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Analogs of human epidermal growth factor which partially inhibit the growth factor-dependent protein-tyrosine kinase activity of the epidermal growth factor receptor

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Three site-directed mutants of human epidermal growth factor, Leu-26→Gly, Leu-47→Ala, and Ile-23→Thr, were examined for their ability to stimulate the protein-tyrosine kinase activity of the epidermal growth factor receptor. The receptor binding affinities of the mutant growth factors were 20- to 50-fold lower, as compared to wild-type growth factor. At saturating concentrations of growth factor, the velocities of the phosphorylation of exogenously added substrate and receptor autophosphorylation were significantly lower with the mutant analogs, suggesting a partial 'uncoupling' of signal transduction. The mutant analogs were shown to compete directly with the binding of wild-type, resulting in a decrease in growth factor-stimulated kinase activity.

Epidermal growth factor analog; Epidermal growth factor receptor kinase; Competitive inhibitors of epidermal growth factor

1. INTRODUCTION

EGF is one of the most highly studied growth factors, yet much remains to be learned about its mechanism of action. The biological effects of EGF are initiated by interaction with its specific cell-surface receptor (see [1,2] for reviews). The binding of EGF to the receptor's extracellular domain stimulates the receptor's cytoplasmically located protein-tyrosine kinase which can phosphorylate itself as well as other cellular proteins [3,4]. The process by which kinase activation is achieved has been explained as resulting either from dimerization of inactive receptor monomers to form activated dimers [5–8] or from dissociation of latent receptor dimers to monomers having high tyrosine kinase activity [9]. A third model invokes a ligand-induced intramolecular conformational change in the receptor's extracellular domain leading to the activation of the kinase activity of the receptor [10,11]. While this issue is still being resolved, it is quite clear that the tyrosine kinase activity of the receptor is essential for the mitogenic response of the cells to EGF [12,13].

Recently our laboratory has generated numerous site-specific mutants of hEGF in an attempt to determine the regions, and/or specific residues, involved in receptor binding ([14]; Campion, S.R., Matsunami, R.K., Engler, D.A. and Niyogi, S.K., submitted for publication; Matsunami, R.K., unpublished observations). The receptor-binding affinity of each mutant, relative to wild type, was measured by both radioreceptor competition and receptor tyrosine kinase stimulation assays. The values obtained by the two methods were generally in agreement for each mutant, and mutant growth factors with relative affinities ranging from 180% to less than 1% have been produced. In this communication we describe three mutants of hEGF, namely, Leu-26 → Gly, Leu-47 → Ala, and Ile-23 → Thr, which stimulate the receptor to a lower V_{max} for the kinase reaction compared to that obtained with wild-type EGF using either $(Glu_4Tyr_1)_n$ as a substrate or by measuring autophosphorylation of the EGF receptor. These mutants are also demonstrated to inhibit competitively the activity of wild-type hEGF in its stimulation of the protein-tyrosine kinase activity of the receptor. Such mutants are interesting in that the lower V_{max} may indicate an altered receptor-ligand complex, making it less active. These mutant hEGFs might be potentially important as growth inhibitors that could block the action of wild type hEGF.

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Abbreviations: EGF, epidermal growth factor; hEGF, human epidermal growth factor; DTT, 1,4-dithiothreitol; WGA, wheat germ agglutinin; HPLC, high pressure liquid chromatography; V_{max}, maximum velocity of a reaction; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (4500 Ci/mmol) was purchased from ICN Radiochemicals and $(Glu_4Tyr_1)_n$ from Sigma Chemical Company.

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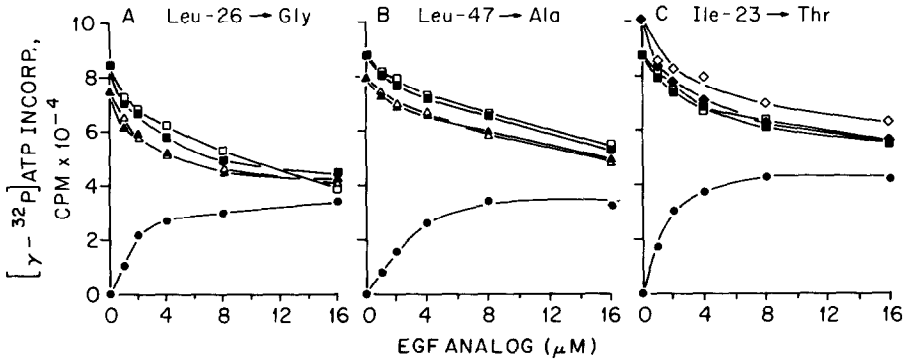

Fig. 1. Dose-response curves for stimulation of EGF receptor tyrosine-kinase activity by mutant EGFs, and competition plots for each of the mutants. ● Dose-response curves; △ actual and ▲ theoretical competition curves against 45 nM wild type; □ actual and ■ theoretical competition curves against 60 nM wild type; ◇ actual and ◆ theoretical competition curves against 75 nM wild type. The receptor tyrosine-kinase assays were done as described in section 2.

2.2. *Production and purification of EGF mutant proteins*

Recombinant wild-type hEGF was made and isolated as reported previously [14]. EGF mutants were generated by site-directed mutagenesis using a modified M13 primer extension method [14]. Purification was the same as that for wild-type hEGF.

2.3. *Isolation of membranes and purification of receptors*

Membrane fractions were isolated from confluent A431 cells and the EGF receptor was solubilized and purified on a WGA agarose column as described previously [14].

2.4. *Competition binding assay*

The binding affinity of each mutant protein was measured by its ability to compete with [^{125}I]hEGF in binding to A431 membrane fractions. Conditions of the binding assay and a description of the determination of dissociation constants have been reported previously [14].

2.5 *Kinase assay*

The activity of each hEGF protein in the protein-tyrosine kinase reaction was measured by its ability to stimulate the phosphorylation of a synthetic polymer, $(Glu_4Tyr_1)_n$, of average molecular mass 35 000. Previous studies have shown that the polymer is a good substrate for the EGF receptor's protein-tyrosine kinase [15,16]. The assay procedure, similar to that of Koland and Cerione [11], was the same as used by Engler et al. [14] and binding affinities were determined as described in the same report. Competition kinase assays were carried out by keeping the concentration of the wild-type protein constant while that of the mutant was varied, as shown in Fig. 1. Each point shown on the curves is an average value determined from duplicate experiments. Analysis of the time courses of the reactions with wild-type hEGF and the mutants showed that they were linear.

2.6 *SDS gel electrophoresis of the kinase reaction mixtures*

To measure the extent of autophosphorylation of the EGF receptor, kinase reactions were performed as described above, with the following modifications: $(Glu_4Tyr_1)_n$ substrate was not added, the specific activity of [γ-^{32}P]ATP was 1 Ci/mmol, and the reactions were stopped by the addition of 3 × Laemmli loading buffer [17]. The samples were then electrophoresed according to the procedure of Laemmli [17]. The amount of each EGF protein used in the autophosphorylation assay was the amount that was saturating as determined from the kinase assay using the $(Glu_4Tyr_1)_n$ substrate. The gel was stained, dried, and processed for autoradiography. The autoradiograph was then scanned using a laser densitometer to determine the amount of radioactivity incorporated into the EGF receptor band. Greater than 95% of the radioactivity on the gel was associated with a 170 kDa band, which was assumed to be the EGF receptor. Each reaction was done in duplicate and the average of the two values obtained by densitometry scans was used to calculate the values shown in Table II.

3. RESULTS AND DISCUSSION

The relative receptor binding affinities of the three mutants of hEGF described in this paper are shown in Table I. The values were obtained by using the radioreceptor competition assay and by measuring their stimulation of the receptor protein-tyrosine kinase activity. Both assays show that the Ile-23 → Thr and Leu-26 → Gly mutants have about 5% of the binding

Table I

Receptor binding affinities of hEGF species as measured by radioreceptor competition and receptor tyrosine kinase stimulation

hEGF species	Relative binding affinity (% of wildtype)	
	Radioreceptor competition assay[a]	Tyrosine kinase stimulation assay[b]
Wild type	100	100
Leu-26→Gly	5	4
Leu-47→Ala	2	2
Ile-23→Thr	3	5

[a]Relative binding affinity = IC_{50} (wild type)/IC_{50} (mutant) × 100%, as described prevously [14].
[b]Relative binding affinity = EC_{50} (wild type)/EC_{50} (mutant) × 100%, as described previously [14].

Table II

V_{max} values obtained for stimulation of the receptor tyrosine-kinase by hEGF analogs

hEGF species	V_{max} of receptor tyrosine kinase (% of wild type)	
	$(Gly_4Tyr_1)_n$ as substrate	Receptor autophosphorylation
Wild type	100	100
Leu-26→Gly	22	23
Leu-47→Ala	24	17
Ile-23→Thr	31	38

affinity of wild type, as determined from the EC_{50} values of the dose response curves, while the Leu-47 → Ala mutant has only about 2% of wild type. Despite their lower binding affinities, each mutant was able to fully displace wild-type hEGF in the competition binding assay, indicating that the mutants bound to the receptor in a specific manner.

As shown in Table II, the V_{max} of the receptor tyrosine kinase activity was markedly less with the three mutants than that obtained with wild-type hEGF. Values, relative to wild-type, were 22% for the Leu-26 → Gly mutant, 24% for the Leu-47 → Ala mutant, and 31% for the Ile-23 → Thr mutant, in the kinase reaction in which $(Glu_4Tyr_1)_n$ was used as a substrate.

The velocity of the EGF receptor autophosphorylation reaction was also measured at saturating concentrations of each mutant. The results show that autophosphorylation of the EGF receptor was significantly lower when stimulated by the mutant hEGFs as compared with wild-type hEGF. The Leu-26 → Gly mutant stimulated autophosphorylation of the EGF receptor band (170 kDa) 23% that of wild-type, the Leu-47 → Ala mutant 17% of wild-type, and the Ile-23 → Thr, 38% (Table II). These values are similar to the V_{max} values obtained with the $(Glu_4Tyr_1)_n$ substrate.

It was decided to examine whether the hEGF mutant proteins, having lower stimulatory capacity as demonstrated in the receptor protein-tyrosine kinase assay, would act as competitive inhibitors of wild-type hEGF under the kinase assay conditions. A modification of the equation from Segel [18] for the velocity of a reaction with one enzyme and mixed alternative substrates was used to evaluate the competitive nature of the mutant hEGF analogs:

$$v_t = \frac{V_{max_A}([A]/K_{d_A}) + V_{max_B}([B]/K_{d_B})}{1 + [A]/K_{d_A} + [B]/K_{d_B}}$$

where A and B are two different activators, and V_{max_A} is the maximum velocity of the reaction with A as the activator, and V_{max_B} is the maximum velocity of the reaction with B as the activator, K_{d_A} and K_{d_B} are the equilibrium constants for activators A and B, respectively, and v_t is the observed velocity under the given conditions.

Fig. 1, panels A, B and C, show the dose-response curves for stimulation by mutant hEGFs of the receptor tyrosine kinase with $(Gly_4Tyr_1)_n$ as a substrate and the competition plots for each of the mutants at two different concentrations of wild-type hEGF. Equilibrium constants and velocity values were obtained from the concentration curves. Also plotted are the theoretical curves predicted by the above equation using the values obtained for the individual mutants. These theoretical curves closely resemble the actual curves and demonstrate that the inhibitory nature of the mutant analogs is purely competitive. A mutant hEGF, Tyr-22 → Asp, that has a lower relative binding affinity (25% that of wild type) but not a lower V_{max}, was tested as a competitor against wild-type hEGF. The competition by this mutant as predicted by the equation resulted in no inhibition of activity (data not shown).

The competitive inhibition of wild-type hEGF-stimulated tyrosine-kinase activity by the mutant hEGF species confirms their lower stimulatory capacity. These results clearly demonstrate a partial 'uncoupling' of growth factor binding and kinase stimulation. The observed decrease in the maximal rate of phosphorylation catalyzed by the EGF/receptor complex in response to the mutant hEGF species suggests that the activated receptor-growth factor complexes formed with these mutants have a conformation somewhat altered from the complex formed with the wild-type growth factor. Obviously, physicochemical studies are necessary to determine the nature of the conformational change(s). Studies examining the structure of the complexes formed between the EGF analogs and the external domain of the EGF receptor as described by Greenfield et al. [19] for wild-type EGF may provide evidence of altered receptor conformations. Our findings also suggest that the design of growth-inhibitory EGF analogs may be feasible.

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