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Biochemical Properties of Site-Directed Mutants of Human Epidermal Growth Factor: Importance of Solvent-Exposed Hydrophobic Residues of the Amino-Terminal Domain in Receptor Binding†

Stephen R. Campion, Risë K. Matsunami, David A. Engler, and Salil K. Niyogi*

The Protein Engineering and Molecular Mutagenesis Program and The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077

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ABSTRACT: Eight analogues of human epidermal growth factor (hEGF) having specific amino acid substitutions in the β-sheet structure (residues 19-31) of the amino-terminal domain were generated by site-directed mutagenesis. Affinity of the epidermal growth factor (EGF) receptor for each of these mutant hEGF analogues was measured by both radioreceptor competition binding and receptor tyrosine kinase stimulation assays. The relative binding affinities obtained by these two methods were generally in agreement for each hEGF species. The results indicate that hydrophobic residues on the exposed surface of the β-sheet structure of the amino-terminal domain of hEGF have an important role in the formation of the active EGF-receptor complex. The substitution of hydrophobic amino acid residues, Val-19 → Gly, Met-21 → Thr, Ile-23 → Thr, and Leu-26 → Gly, resulted in decreased binding affinity, with the most severe reductions observed with the last two mutants. The mutations Ala-25 → Val and Lys-28 → Arg introduced amino acid residues resulting in slightly increased receptor binding affinity. Similar to previous results with acidic residues in this region [Engler, D. A., Matsunami, R. K., Campion, S. R., Stringer, C. D., Stevens, A., & Niyogi, S. K. (1988) *J. Biol. Chem. 263*, 12384-12390], removal of the positive charge in the Lys-28 → Leu substitution had almost no effect on binding affinity, indicating the lack of any absolute requirement for ionic interactions at this site. Substitution of Tyr-22, which resulted in decreased receptor binding affinity, provides further indication of the importance of aromatic residues in this region of the molecule, as found earlier with Tyr-29 (cf. reference above). These results provide direct evidence suggesting the importance of aliphatic and aromatic amino acid side chains, of the amino-terminal β-sheet of the EGF molecule, in receptor-ligand interactions.

Recent studies using site-directed mutagenesis have attempted to determine those amino acid residues of EGF[1] and the related transforming growth factor (TGF-α) which are directly involved in intermolecular interactions with the EGF receptor. For the most part, these studies have concentrated on altering amino acid residues in the carboxy-terminal domain (residues 32-53) of the growth factor peptide. The substitution of the highly conserved hydrophobic residue Leu-47 (Leu-48 in TGF-α) near the carboxy-terminal end of the peptide resulted in a marked reduction in the affinity of the receptor for hEGF (Engler et al., 1988; Dudgeon et al., 1989),[2] mEGF (Ray et al., 1988; Moy et al., 1989), and hTGF-α (Lazar et al., 1988). Other amino acids which have been examined by mutagenesis include the highly conserved Tyr-38, Arg-42, and Asp-47 residues of hTGF-α (Lazar et al., 1989; Defeo-Jones et al., 1988, 1989) as well as Tyr-37 and Arg-41 of hEGF (Engler et al., 1990). The results of those studies have demonstrated a nearly absolute requirement for Arg-41 (Arg-42

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* To whom correspondence should be addressed.

[1] Abbreviations: EGF, epidermal growth factor; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; TGF-α, transforming growth factor α; hTGF-α, human transforming growth factor α; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; 2D NMR, two-dimensional nuclear magnetic resonance spectroscopy; 1D NMR, one-dimensional nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography.

[2] R. K. Matsunami, G. T. Montelione, N. Greenfield, M. L. Yette, A. Stevens, S. K. Niyogi, Mutational Analysis of Leucine 47 in Human Epidermal Growth Factor (manuscript in preparation).

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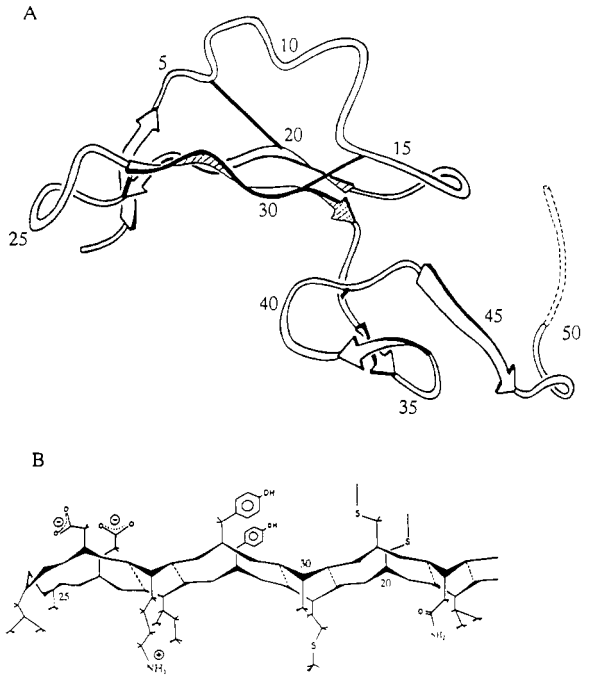

FIGURE 1: (A) Representation of the three-dimensional structure of mEGF [adapted from Kohda et al. (1988)]. (B) Structure of the large antiparallel β-sheet of the amino-terminal domain. This model is provided to demonstrate the relative orientation of amino acid side chains predicted for this region of the molecule and does not represent molecular dimensions.

in TGF-α) and the importance of the hydrophobic Leu-47 (Leu-48 in TGF-α) in receptor binding. The importance of these highly conserved residues has seemed to imply that the carboxy-terminal domain of the growth factor peptide contains the receptor binding site(s). The participtation of residues outside of the carboxy-terminal domain in receptor binding, however, has not been fully investigated.

Models of EGF structure (Figure 1A) derived from 2D NMR data (Cooke et al., 1987; Montelione et al., 1987; Kohda et al., 1988) predict that residues 19–32 in the amino-terminal domain of the protein exist in an antiparallel β-sheet conformation, with amino acid side chains locked into positions either above or below the plane of the β-sheet (Figure 1B). Amino acid side chains on one face of the β-sheet appear to be involved in intramolecular interactions with other residues in the peptide (residues 6-13). The clustering of the aromatic side chains in aqueous solution (Mayo et al., 1986), in concert with the physical constraints imposed by the three intramolecular disulfide bonds, functions to establish an extremely stable protein structure (Holliday et al., 1976), which allows a group of hydrophobic residues to remain in a conformation relatively exposed to the aqueous solvent environment. In a previous study involving this region, mutations were generated at residues Glu-24, Asp-27, and Tyr-29; only the replacement of the tyrosine residue caused a major reduction in the receptor binding affinity (Engler et al., 1988). In the present study, seven additional residues (Val-19, Met-21, Tyr-22, Ile-23, Ala-25, Leu-26, and Lys-28) in the predicted β-sheet of the amino-terminal domain were targeted for mutagenesis to evaluate more thoroughly the role of amino acid residues in this region of the protein. The effects of specific amino acid substitutions of individual β-sheet residues on the receptor affinity were examined by both radioreceptor compeititon binding and tyrosine kinase stimulation assays.

The results presented here suggest that residues on the solvent-exposed surface of the β-sheet structure of the amino-terminal domain may play an important role in the formation of the active EGF–receptor complex. The observed changes in receptor binding affinity are consistent with an entropy-driven binding model and further suggest the importance of hydrophobic EGF–receptor interactions.[3]

Experimental Procedures

Membrane Preparation. Membrane-bound EGF receptors were prepared according to a modification of the procedure described by Akiyama et al. (1985). Confluent A431 (human epidermoid carcinoma) cell cultures (grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) were washed twice with ice-cold phosphate-buffered saline and removed from the surface of the plates with a rubber policeman. The cell suspension was kept on ice, the cells were allowed to settle, and the buffer was discarded. The cells were rinsed with several volumes of hypotonic KMP buffer (20 mM Pipes, pH 7.2, 1 mM $MgCl_2$, and 5 mM KCl), placed on ice, and allowed to settle, and the buffer was again discarded. The cells were allowed to swell in 4 volumes of KMP buffer and homogenized by 25–30 strokes in a glass Dounce homogenizer. The cell lysate was centrifuged at 15000*g* for 5 min at 4 °C, the pellet was resuspended in KMP buffer and centrifuged, and the supernatants were combined. Cell lysis and fractionation were monitored by microscopic examination. The combined supernatant was centrifuged at 100000*g* for 30 min at 4 °C and the membrane pellet resuspended in 20 mM Hepes, pH 7.4, containing 0.1% BSA, for use in the receptor binding assays.

Soluble Receptor Isolation. A431 cell membranes were resuspended in solubilization buffer [40 mM Hepes, pH 7.2, 0.5 M NaCl, 10% glycerol (v/v), 1 mM EGTA, and 10 μg/mL leupeptin], and the receptor was solubilized by addition of Triton-X-100 to a concentration of 1% (v/v). The membrane suspension was kept at room temperature for 20 min followed by centrifugation for 30 min at 100000*g*. The soluble fraction was loaded onto a wheat germ agglutinin–agarose column, equilibrated in solubilization buffer containing 0.05% Triton-X-100, and the EGF receptor eluted with 0.3 M *N*-acetylglucosamine. Active receptor fractions were stored at −80 °C for use in the tyrosine kinase stimulation assays.

Radioreceptor Competition Binding Assay. The binding of EGF to its receptor was measured by the radioreceptor competition method described by Carpenter (1985) and is described briefly below. ^{125}I-hEGF was prepared by the chloramine-T method (Hunter & Greenwood, 1962) to an average specific activity of ~150000 cpm/pmol. The receptor-containing A431 membrane preparation (approximately 0.2 μg/mL total membrane protein) was incubated with radioiodinated EGF in a mixture containing 20 mM Hepes, pH 7.4, 0.1% BSA, and the unlabeled competing hEGF species. The mixtures were allowed to reach equilibrium after which the receptor-bound ^{125}I-EGF was collected on cellulose acetate filters (Millipore GVWP). Unbound ligand was removed by washing filters with 20 mM Hepes, pH 7.4, containing 0.1% BSA. Filters were dried, and the radioactivity was quantitated by liquid scintillation spectrometry.

Receptor Tyrosine Kinase Stimulation Assay. The rate of EGF receptor catalyzed incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic polypeptide substrate $(Glu_4,Tyr_1)_n$ (Sigma Chemical Co.) was measured as a function of growth factor concentration. Solubilized and partially purified EGF receptors (Akiyama et al., 1985) from A431 cell membranes

[3] S. R. Campion and S. K. Niyogi, The Hydrophobic Nature of Epidermal Growth Factor Receptor-Ligand Interactions (submitted for publication).

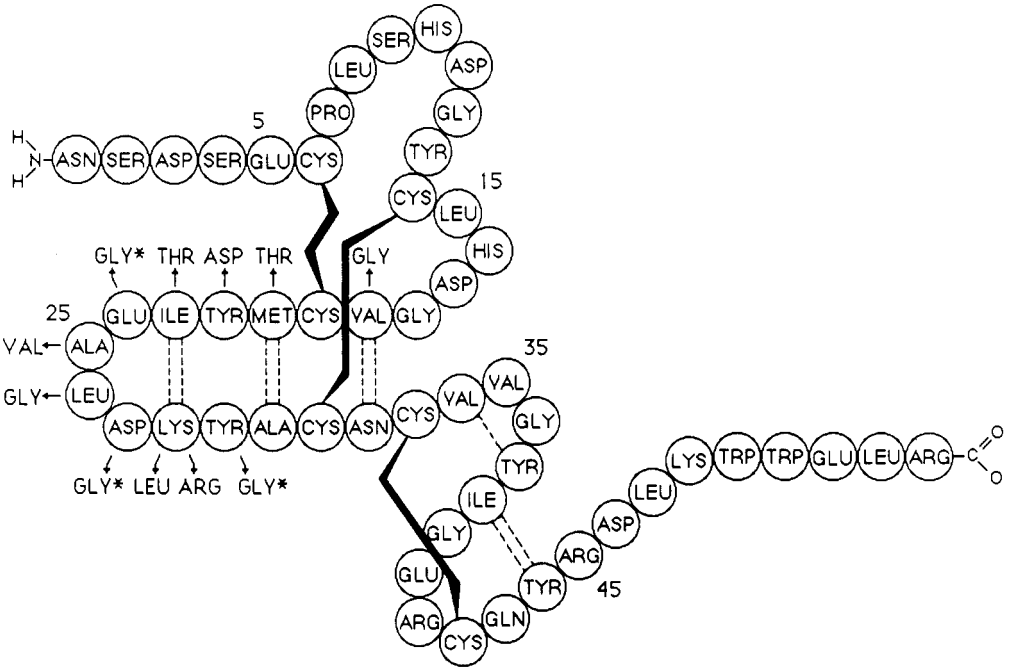

FIGURE 2: Substitution of amino acid residues in the β-sheet structure of the amino-terminal domain of hEGF [adapted from Cooke et al. (1987)]. Hydrogen bonding in the peptide backbone is indicated by dashed lines. The substitutions of amino acid residues by site-directed mutagenesis were as indicated. Those substitutions indicated by asterisks were analyzed previously [see Engler et al. (1988)].

were preincubated with increasing concentrations of wild-type or mutant EGF protein for 15 min at room temperature under conditions similar to those described by Koland and Cerione (1988). The tyrosine kinase reaction was initiated by the addition of [γ-^{32}P]ATP (0.5 Ci/mmol) and $(Glu_4,Tyr_1)_n$ substrates to final concentrations of 75 μM and 0.5 mg/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 stopped by 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, washed extensively with the same solvent, and dried, and the incorporated radioactivity was quantitated by liquid scintillation spectrometry. Greater than 98% of the acid-insoluble radioactivity was found to be incorporated into the exogenously added $(Glu_4,Tyr_1)_n$ substrate under these conditions.

Expression of hEGF Protein in Escherichia coli. Production of wild-type or mutant hEGF as a periplasmic protein in *E. coli* has been previously described (Engler et al., 1988). Protein expression was induced by the addition of isopropyl thiogalactoside at a concentration of 0.1 mM to late log phase cultures growing at 37 °C in LB medium containing 25 μg/mL ampicillin and 1–5 μg/mL chloramphenicol. Incubation was continued for 12 h at which time cells were harvested by centrifugation and the recombinant growth factor protein was extracted and purified as described below.

Isolation and Purification of Recombinant hEGF. Wild-type or mutant protein was routinely liberated from the bacterial periplasm by the alkaline Tris-HCl extraction procedure described in Engler et al. (1988). After removal of the cells by centrifugation, protein was precipitated from the extract by the addition of $(NH_4)_2SO_4$ to 80% saturation. The protein pellet, collected by centrifugation, was resuspended and dialyzed against 25 mM sodium phosphate, pH 7.2, prior to purification. Wild-type or mutant analogues of hEGF were first separated by gel filtration chromatography on a Sephadex G-75 column (1 × 90 cm) equilibrated and eluted with 25 mM sodium phosphate, pH 7.2. Fractions containing hEGF protein were loaded directly onto a Vydac 218 TPS reverse-phase column (4.6 × 250 mm) and eluted, with a 15–34% (v/v) linear gradient of acetonitrile in 10 mM sodium phosphate, pH 7.2, on a Waters Model 840 HPLC system. This process resulted in the isolation of homogeneous hEGF protein which was lyophilized and stored at −80 °C.

Site-Directed Mutagenesis. Specific genetic alterations were made in the *hEGF* gene (Engler et al., 1988) with mutant oligonucleotide primers and single-stranded M13 DNA containing the *hEGF* gene insert according to protocols described earlier (Niyogi et al., 1986; Enger et al., 1988). All engineered mutations were confirmed by DNA sequencing (Sanger et al., 1977). Each altered *hEGF* gene was then inserted into the expression vector, and the mutant gene product was produced, isolated, and purified as described above. As found earlier (Engler et al., 1988), the recombinant *hEGF* gene was properly expressed and the protein product correctly processed and secreted into the *E. coli* periplasm. In *E. coli* cells lacking the *hEGF* gene, no protein eluting with a retention time similar to that of the recombinant hEGF protein product was detected during HPLC purification.

Results

Initial studies on the mutagenesis of residues in this region of the protein demonstrated that the acidic residues Glu-24 and Asp-27 in the β-sheet of the amino-terminal domain were not important for the binding of EGF to the receptor (Engler et al., 1988). In that study, the removal of the aromatic side chain of Tyr-29, by replacement with glycine, significantly lowered the ability of the growth factor to bind to the receptor, suggesting an important role for Tyr-29 in receptor binding. In this report, seven additional sites in the β-sheet structure of the amino-terminal domain of hEGF were targeted for site-directed mutagenesis. The amino acid substitutions which were generated are shown in Figure 2 and include the alterations, mainly of those residues having side chains oriented on the solvent-exposed surface of the β-sheet region, as well as the conserved Tyr-22. The ability of each of these mutant hEGF analogues to displace wild-type hEGF from the re-

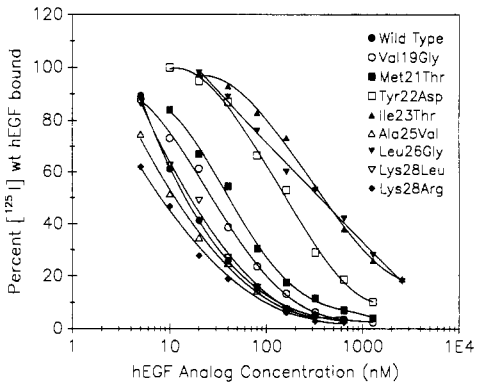

FIGURE 3: Displacement of receptor-bound wild-type hEGF by competing hEGF species. The binding of radioiodinated wild-type hEGF was measured in the presence of increasing concentrations of mutant hEGF species, as described under Experimental Procedures. Curves which appear to the right of the wild-type competition curve are for those mutants having decreased receptor binding affinity; curves for the hEGF analogues having greater binding affinity, relative to that of wild-type hEGF, appear to the left.

Table I: Comparison of Receptor Binding Affinity As Measured by Radioreceptor Competition and Receptor Tyrosine Kinase Stimulation Assays[a]

hEGF species	relative binding affinity (% of wild type)	
	radioreceptor competition assay	tyrosine kinase stimulation assay
wild type	100	100
Val-19 → Gly	58	35
Met-21 → Thr	36	19
Tyr-22 → Asp	8	25
Ile-23 → Thr	4	4
Glu-24 → Gly[b]	86	84
Ala-25 → Val	136	106
Leu-26 → Gly	5	5
Asp-27 → Gly[b]	48	71
Lys-28 → Leu	79	95
Lys-28 → Arg	188	180
Tyr-29 → Gly[b]	17	24

[a] Relative binding affinity = IC_{50}(wild type)/IC_{50}(hEGF analogue) × 100 for the radioreceptor competition binding assay and EC_{50}(wild type)/EC_{50}(hEGF analogue) × 100 for the tyrosine kinase stimulation assay. See text under Results describing the determination of IC_{50} and EC_{50} values. [b] See Engler et al. (1988).

ceptor–ligand complex and to stimulate the receptor's intrinsic protein-tyrosine kinase activity was examined.

Determination of Relative Affinities of hEGF Analogues by Radioreceptor Competition. The binding of radioiodinated wild-type hEGF to its receptor was measured in the presence of increasing concentrations of each of the eight new mutant hEGF analogues. The amount of bound radioiodinated wild-type hEGF decreased with increasing concentration of the competing hEGF species and generated the competition binding curves shown in Figure 3. The amount of wild-type and mutant hEGF protein required for 50% displacement of radioiodinated wild-type hEGF (IC_{50}) was determined for each hEGF analogue. A ratio comparing wild-type and mutant IC_{50} values was used as a measure of the relative affinity of the receptor for each mutant hEGF peptide (Table I). The relative binding affinities of the mutant hEGF species ranged from slightly higher to 25-fold lower than that of wild-type hEGF. All of the mutations that removed or replaced hydrophobic amino acid side chains resulted in decreased binding affinity. The most severe effects on the binding affinity were observed with the Ile-23 → Thr and Leu-26 → Gly analogues. Replacement of either of the two aromatic (tyrosine) residues

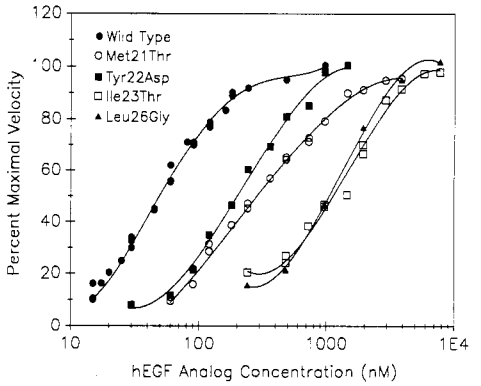

FIGURE 4: Stimulation of EGF receptor tyrosine kinase activity by wild-type and mutant hEGF species. The stimulation of the rate of EGF-dependent tyrosine phosphorylation of exogenously added $(Glu_4,Tyr_1)_n$ substrate was examined as described under Experimental Procedures by use of solubilized and partially purified EGF receptors. Curves are for the activation of the receptor tyrosine kinase by selected hEGF mutants having decreased receptor binding affinity. Some variability in the absolute amount of growth factor peptide required for kinase stimulation was observed when curves were generated with different receptor preparations and prevented the presentation of all the mutants in this figure. However, each mutant was assayed relative to wild type, and those values appear in Table I.

also resulted in decreased receptor affinity. Each of the mutants tested was able to fully displace wild-type hEGF in the competition binding assay, indicating that all of the mutant hEGF species bound in a specific manner to the EGF binding site on the receptor, despite any decrease in affinity.

Determination of Relative Binding Affinities of Mutant hEGF Analogues Using Receptor Tyrosine Kinase Stimulation Assays. The relative binding affinity of the receptor for each hEGF analogue was also measured directly by examining the ability of the mutant hEGF species to stimulate the receptor's protein-tyrosine kinase activity by use of solubilized and partially purified EGF receptors. The velocity of the EGF-dependent kinase reaction was measured as a function of the concentration of hEGF species present in the assay. The kinase stimulation curves for several representative mutants, including the Ile-23 → Thr and Leu-26 → Gly hEGF analogues, are shown in Figure 4. The protein concentration required for half-maximal stimulation of the receptor kinase activity (EC_{50}) was determined for wild-type hEGF and the mutant analogues. The ratio of the EC_{50} value of wild type compared with that for each mutant was used to assess the relative affinity of the receptor for the mutant proteins under the kinase assay conditions. Although slight variability in the concentration of EGF required for half-maximal stimulation of receptor kinase activity was observed with different receptor preparations, the affinity of mutant hEGF analogues measured relative to that of wild type remained internally consistent with each receptor preparation. The relative affinity of the receptor for each mutant growth factor, as determined by the receptor tyrosine kinase stimulation assay, was generally observed to be in agreement with the value found by radioreceptor competition binding. The alteration of specific residues in the growth factor peptide resulted in similar changes in relative affinity under both assay conditions and clearly indicated the importance of the hydrophobic residues, Ile-23 and Leu-26, as well as the aromatic residues, Tyr-22 and Tyr-29, in the formation of an active receptor–ligand complex.

Discussion

The substitution of amino acids by site-directed mutagenesis is a useful technique for the examination of the contribution of specific residues to protein structure and function. This

study, specifically, involved substitutions of amino acid residues in the β-sheet structure of the amino-terminal domain of hEGF in an attempt to identify those residues in the growth factor peptide that participate in the molecular interactions required for the formation of the EGF–receptor complex. We had previously examined the nature of the chemical "bonding" responsible for the formation of the highly stable EGF–receptor complex and found it to be the result of an entropy-based change in free energy characteristic of hydrophobic protein–protein interactions, suggesting the potential importance of residues with nonpolar side chains in the association of these molecules.[3]

The structure of EGF established by 2D NMR (Cooke et al., 1987; Montelione et al., 1987; Kohda et al., 1988) has predicted the existence of a relatively large region of solvent-exposed hydrophobic amino acid side chains in the β-sheet structure of the amino-terminal domain of the growth factor peptide. Nearly all of the residues in this region of the protein have been the target of site-directed mutagenesis in our laboratory. The effects of removal or replacement of the acidic and basic functional groups, polar aromatic tyrosine residues, and hydrophobic amino acids in this region of the protein have now been examined.

In a previous study (Engler et al., 1988), mutations involved residues in the amino-terminal β-sheet structure that were all above the plane of the β-sheet. Substitution of Glu-24 with glycine resulted in only a slight decrease in receptor binding affinity, retaining almost 90% relative binding affinity. A similar substitution of Asp-27 with glycine still retained about 50% of the receptor binding affinity. The aromatic side chains of Tyr-22 and Tyr-29, also predicted to be oriented above the hydrogen-bonded β-sheet structure, have been demonstrated by 2D NMR to be in close proximity with other aromatic residues in the amino-terminal domain and may in part be responsible for the correct folding and stabilization of the protein in solution (Mayo et al., 1986). Substitution of Tyr-22 and Tyr-29 with aspartate and glycine, respectively, resulted in the loss of ~80% of relative receptor binding affinity. However, the reduced affinity observed following the removal of either of these aromatic side chains may be the result of decreased protein stability and/or altered protein folding or conformation. This is indicated by the appearance of multiple forms of these otherwise full-length mutant proteins during their final purification by reverse-phase HPLC (results not shown). Precise characterization of possible structural roles of Tyr-22 and Tyr-29 in hEGF must await detailed 2D NMR studies of mutants at these sites.

The substitutions Val-19 → Gly, Met-21 → Thr, Ile-23 → Thr, and Leu-26 → Gly resulted in decreased receptor binding affinity for these mutants. The decrease in affinity upon substitution of hydrophobic residues with glycine or an amino acid having a more polar side chain, such as threonine, suggests that it is the hydrophobic property of these side chains which is required for the proper formation of the EGF–receptor complex. Conversely, the effect of substitution of amino acids with residues having greater "water-immobilizing" capacity was also examined. Substitution with a more hydrophobic residue in the mutation Ala-25 → Val resulted in a slight increase in the affinity of the receptor for this hEGF species. Introduction of the alternate basic amino acid in the Lys-28 → Arg mutation resulted in an hEGF analogue with almost double the receptor affinity of wild type. The mutation Lys-48 → Arg near the carboxy-terminal tail also demonstrated a slight increase in binding affinity (data not shown). The substitution of arginine for lysine, like the valine substitution for alanine, introduces an amino acid which apparently increases the entropy gained by the system upon receptor–ligand association and thereby enhances the thermodynamic stability of the complex. The presence of the basic amino acid side-chain at position 28 was not however required for receptor binding. The amino acid substitution Lys-28 → Leu generated an hEGF analogue which had essentially no decrease in binding affinity. These results appear to support the evidence for an entropy-driven EGF binding and the belief that hydrophobic protein–protein interactions provide the energy which drives the formation of the EGF–receptor complex.[3]

The question of the effect of mutation(s) on the structure of the peptide must be considered. For hydrophobic interactions to occur, it is necessary for the growth factor and the receptor to interact in a way in which the water-immobilizing side chains at the surface of these molecules become sequestered following association. The importance of the protein structure for the establishment of the necessary protein–protein and protein–solvent interaction cannot be overstated. The thermodynamically stable formation of a receptor–ligand complex must require complementary growth factor and receptor three-dimensional structures to ensure that the hydrated surfaces of these molecules can associate to form the critical solvent-excluded region of protein–protein "bonding". Preliminary evaluation of the 1D NMR spectra, at both pH 3 and pH 7, of the most severely affected mutant analogues, Ile-23 → Thr and Leu-26 → Gly, indicates that some minor structural and dynamic differences exist between mutants and wild-type hEGF. The NMR data demonstrate a similar degree of stability in the β-sheet hydrogen bonding of mutant and wild-type hEGF proteins, indicating similar overall peptide folding and conformation (G. T. Montelione, personal communication). The effect of the individual amino acid substitutions on EGF receptor–ligand interactions cannot yet be related directly to well-characterized alterations in protein structure and must await detailed 2D NMR analysis. The changes in receptor binding affinity of the EGF mutants described here are presently considered to be due to the effect of localized changes in the interactions of the EGF molecule with the solvent and the receptor and not alterations in critical intramolecular interactions required for proper growth factor folding or tertiary structure.

In addition to further suggesting the general hydrophobic nature of EGF–receptor interactions, this report provides evidence demonstrating the importance of residues outside of the highly conserved carboxy-terminal domain of EGF in the formation of the active EGF–receptor complex. From our studies it appears that the interactions between growth factor and receptor require the involvement of regions encompassing both domains of the growth factor peptide. Furthermore, studies in our laboratory have shown that the effects of Ile-23 → Thr and Leu-26 → Gly mutations, as well as mutations of the highly conserved Leu-47, on the strength of the receptor–ligand interactions are translated into a decreased ability to fully activate the receptor tyrosine kinase activity, and these hEGF analogues have also been demonstrated to be partial antagonists of the EGF-dependent receptor kinase activity (Matsunami et al., 1990). A better understanding of both the chemical nature of the molecular association and the role of specific amino acid residues in the formation of the EGF-receptor complex should aid in the design of growth factor analogues having desired biochemical properties.

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