amino acids: Asn_1-Ser_2. Attempts to cleave murine EGF with leucine aminopeptidase or prolidase were unsuccessful [the digestion was monitored by RP-HPLC under conditions which can separate $EGF_{\alpha 1}$ from EGFβ (Burgess et al., 1982)]. As a consequence an N-terminal-truncated EGF-T was prepared with a β-galactosidase–EGF-T fusion protein (Ruther & Müller-Hill, 1985). So that the EGF-T could be released intact with cyanogen bromide, Met_{21} was replaced with a lysine residue, and the oligonucleotides were ligated to attach $EGF_{(3-48)}$.Met_3.Lys_{21} to β-galactosidase. β-Gal–$EGF_{(3-48)}$-Met_3.Lys_{21} was present as a soluble protein in the cytoplasm of *E. coli*. The fusion protein was active in both the β-galactosidase assay and the EGF receptor binding assay. After enriching for the fusion protein by precipitation with 28% saturated ammonium sulfate, $EGF_{(4-48)}$.Lys_{21} was cleaved from the fusion protein with cyanogen bromide. After the cyanogen bromide was removed by freeze-drying and Sephadex G-25 chromatography (at pH 7.4 this analogue eluted at the void volume of the column), EGF receptor binding activity was still detectable. $EGF_{(4-48)}$.Lys_{21} was purified to homogeneity by two sequential steps of reversed-phase HPLC (Figure 6). Approximately 200 μg of this purified EGF analogue was obtained from each liter of induced bacterial culture. The amino acid composition and N-terminal amino acid sequence determination (36 cycles of Edman degradation) confirmed the purity and identity of the product. A quantitative comparison of the mitogenic potency of $EGF_{(4-48)}$.Lys_{21} with that of EGF (Figure 7A) indicated that the analogue was equipotent. Similarly, the receptor binding characteristics of ^{125}I-labeled $EGF_{(4-48)}$.Lys_{21} indicated that the analogue bound with high affinity to the EGF receptor (Figure 7B). Thus, neither the first three residues nor the last five residues of EGF are required for the folding or in vitro biological activity of EGF.

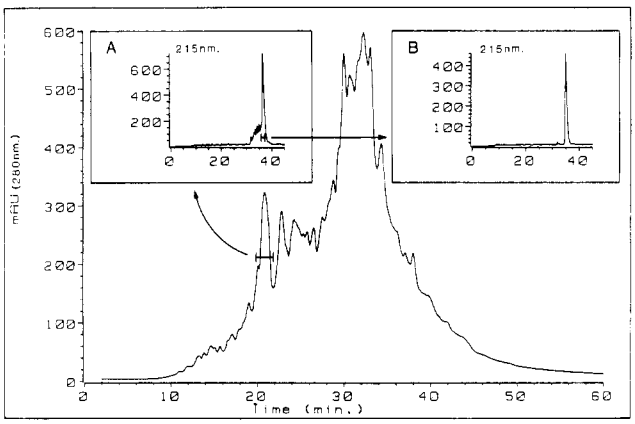

FIGURE 6: Reversed-phase HPLC purification of $EGF_{(4-48)}$.Lys_{21}. (Main panel) The active fractions from a Sephadex G-25 column were loaded onto a Brownlee RP-300 column (30 × 2.1 mm i.d.), previously equilibrated with a primary buffer of 0.15% (v/v) aqueous TFA, at a flow rate of 2 mL/min. Retained proteins were eluted with a linear 60-min gradient between the primary buffer and 60% CH_3CN/40% H_2O/0.125 % (v/v) TFA at a flow rate of 100 μL/min. The column temperature was 45 °C. Eluting proteins were monitored at 280 nm, and 1-min fractions were recovered automatically with a Pharmacia FRAC 100 collector. Biological activity of eluent fractions was monitored by a radioreceptor assay. (Panel B) Fractions 19 and 20 from above (peak fractions in the bioassay) were pooled. A 40-μL aliquot (20% of the sample) was diluted 5-fold with 0.15% (v/v) aqueous TFA and loaded onto an ODS-Hypersil column (100 × 2.1 mm i.d.). Chromatographic conditions were as above except that the chromatogram was monitored at 215 nm. The peak fraction was recovered manually and chromatographed under identical conditions (panel B) prior to N-terminal sequence, amino acid analysis, and biological assays.

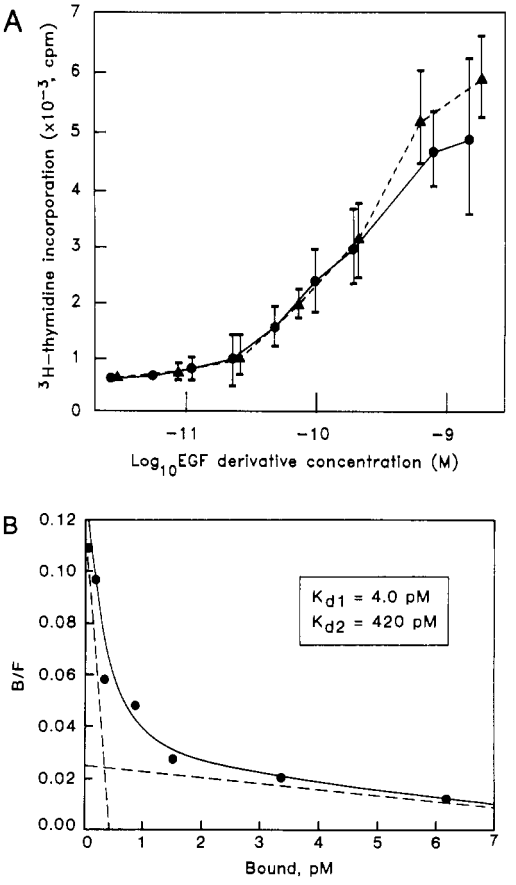

FIGURE 7: (Panel A) Mitogenic activity of purified $EGF_{(4-48)}$.Lys_{21}. Stimulation of [^{3}H]thymidine uptake by confluent Balb/c 3T3 cells by EGF (●) or $EGF_{(4-48)}$.Lys_{21} (▲). Points represent the mean and standard deviation for three [$EGF_{(4-48)}$.Lys_{21}] or six (EGF) replicates at each concentration. (Panel B) Scatchard analysis of ^{125}I-labeled $EGF_{(4-48)}$.Lys_{21} binding to 3T3 cells. Confluent Balb/c 3T3 were allowed to bind ^{125}I-labeled $EGF_{(4-48)}$.Lys_{21} (2 pM to 1.5 nM) for 30 min at 37 °C. Nonspecific binding was determined in the presence of a 50-fold excess of unlabeled EGF. Data analysis was performed with the LIGAND program (Munson, 1981).

Cyanogen Bromide Treatment of EGF. It has been reported previously that cyanogen bromide treatment of murine EGF produces a derivative capable of binding to the EGF receptor, but which is not mitogenic (Yarden et al., 1982). Although less potent than EGF, CNBr-treated EGF is reported to stimulate the induction of ornithine decarboxylase in 3T3 cells, the enhancement of endogenous membrane protein phosphorylation, and the reorganization of the microfilament system in A431 epidermoid carcinoma cells (Yarden et al., 1982). However, even at 16 nM CNBr-treated EGF stimulated only 10% of the maximal EGF-induced enhancement of DNA synthesis in human foreskin fibroblasts (Yarden et al., 1982).

Our initial attempts to study the biological properties of CNBr-treated EGF were thwarted by contaminating EGF. After treatment of EGF with CNBr the RP-HPLC elution profile for the cleaved EGF was clearly broader than that of EGF, but it still eluted from the Brownlee RP-300 column (30 × 2.1 mm) at 30 min and was clearly not fully resolved from EGF. It was not usually possible to resolve the unmodified EGF peak from the CNBr-treated EGF on the reversed-phase system; however, by neutralizing and reducing the CNBr-EGF reaction mixture with dithiothreitol and rechromatographing in the standard reversed-phase system it was possible to detect reduced EGF as a well-resolved peak (at 35 min) clearly separated from the native EGF molecule (30 min) and the CNBr-cleaved EGF peptides (i.e., 1–21 eluting as two isomers at 23 and 25 min and 22–53 eluting at 31 min). Only after chromatographing the reversed-phase-purified CNBr-treated EGF on a Pharmacia Mono Q column (under identical conditions with those described for Figure 5) was it possible to separate CNBr-EGF (21 min) from the unmodified EGF (23 min). Once the CNBr-treated EGF had been purified by both reversed-phase HPLC and Mono Q anion-exchange chromatography, no detectable native EGF remained. Amino acid analysis failed to detect any residual methionine, and N-terminal sequence analysis detected both the N-terminal sequence and the sequence from the cleavage point in equal amounts (Table II). By use of this preparation of CNBr-treated EGF for receptor competition studies, a single binding site with a K_d of almost 100 nM was observed. The results of the receptor competition experiments for two separate preparations of CNBr-treated EGF are given in Figure 8. The concentration for 50% inhibition of ^{125}I-labeled EGF (200 pM) binding was almost 100 times greater than that for EGF. This reduced potency was also reflected in the ability of CNBr-treated EGF to stimulate DNA synthesis in 3T3 fibroblasts—half-maximal stimulation required almost 1 nM CNBr-treated EGF (Figure 9). In the same experiment EGF stimulated half-maximal enhancement of DNA synthesis at 10 pM (Figure 9).

As mentioned above, the two halves of CNBr-treated EGF (namely, residues 1–21 and 22–53) can be separated by reversed-phase chromatography following reduction with dithiothreitol. The mEGF peptide 1–21 eluted as two species (the different isomers of the lactone are presumably the reason for these two species), and the mEGF peptide eluting at 31 min corresponded to residues 22–53. Only the C-terminal half (residues 22–53) exhibited any residual activity in the mitogenic assay (Figure 9), but the potency of this peptide was

Table II: Sequence Analysis of CNBr-Treated Mouse Epidermal Growth Factor[a]

cycle no.	phenylthiohydantoin amino acid released (pmol)
1	Asn (20), His (30)
2	Ser (5), Ile (40)
3	Tyr (39), Glu (26)
4	Pro (19), Ser (3)
5	Gly (18), Leu (16)
6	*b*, Asp (8)

[a] Values in parentheses are yields of phenylthiohydantoin amino acid in picomoles. Predicted sequences for EGF residues 1–6 = Asn-Ser-Tyr-Pro-Gly-Cys and EGF residues 22–27 = His-Ile-Glu-Ser-Leu-Asp (Savage et al., 1972). [b] Cys was not detected since the protein was not reduced and alkylated.

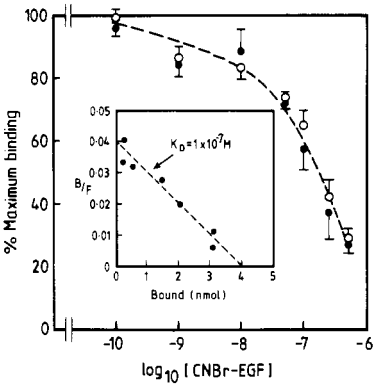

FIGURE 8: Competition between CNBr-treated EGF and ^{125}I-EGF (200 pM) for binding to the EGF receptor. The results of two separate experiments are shown with the solid and open circles. The bars represent the standard deviation for triplicate incubations. The insert shows the Scatchard analysis for the first experiment.

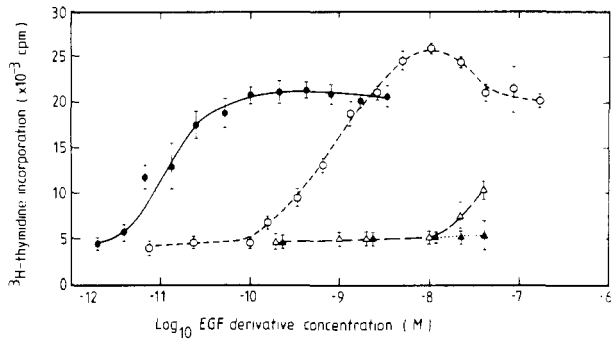

FIGURE 9: Stimulation of [^{3}H]thymidine uptake in Balb/c 3T3 cells by EGF (●) CNBr-treated EGF (○), and the two separate fragments from reduced CNBr-treated EGF [(△) residues 22–53; (▲) residues 1–21]. Results represent the means and standard deviations for triplicate incubations of each derivative.

more than 1000-fold lower than that of EGF. Heath and Merrifield (1986) found some mitogenic activity associated with a synthetic fragment (residues 15–53) of murine EGF, but the potency was less than $1/10^4$ that of native EGF. Komoriya et al. (1984) also synthesized fragments of murine EGF and found some binding activity with a cyclized fragment equivalent to the residues between 14 and 31. Again this potency was less than $1/10^4$ that of native EGF. The three-dimensional structure of murine EGF (Montelione et al., 1986, 1987; Cooke et al., 1987) indicates that the residues between 18 and 33 are involved in an antiparallel β-pleated sheet. Cleavage of the EGF polypeptide chain at position 21 appears to disrupt this β structure, and it is this disruption which is the likely cause of the lowered affinity of CNBr-treated EGF for the EGF receptor. Since Met_{21} can be replaced by a leucine without seriously compromising the activity of human EGF (Sumi et al., 1985), it is unlikely that the Met_{21} side chain is critical for receptor binding.

The third disulfide-bonded loop of EGF is the most conserved domain in the molecule (Simpson et al., 1985) and has been indicated as an important domain for the binding of EGF to its receptor. We have prepared third loop analogues of EGF, some of which were extended to residue 53 (D. B. Scanlon and A. W. Burgess, personal communication) but were not able to demonstrate significant receptor binding or mitogenic activity for any of these analogues. Similar results were reported by Heath and Merrifield (1986). It is not clear why there is a discrepancy between these results and those of Eppstein et al. (1985) and Nestor et al. (1985) with the TGFα analogues and the vaccinia virus third loop peptides. The folding of the residues between 34 and 46 of EGF is quite complex (Cooke et al., 1987; Montelione et al., 1987) and is presumably influenced by long-range interactions with other parts of the peptide chain (e.g., the regions between 14 and 18). EGF analogues capable of high-affinity binding to the receptor may require several noncontiguous regions of the polypeptide chain.

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Guanosine 5′-Triphosphate Binding Protein (G_i) and Two Additional Pertussis Toxin Substrates Associated with Muscarinic Receptors in Rat Heart Myocytes: Characterization and Age Dependency†

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ABSTRACT: The coupling of muscarinic receptors with G-proteins was investigated in cultured myocytes prepared from the hearts of newborn rats. The coupling was investigated in both young (5 days after plating) and aged (14 days after plating) cultures, in view of the completly different effects of 5′-guanylyl imidodiphosphate [Gpp(NH)p] on muscarinic agonist binding to homogenates from young vs aged cultures [Moscona-Amir, E., Henis, Y. I., Yechiel, E., Barenholz, Y., & Sokolovsky, M. (1986) *Biochemistry 25*, 8118–8124]. Pretreatment of cultures from both ages by *Bordetella pertussis* toxin (IAP) was found to eliminate any Gpp(NH)p effect on carbamylcholine binding. IAP by itself induced a rightward shift in the carbamylcholine competition curve in homogenates from aged cultures, but no such effect was observed in homogenates from young cultures. IAP-catalyzed [^{32}P]ADP-ribosylation of membrane preparations from young and aged cultures revealed major differences between them. Young cultures exhibited a major IAP substrate at 40 kDa, which was also recognized by anti-α_i antibodies, and two novel IAP substrates at 28 and 42 kDa, which were weakly ADP-ribosylated by the toxin and were not recognized with either anti-α_i or anti-α_o antibodies. In aged cultures, only the 40-kDa band (ribosylated to a lower degree) was detected. The parallel age-dependent changes in the three IAP substrates (28, 40, and 42 kDa) and in the interactions of the G-protein(s) with the muscarinic receptors strongly suggest close association between the two phenomena. All of these age-dependent changes in the G-protein related parameters were prevented by phosphatidylcholine–liposome treatment of the aged cultures. The role of the membrane lipid composition in these phenomena is discussed.

Many cell surface receptors exert their action through specific guanine nucleotide regulatory proteins (G-proteins) which couple receptors to signal generating systems in the plasma membrane. A growing body of evidence suggests that a family of G-proteins is involved in different cellular functions: inhibition (G_i) and stimulation (G_s) of adenylate cyclase in the heart (Sternweis et al., 1981; Endoh et al., 1985; Liang et al., 1986), control of K^+ channels (G_k) in mammalian and avian heart (Martin et al., 1985; Logothetis et al., 1987; Yatani, 1987), mediation (via G_o) of muscarinic receptor–Na^+ channel interactions in rat brain and heart (Cohen-Armon & Sokolovsky, 1986; Cohen-Armon et al., 1988), inhibition of inward Ca^{2+} currents (probably via G_o) (Halvorsen & Nathanson, 1984), stimulation of phosphoinositide metabolism (via G_i or G_p) (Nakamura & Ui, 1985; Hepler & Harden, 1986), stimulation of the activity of membrane-bound phospholipase A_2 (Burch et al., 1986; Jelsema, 1987; Jelsema & Axelrod, 1987; Axelrod et al., 1987), and stimulation of cGMP-dependent phosphodiesterase (G_T) (Fung, 1983). While functionally diverse, G-proteins share several structural and mechanistic features. Each G-protein consists of α, β, and γ subunits and is most clearly distinguished from other G-proteins by the biological and biochemical attributes of its GTP binding α subunit (Michel et al., 1985; Roof et al., 1985). The α subunit of some G-proteins, for example, G_i (Kurose et al., 1983; Bokoch et al., 1984), G_o (Sternweis & Robishaw, 1984), and G_T (van Dop et al., 1984), is specifically ADP ribosylated by *Bordetella pertussis* toxin (IAP).[1]

The binding of guanine nucleotides to G-proteins strongly affects the binding of agonists to a wide variety of receptors, particularly those linked to adenylate cyclase. Muscarinic receptors mediate inhibition of adenylate cyclase in heart (Watanabe et al., 1978; Endoh et al., 1985; Liang et al., 1986) and brain (Onali et al., 1983), and the effect of guanine nu-

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[1] Abbreviations: IAP, *Bordetella pertussis* toxin; PC, phosphatidylcholine; [^{3}H]4NMPB, *N*-[^{3}H]methyl-4-piperidyl benzilate; Gpp(NH)p, 5′-guanylyl imidodiphosphate; DTT, dithiothreitol.

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