The Functional Importance of Hydrophobicity of the Tyrosine at Position 13 of Human Epidermal Growth Factor in Receptor Binding*

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The tyrosine at position 13 of epidermal growth factor (EGF) has been implicated as playing a role in receptor binding due to its close proximity to the critical arginine 41 residue as well as its high degree of conservation in EGF and EGF-like proteins that can bind to the EGF receptor. Site-directed mutagenesis of tyrosine 13 in human EGF (hEGF) was employed to examine the role of this residue in ligand-receptor interaction. The removal of the hydroxyl moiety of the tyrosine by substitution with phenylalanine had little effect on the binding, indicating that it is not involved in any crucial hydrogen bonds with either the receptor or with other regions of the EGF molecule. The substitution of the aromatic tyrosine side-chain with the nonpolar leucine side-chain caused the receptor affinity to decrease only slightly, indicating that aromaticity of the amino acid at this site is also not critical. Substitutions with other hydrophobic residues, isoleucine, valine, and alanine, resulted in a significant decrease in receptor affinity as a function of decreasing hydrophobicity. Substitution of tyrosine 13 with the polar residues histidine and arginine markedly decreased receptor binding affinity, and complete removal of the side-chain by substitution with glycine dramatically lowered the binding affinity to 0.3% as compared to wild type. Analysis of three hEGF mutants, Tyr13→Leu, Tyr13→Arg, and Tyr13→Gly, by circular dichroism showed that the major structural features of hEGF were not significantly altered. The results demonstrate that the decreased receptor affinities of these hEGF mutants are due to disruption of the functional contribution(s) of the tyrosine 13 residue rather than alteration(s) in the overall structural integrity. Overall, the results suggest that the tyrosine 13 side-chain plays a critical role in receptor binding by contributing to hydrophobic receptor-ligand interactions.

EGF is a protein that plays an important role in the regulation of cell growth and proliferation. EGF acts by binding with high affinity to its specific cell surface receptor thereby stimulating the intrinsic protein-tyrosine kinase activity of the receptor. The tyrosine kinase activity of the receptor in turn initiates a signal transduction cascade which ultimately leads to DNA synthesis and cell proliferation. A variety of biochemical changes occurs within the cell in response to the signal cascade; these changes include a rise in intracellular calcium levels, increased glycolysis and protein synthesis, and increases in the expression of certain genes including the gene for the EGF receptor (for recent reviews on the above, see Carpenter and Cohen, 1990; Ulrich and Schlessinger, 1990). The EGF molecule is composed of 53 amino acid residues and contains three intramolecular disulfide bonds. Models of EGF structure derived from two-dimensional NMR data (Montelione et al., 1986, 1987, 1992; Cooke et al., 1987; Kohda et al., 1988) predict that the protein can be subdivided into two slightly overlapping structural domains (Fig. 1). The amino-terminal domain (Asn1 to Val30) contains a major antiparallel β-sheet structure (residues 19–31). The carboxyl-terminal domain (Ala37 to Arg97) contains a double hairpin structure as well as a minor antiparallel β-sheet between residues 37–38 and 44–45. NMR and NOE studies (Mayo et al., 1986) predict a clustering of the aromatic groups which, in concert with the physical constraints imposed by the three intramolecular disulfide bonds, functions to establish an extremely stable protein structure (Holladay et al., 1976).

Site-directed mutagenesis has been used extensively to examine the effects of specific amino acid substitution of residues in the EGF protein on receptor-ligand interactions. These studies have shown that EGF receptor-ligand association requires the involvement of amino acid residues in both the amino- and carboxyl-terminal regions of the EGF protein (see Fig. 1). Several of these important residues are hydrophobic in nature. Substitution of the highly conserved Leu47 in the carboxyl-terminal region of the peptide resulted in a marked reduction in the affinity of the receptor for hEGF (Engler et al., 1988; Dudgeon et al., 1990; Matsunami et al., 1991) and mEGF (Ray et al., 1988; Moy et al., 1989). Examination of mutant proteins by NMR indicated only minor structural perturbations that were insufficient to account for the drastic loss of activity (Moy et al., 1989; Dudgeon et al., 1990, 1996; Matsunami et al., 1991). Replacement of either Ile23 or Leu26 in the amino-terminal domain of hEGF resulted in decreased receptor binding affinity (Campion et al., 1990). The importance of Ile23 has recently been confirmed by Koide et al. (1992) with a series of mutations at this site. Here again, structural analysis of mutant proteins by NMR indicated only slight perturbations that were unable to account for the
dramatic loss of biological activity (Koide et al., 1992). The cumulative effect of simultaneous mutations at several of the above sites on relative receptor affinity confirmed the importance of these residues in receptor binding and indicated that each of the separate sites functions essentially independently in the interaction of the EGF molecule with its receptor (Campion et al., 1993).

In contrast to the majority of other electrostatic residues which play only a minor role in EGF receptor-ligand association (Campion et al., 1992) the highly conserved Arg<sup>13</sup> of hEGF was shown to be crucial for the formation of a stable EGF-receptor complex (Engler et al., 1990, 1992). Even a mutation to lysine, which conserves the positive charge, reduced the affinity to <0.5% of wild type (Engler et al., 1990). Chemical modification of the lysine mutation to restore the guanidinium moiety restored full activity (Engler et al., 1992). The position 41 mutants, when examined by NMR, showed very minor perturbations that could not explain their drastic loss in activity (Engler et al., 1990; Hommel et al., 1991).

The importance of the highly conserved tyrosine at position 13 in receptor-ligand interaction was suggested due to its close proximity to the arginine 41 site (see Fig. 1) as revealed by NMR studies (for example, see Kohda et al. (1988) and Montelione et al. (1992)) and its possible role in contributing to the hydrophobic surface on the EGF molecule (Mayo et al., 1986). The role of tyrosine 13 in receptor binding was investigated by site-directed mutagenesis. The results show that a tyrosine at position 13 is not critical for overall binding of EGF to its receptor; rather, hydrophobicity appears to play a significant role in the formation of a stable ligand-receptor complex. Analysis of the gross structure of mutant hEGF proteins shows no major structural alterations that could account for the changes in binding affinity, suggesting a functional role of tyrosine 13 in receptor binding. The possible importance of position 13 of hEGF in receptor binding was suggested in a preliminary study with four substitutions, Phe, Leu, Ala, and Gly, at that position (Tadaki and Niyogi, 1991).

**MATERIALS AND METHODS**

Oligonucleotide Synthesis—Synthetic oligonucleotides containing the desired mutations (see Table I) were synthesized on an Applied Biosystems 391 DNA synthesizer utilizing phosphoramidite chemistry (Sinha et al., 1984). Phosphorylation of oligonucleotides at their 5' termini was performed as described before (Engler et al., 1988). The phosphorylated product was purified on a Biogel P-10 (Bio-Rad) column equilibrated in 10 mM tetraethylammonium acetate, pH 7.0, and eluted with the same buffer (Engler et al., 1988).

Oligonucleotide-directed Site-specific Mutagenesis—Two methods of site-directed mutagenesis were employed. The majority of the mutations were made using single stranded M13 mp19 phage DNA containing the cloned hEGF gene as a template for mutagenesis as described by Engler et al. (1988). The oligonucleotides primers used for these mutagenesis were shown in Table I. All mutations were confirmed by DNA sequencing by the dye-sequencing termina-tion method (Sanger et al., 1977) and to confirm the absence of any other genetic alterations. The mutant hEGF gene was excised from the M13 phage DNA and inserted into the expression vector pEGF-1 (Engler et al., 1988). Proper insert orientation was confirmed by restriction enzyme digestion and analysis by gel electrophoresis.

Site-directed mutagenesis by PCR was also used to generate one of the mutations (Tyr<sup>13</sup>→Val). Double stranded DNA of the expression vector containing the hEGF insert was used as template. Two oligonucleotides (see Table I) were utilized in a “back to back” configuration with one of the oligonucleotides having the desired mutation (Heimley et al., 1989). PCR using a thermostable polymerase isolated from *Pyrococcus furiosus* (Stratagene) was used to incorporate and amplify the hEGF gene-containing vector DNA with the proper mutation. The PCR reactions were carried out in standard buffer (20 mM Tris•Cl, pH 8.2, 10 mM KCl, 6 mM (NH_4)_2SO_4, 2 mM MgCl_2, 0.1% Triton-X-100, and 10 μg/ml BSA) supplied with the enzyme with the addition of 0.25 mM dNTPs. Conditions for the first PCR cycle were as follows: denaturation at 94 °C for 3 min, annealing at 40 °C for 1 min, and primer extension at 72 °C for 15 min. Twenty-five successive cycles were performed under the same conditions except that the denaturation time was reduced to 1 min. The linear PCR product was isolated and eluted from a 1% agarose gel. After confirmation of the mutation by DNA sequencing (Sanger et al., 1977) the PCR product was treated with Klenow polymerase (New England BioLabs) to form blunt-ended and circularized by incubation overnight with T4 DNA ligase (New England BioLabs) at 14 °C.

**Expression of hEGF Protein in Escherichia coli**—Wild-type hEGF and its analogues were produced as periplasmic proteins in *E. coli* JM107 as described before (Engler et al., 1988; Campion et al., 1990). The cells were grown at 37 °C in LB medium containing 25 μg/ml ampicillin. Chloramphenicol (0.1 mg/ml final concentration) was added at early log phase of growth and protein production was induced with 0.1 mM isopropylthiogalactoside at late log phase of the culture.

**Table I**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Length</th>
<th>Sequence</th>
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<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Gly</td>
<td>21</td>
<td>5'-CACGAGGCGGGCCGCTGAC-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Phe</td>
<td>21</td>
<td>5'-CACGAGGCGGCTTGTGCTGAC-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→His</td>
<td>21</td>
<td>5'-CACGAGGCGGCCATGCTGAC-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Arg</td>
<td>21</td>
<td>5'-TGTCCAGGCAAGGTGGCTGAC-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Leu</td>
<td>21</td>
<td>5'-TGTCCAGGCAAGGCTGCTGAC-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Ala</td>
<td>21</td>
<td>5'-TGTCCAGGCAAGGGCTGCTGAC-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Val</td>
<td>20</td>
<td>5'-GGGCTGCGCTGACGACGG-C' 3'</td>
</tr>
<tr>
<td>Tyr&lt;sup&gt;13&lt;/sup&gt;→Val (reverse)</td>
<td>16</td>
<td>5'-CTGCGAGACCCGG-C' 3'</td>
</tr>
</tbody>
</table>

* The Tyr<sup>13</sup>→Val mutation was introduced by PCR as described under "Materials and Methods."
cells were incubated for 12 h, then harvested as described previously (Engler et al., 1988).

Isolation and Purification of Recombinant hEGF Protein—The recombinant hEGF protein was liberated from the periplasm by alkaline Tris-EDTA (Engler et al., 1988). Purification was according to Engler et al. (1988) and Campion et al. (1990) and is briefly outlined below. After removal of the cells by centrifugation, protein was precipitated from the supernatant with the addition of (NH_4)_2SO_4 to 80% saturation. The protein was collected by centrifugation, then resuspended and dialyzed against 25 mM sodium phosphate, pH 7.2. The wild-type or mutant hEGF analogue was first separated by gel filtration chromatography on a Sephadex G-75 column (1 x 30 cm) using 25 mM sodium phosphate, pH 7.2. The fractions containing the hEGF protein were loaded onto a reversed-phase HPLC column (Vydac 218 TPS, 4.6 x 250 mm) and eluted with a 15-34% linear gradient of acetonitrile in 10 mM sodium phosphate, pH 7.2, on a Waters Model 540 HPLC system. As observed before (Campion et al., 1990) this process resulted in the isolation of homogeneous hEGF protein, as checked by gel electrophoresis (Engler et al., 1988).

The appropriate amino acid substitution for each mutant protein was verified by analysis of amino acid composition according to Balding-meyer et al. (1994). The proteins were lyophilized and stored at -80°C. 400-400 µg protein in a final volume of 1 ml, containing 20 mM Hepes, pH 7.4, and 0.1% BSA, was used for all experiments. Culture, were obtained for wild-type and mutant hEGF proteins.

Radioactive Competition Binding Assay—Membrane-bound EGFR proteins were prepared according to Akiyama et al. (1985). The binding of hEGF to its receptor was measured using the procedure described by Akiyama et al. (1985) for EGFR binding to membrane receptors in cell-free extracts. 125I-labeled wild-type hEGF was prepared by the chloramine-T method (Hunter and Greenwood, 1962) to a specific activity of ~150,000 cpm/nmol. The receptor-containing A431 membrane preparation (~0.2 µg/ml total protein) was incubated with radiiodinated wild-type hEGF in a mixture containing 20 mM Hepes, pH 7.4, and 0.1% BSA (w/v) in the presence of an increasing concentration of unlabeled wild-type or mutant hEGF protein. The mixtures were allowed to reach equilibrium (30 min at 30°C) after which the 125I-hEGF was collected on cellulose-acetate filters (Millipore X-AF, 25 mm) and eluted with a 15-34% linear gradient of acetonitrile in 10 mM sodium phosphate, pH 7.2. Filters were dried and the radioactivity quantitated by liquid scintillation spectrometry.

Receptor Tyrosine Kinase Stimulation Assay—The ability of wild-type and mutant hEGF proteins to stimulate the tyrosine kinase activity of the EGFR receptor was determined by measuring the incorporation of 32P from [γ-32P]ATP into the synthetic polypeptide substrate (Glu Tyr), (Sigma). Solubilized and partially purified EGFR receptors (Akiyama et al., 1985) from A431 cell membranes were preincubated with increasing concentrations of wild-type or mutant hEGF protein for 15 min at room temperature under conditions similar to those described by Akiyama et al. (1985) with modifications by Campion et al. (1990). Precipitation in the absence ofEGF served as a control. The reaction mixture contained 20 mM Hepes (pH 7.2), 250 mM NaCl, 2 mM MnCl_2, 10 mM MgCl_2, 1 mM DTT, 250 mM (NH_4)_2SO_4, 100 µM Na_2VO_4, 5% glycerol, 0.05% Triton X-100, and ~10 µg of receptor protein. The tyrosine kinase reaction was initiated by the addition of [γ-32P]ATP (0.5 Ci/mmol) and (Glu Tyr), substrates to final concentrations of 75 µM and 0.5 µg/ml, respectively, in a final volume of 0.1 ml. The complete reaction mixture was incubated at room temperature for 10 min, and the reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The acid-insoluble material was collected on 25-mm Millipore HAWP filters which were then washed extensively with 5% trichloroacetic acid containing 10 mM sodium pyrophosphate and dried, and the incorporated radioactivity was quantitated by liquid scintillation spectrometry. The radioactivity incorporated in the absence of hEGF was subtracted from the hEGF-stimulated values. The kinase activities reported here include the incorporation of 32P into both the polypeptide substrate and the receptor. The contribution of the latter, as determined by assaysing the receptor in the absence of the peptide substrate, was found to be less than 2% of the total activity.

CD Measurements—Wild-type and selected hEGF mutant proteins were examined by CD measurements. Five hundred µg of each protein were suspended in 3 ml of 10 mM sodium phosphate, pH 7.2, and pipetted into a 1-cm path length cylindrical quartz cuvette. All of the proteins were quantified from their absorption at 280 nm. The amphetamines, thus obtained, were multiplied by 1.072 to compensate for the substituted tyrosine residue at position 13. The Tyr13-Leu protein was also used as a negative control by reduction with 10 mM DTT (final concentration) overnight at room temperature. The DTT-treated protein was passed through a Bio-Gel P-2 column (equilibrated in and eluted with 10 mM sodium phosphate, pH 7.2) to remove the DTT. The CD spectra were scanned from 260 to 215 nm on a Jasco J-40A CD spectropolarimeter.

RESULTS

Relative Affinities of hEGF Analogues Determined by Radioreceptor Competition Binding Assay—Radiiodinated hEGF binds specifically to receptors in the enriched membrane particulate fraction of A431 cell lysates (Carpenter, 1988). We have previously shown that receptor-bound 125I-hEGF can be fully displaced by unlabeled hEGF, demonstrating the high specificity of the ligand's interaction with the membrane-bound receptor used in this study (Engler et al., 1988; Campion et al., 1990). In competition assays the binding of radiiodinated wild-type hEGF was examined in the presence of increasing concentration of wild-type and each of the mutant hEGF analogues. The concentration of hEGF protein required for 50% displacement of radiiodinated wild-type hEGF (IC50) was determined for wild-type and for each mutant analogue. A ratio comparing wild-type and mutant IC50 values was used as a measure of the relative affinity for each mutant hEGF analogue. These values are shown in Table II. Representative displacement curves for wild-type, Tyr13→Leu, Tyr13→Ile, Tyr13→Val, and Tyr13→Ala hEGF are shown in Fig. 2A, whereas those for the Tyr13→Phe, Tyr13→His, Tyr13→Arg, and Tyr13→Gly hEGF are shown in Fig. 2B. The Tyr13→Phe mutant had a binding affinity nearly identical to that of the wild-type protein. The Tyr13→Leu analogue showed a small decrease in relative binding affinity to 78% of wild type. The Ile13, Val13, and Ala13 variants had receptor binding affinities down to 22, 20, and 8%, respectively, as compared to wild type.

Substitutions with polar residues led to a marked decrease in the relative binding affinity as seen in Fig. 2B. The Tyr13→Leu, which maintains a ring structure on the side-chain, had a binding affinity reduced to 16%. The introduction of a charged residue, Tyr13→Arg, dropped the binding affinity to 5.5% as compared to wild type. Complete removal of the side chain by replacement with glycine dramatically lowered the binding to 0.3%.

Relative Agonist Activities of hEGF Analogues Determined by Tyrosine Kinase Stimulation Assays—The mitogenic effect of EGF is mediated through the stimulation of the receptor's intrinsic tyrosine kinase activity, and this agonist activity in TABLE II

<table>
<thead>
<tr>
<th>hEGF species</th>
<th>Relative receptor affinitya</th>
<th>Relative agonist activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tyr13→Phe</td>
<td>97 ± 10</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>Tyr13→Leu</td>
<td>78 ± 8</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>Tyr13→Ile</td>
<td>22 ± 3</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Tyr13→Val</td>
<td>20 ± 3</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Tyr13→Ala</td>
<td>8.3 ± 0.8</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Tyr13→His</td>
<td>5.0 ± 1</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Tyr13→Arg</td>
<td>5.5 ± 0.5</td>
<td>2 ± 0.7</td>
</tr>
<tr>
<td>Tyr13→Gly</td>
<td>0.3 ± 0.05</td>
<td>2 ± 0.7</td>
</tr>
</tbody>
</table>

* Based on radioreceptor competition binding assays (see "Materials and Methods"). See "Results" for determination of relative receptor affinities. The values given are the averages of four determinations.

* Based on receptor tyrosine kinase stimulation assays (see "Materials and Methods"). See "Results" for determination of relative agonist activities. The values given are the averages of four determinations.
type was beyond the useful range of concentrations for this investigation. Above 15-20 nm, the CD profile is shown in Fig. 1. The potential importance of aromatic residues in EGF was first demonstrated in the studies of Mayo et al. (1986) using NMR and NOE measurements. Their studies predicted that the aromatic residues were clustered together on the surface of the protein. These observations suggested that the aromatic residues might be involved in ligand-receptor interactions by providing a hydrophobic surface on the EGF protein. In previous studies from this laboratory (Engler et al., 1988; Campion et al., 1990) the individual replacements of the aromatic residues, Tyr<sup>13</sup> and Tyr<sup>72</sup>, led to severe decreases in receptor binding affinity. Our preliminary evidence suggested that the loss in activity was at least partly due to structural alteration(s). It should be pointed out, however, that these substitutions, leucine, isoleucine, valine, and alanine, had agonist activities of 75, 7, 7, and 2%, respectively, as compared with wild type. Replacement with the polar residues histidine and arginine lowered the relative agonist activity to 5 and 2%, respectively. Due to the extremely low concentration needed to reach the same degree of stimulation for half-maximal stimulation of the receptor's kinase activity was determined for wild-type hEGF, and the protein concentration needed to reach the half-maximal value of wild-type hEGF by competing hEGF analogues. The Tyr<sup>13</sup>→Leu, Tyr<sup>13</sup>→Arg, and Tyr<sup>13</sup>→Gly mutants were also examined. Wild-type hEGF protein was used to zero the instrument base line. The mutants were scanned against this base line to generate the difference spectrum for each mutant (Fig. 4B). A difference spectrum for DTT-treated Tyr<sup>13</sup>→Leu hEGF (Fig. 4B) was generated in a control experiment, which showed a profile considerably shifted from that of wild-type protein and with a major negative peak at ~226 nm, similar to the results obtained by Holladay et al. (1976) with unfolded (by 8.5 M guanidinium hydrochloride) mEGF. The three native mutant proteins showed only minor differences from wild type in the spectrum between 225 to 235 nm, but displayed significant changes in the spectrum between 215 to 225 nm.

**FIG. 3.** Stimulation of receptor tyrosine kinase activity by wild-type and mutant hEGF analogues. The rate of EGF-dependent phosphorylation of exogenously added (Glu<sub>3</sub>Tyr<sub>6</sub>) substrate by partially purified EGF receptor was measured as described under "Materials and Methods." These plots are the results from a single experiment. A, stimulation of the kinase activity by the hydrophobic mutations; B, stimulation by aromatic and polar mutations. In 1976 there is a major positive peak between 220 to 237 nm, which is indicative of the overall native structure of EGF. CD spectra of three mutant proteins, Tyr<sup>13</sup>→Leu, Tyr<sup>13</sup>→Arg, and Tyr<sup>13</sup>→Gly, were also examined. Wild-type hEGF protein was used to zero the instrument base line. The mutants were scanned against this base line to generate the difference spectrum for each mutant (Fig. 4B). A difference spectrum for DTT-treated Tyr<sup>13</sup>→Leu hEGF (Fig. 4B) was generated in a control experiment, which showed a profile considerably shifted from that of wild-type protein and with a major negative peak at ~226 nm, similar to the results obtained by Holladay et al. (1976) with unfolded (by 8.5 M guanidinium hydrochloride) mEGF. The three native mutant proteins showed only minor differences from wild type in the spectrum between 225 to 235 nm, but displayed significant changes in the spectrum between 215 to 225 nm.

**DISCUSSION**

The potential importance of aromatic residues in EGF was first demonstrated in the studies of Mayo et al. (1986) using NMR and NOE measurements. Their studies predicted that the aromatic residues were clustered together on the surface of the protein. These observations suggested that the aromatic residues might be involved in ligand-receptor interactions by providing a hydrophobic surface on the EGF protein. In previous studies from this laboratory (Engler et al., 1988; Campion et al., 1990) the individual replacements of the aromatic residues, Tyr<sup>13</sup> and Tyr<sup>72</sup>, led to severe decreases in receptor binding affinity. Our preliminary evidence suggested that the loss in activity was at least partly due to structural alteration(s). It should be pointed out, however, that these substitutions, leucine, isoleucine, valine, and alanine, had agonist activities of 75, 7, 7, and 2%, respectively, as compared with wild type. Replacement with the polar residues histidine and arginine lowered the relative agonist activity to 5 and 2%, respectively. Due to the extremely low concentration needed to reach the half-maximal value of wild type was beyond the useful range of concentrations for this investigation. Above 15-20 nm, the CD profile is shown in Fig. 1. The potential importance of aromatic residues in EGF was first demonstrated in the studies of Mayo et al. (1986) using NMR and NOE measurements. Their studies predicted that the aromatic residues were clustered together on the surface of the protein. These observations suggested that the aromatic residues might be involved in ligand-receptor interactions by providing a hydrophobic surface on the EGF protein. In previous studies from this laboratory (Engler et al., 1988; Campion et al., 1990) the individual replacements of the aromatic residues, Tyr<sup>13</sup> and Tyr<sup>72</sup>, led to severe decreases in receptor binding affinity. Our preliminary evidence suggested that the loss in activity was at least partly due to structural alteration(s). It should be pointed out, however, that these
conclusions are based on a single mutation at each site; a more in-depth study with multiple mutations at each site is needed to truly determine the role(s) of these tyrosine residues in receptor binding. The highly conserved tyrosine 37 residue of hEGF was previously investigated in this laboratory. It was found that neither a tyrosine nor an aromatic group was involved in critical hydrogen bonding with the receptor nor with other regions of the EGF ligand (Engler et al., 1990; Engler et al., 1991). That Tyr 13 might participate in some nonreceptor recognition feature(s) common to all EGF-like proteins is suggested by the fact that an aromatic residue in the differential spectrum, corresponding to the major peak region of wild-type hEGF (Fig. 4A) indicating the loss of the tyrosine 13 chromophore, rather than the reduction of disulfide bonds, and the profile is similar to that obtained with unfolded wild-type EGF by Holladay et al. (1976). A comparison of the CD spectra of the three mutants (Fig. 4B) to the wild-type spectrum shows some changes above 225 nm in the major peak region. These changes are similar among the three mutants and are considerably smaller than the large fluctuation(s) observed in this region for the DTT-treated protein. These changes could possibly be accounted for by the loss of the tyrosine 13 chromophore, rather than major disruption of overall native structure.

The three mutants displayed more striking spectral changes below 225 nm and particularly between 215 to 220 nm. These could be indicative of structural changes in peptide groups, including the $\beta$-sheet residues (Holladay et al., 1976). NMR studies with Tyr 13-Leu hEGF revealed significant spectral changes for many residues distant from the mutated site, although the gross overall structure was not significantly altered (Hommel et al., 1991). Most of the altered residues resided in the major $\beta$-sheet of the amino-terminal motif of hEGF. The CD spectral changes seen between 215 and 220 nm in our studies probably correspond to the structural changes observed by Hommel et al. (1991). It is noteworthy that the CD spectral changes between 215 and 220 nm were most pronounced with the Tyr 13-Leu mutant, although it had an activity similar to that of wild-type hEGF. Tyr 13-Arg and Tyr 13-Gly, which displayed smaller spectral changes between 215 and 220 nm, had greatly reduced receptor binding affinity. These results suggest that the structural changes, mainly in the $\beta$-sheet residues, introduced by substitution of Tyr 13 cannot account for the loss of receptor binding affinity. Taken together, the available evidence suggests that the decreases in receptor binding affinity of the mutant proteins are
not due to changes in the three-dimensional structure of hEGF. Rather, the tyrosine 13 residue appears to play a "functional" role in receptor binding by contributing to hydrophobic receptor-ligand interactions.

Site-directed mutagenesis studies from this laboratory have implicated several residues of hEGF in receptor recognition. These sites include Tyr13, Ile23, and Leu26 in the aminoterminal domain, and Arg41 and Leu6 in the carboxy-terminal domain (see Fig. 1). The residues Tyr13 and Ile23, although in the same domain, are located within separate disulfide loops. In recent studies from this laboratory, double-site mutations were generated at some of the above sites to assess potential effects on protein structure and to evaluate their participation in receptor-ligand interactions as well as any positive or negative cooperativity in the binding of separate regions of the peptide ligand (Campion et al., 1993). These studies have demonstrated, in general, a cumulative effect of mutations in separate regions of the hEGF molecule, indicating that individual sites on the growth factor peptide, for the most part, interact independently with the receptor and that substitution of an important residue at one site fails to substantially alter the receptor-ligand interactions associated with residues in other regions of the growth factor peptide. However, the relative affinity of the double-site mutant analogue combining Ile23→Thr with Tyr13→His was somewhat less than that predicted for purely independent sites and may indicate a small degree of cooperativity between these two sites. Another structural consideration involves the location of the aromatic side-chain of Tyr13 which is predicted to be in close proximity to the Arg41 side-chain and specifically the aromatic side-chain of Tyr13 may affect the native structure of the receptor-ligand interactions as well as any positive or negative cooperativity in the binding of separate regions of the hormone. However, the tyrosine residue is mutated indirectly as the result of a change in the interaction between the amino- and carboxyl-terminal domains via the interaction between the 13 site and its neighbor Arg41. It should be noted, however, that a direct interaction between the Arg41 side-chain and specifically the aromatic side-chain of Tyr13 is not required, as the nonpolar leucine side-chain can substitute for Tyr13 with little effect on affinity. Overall, the effects of simultaneous mutation of Tyr13 and other residues of hEGF suggest that Tyr13 may be functionally associated with the carboxyl-terminal EGF receptor-binding domain as well as being in close proximity structurally.

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