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Evaluation of the Role of Electrostatic Residues in Human Epidermal Growth Factor by Site-Directed Mutagenesis and Chemical Modification

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Abstract Four residues in the carboxy-terminal domain of human epidermal growth factor (hEGF), glutamate 40, glutamine 43, arginine 45, and aspartate 46 were targeted for site-directed mutagenesis to evaluate their potential role in epidermal growth factor (EGF) receptor-ligand interaction. One or more mutations were generated at each of these sites and the altered recombinant hEGF gene products were purified and evaluated by radioreceptor competition binding assay. Charge-conservative replacement of glutamate 40 with aspartate resulted in a decrease in receptor binding affinity to 30% relative to wild-type hEGF. On the other hand, removal of the electrostatic charge by substitution of glutamate 40 with glutamine or alanine resulted in only a slightly greater decrease in receptor binding to 25% relative receptor affinity. The introduction of a positive charge upon substitution of glutamine 43 with lysine had no effect on receptor binding. The substitution of arginine 45 with lysine also showed no effect on receptor binding, unlike the absolute requirement observed for the arginine side-chain at position 41 [Engler DA, Campion SR, Hauser MR, Cook JS, Niyogi, SK: J Biol Chem 267:2274–2281, 1992]. Subsequent elimination of the positive charge of lysine 45 by reaction with potassium cyanate showed that the electrostatic property of the residue at this site, as well as that at lysine 28 and lysine 48, was not required for receptor-ligand association. The most highly conserved of the four residues studied in this report, aspartate 46, was replaced with alanine, tyrosine, and arginine, resulting in a decrease in relative receptor affinity to 23, 14, and 4 percent, respectively, and suggests the importance of an acidic group at this site of EGF. The ability to generate sufficient yields of mutant recombinant EGF protein was sensitive to the type of side-chain substitutions generated at the sites described in this report and may indicate a role for these residues in the formation of the EGF structure apparently required for productive yields of EGF proteins in the expression system used in this study.

Key words: hEGF, ionic residues, site-directed mutagenesis, chemical modification, receptor affinity

Alteration of EGF by site-directed mutagenesis has implicated several sites in the molecule as candidates for participating in critical receptor–growth factor interactions. The mutagenesis data provide evidence that EGF-receptor association requires the involvement of regions in both the amino- and carboxy-terminal domains of the growth factor peptide. Replacement of selected hydrophobic residues in the amino-terminal domain greatly decreased receptor binding affinity [1]. Mutagenesis studies have shown that the non-polar leucine 47 side-chain is essential for high affinity binding [2–6] and optimal receptor kinase activation [7]. The removal of acidic glutamate 24 and aspartate 27 side-chains in the amino-terminal domain [2] or replacement of the positively charged residue lysine 28 with the uncharged leucine [1] had little effect on receptor binding. In contrast, the electrostatic residue arginine 41 has been shown to be of great importance for the optimal binding of EGF to its receptor [8], and it has recently been demonstrated that the formation of the EGF receptor-ligand complex requires, specifically, the guanidinium group of the arginine 41 side-chain

Abbreviations used: BSA, bovine serum albumin; EGF, epidermal growth factor; hEGF, human epidermal growth factor; Hepes, N-[2-hydroxyethyl]piperizine-N′-[2-ethanesulfonic acid]; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance.

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of EGF in a critical growth factor–receptor interaction [9].

Both arginine 41 and leucine 47, located in the carboxy-terminal domain of EGF, are highly conserved through a number of homologous proteins from a variety of species [10,11]. The importance of any residue, however, cannot always be predicted based on structural considerations and/or sequence homology, and the carboxy-terminal domain of EGF contains a number of other residues with somewhat lower degrees of sequence conservation which might also participate in receptor-ligand association. In this study, glutamate 40, glutamine 43, and aspartate 46 were altered by site-directed mutagenesis, and arginine 45 by mutagenesis and chemical modification, in an attempt to evaluate the possible role of these residues in receptor binding in light of their close proximity to the critical residues arginine 41 and leucine 47. The results demonstrate the lack of a strict requirement for the electrostatic side-chains of glutamate 40 or arginine 45, as well as a tolerance for an additional positive electrostatic charge in place of glutamine 43, which had been previously predicted to be important for EGF structure/function [12]. The decreased receptor affinity of hEGF analogs following replacement of the aspartate 46 side-chain indicates some preference for an acidic residue at this site in the molecule. Thus, while there is a stringent requirement for arginine 41 and perhaps aspartate 46, the participation of other electrostatic residues in the formation of the receptor-ligand complex appears not to be critical.

MATERIALS AND METHODS

Production of Mutant EGF Proteins

The materials and methods pertaining to the production, purification, and characterization of the wild-type and mutant recombinant hEGF proteins including the reverse-phase HPLC methodology used in this study were previously described [1,2]. Introduction of each mutation was confirmed by DNA sequencing [13]. Quantitation of EGF proteins was estimated from their absorbance at 280 nm relative to the extinction coefficient standardized for wild-type EGF. Appropriate adjustments to the EGF extinction coefficient were made for mutations involving substitution with aromatic residues.

Chemical Modification of EGF

Lyophilized samples of highly purified wild-type or mutant hEGF (50 μg) were incubated for 24 h at 37°C in 50 μl of 1 M potassium cyanate (Aldrich Chemical Co.) containing 0.5 M sodium borate, pH 8.9. The reaction products were isolated using reverse-phase HPLC, and the amino acid modifications leading to neutralization were verified by non-denaturing gel electrophoresis (results not shown).

Receptor Binding Assay

Membrane-bound EGF receptors were prepared using a modification [1] of the procedure described by Akiyama et al., [14]. The binding of EGF to its receptor was measured using the method described by Carpenter [15] for EGF binding to membrane-bound receptors in cell-free extracts. Radioiodinated hEGF was prepared by the chloramine-T method [16] to an average specific activity of 150,000 cpm/pmol. The receptor-containing A431 membrane preparation (approximately 0.2 μg/ml total membrane protein) was incubated with radioiodinated wild-type hEGF in a mixture containing 20 mM Hepes, pH 7.4, and 0.1% (w/v) BSA in the presence of an increasing concentration of wild-type hEGF or of either the genetically or chemically altered growth factor analogues. The mixtures were allowed to reach equilibrium (30 min) after which the receptor-bound ^{125}I-hEGF 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 quantitated by liquid scintillation spectrometry.

The binding of radioiodinated wild-type hEGF was measured in the presence of an increasing concentration of the wild-type or the altered hEGF analog. The concentration of EGF protein required for displacement of 50 percent of the radiolabeled hEGF ([IC_{50}]) was estimated for each EGF species and used to obtain a relative measure of receptor binding affinity.

RESULTS

Nature of the Substitutions

To evaluate the nature of potential interactions involving hEGF residues glutamate 40, glutamine 43, arginine 45, and aspartate 46, we have produced hEGF analogs using site-directed

mutagenesis and chemical modification, and the effects of specific hEGF amino acid alterations were evaluated using radioreceptor competition binding assays. Mutations were generated which substituted glutamate 40 with the amino acids alanine, aspartate, and glutamine, while the neighboring residue glutamine 43 was substituted with lysine. Arginine 45 was replaced in a charge-conservative substitution with lysine and subsequently neutralized by reaction with potassium cyanate. In control experiments the amino groups of wild-type hEGF, which include the amino-terminal α-amine, lysine 28, and lysine 48, were also modified by reaction with potassium cyanate. The relatively conserved residue aspartate 46 was substituted with alanine, arginine, and tyrosine.

Effects of Substitution of Glutamate 40 and Glutamine 43

The effect of substitution of glutamate 40 on the ability of hEGF to displace wild-type ^{125}I-hEGF is shown in Figure 1A. Substitution of glutamate 40 with aspartate, which moves the acidic functional group closer by one methylene unit, resulted in a decrease in receptor binding affinity to 30 percent relative to wild-type hEGF. The amino acid substitutions in both the Glu-40→Gln and Glu-40→Ala mutations completely eliminate the negative charge of the residue at position 40; however, replacement of the electrostatic side-chain did not decrease the affinity significantly further than was observed in the charge-conservative mutation to aspartate. The neutral polar side-chain of glutamine 43 was replaced with the positively charged amine side-chain of lysine with no effect on the relative affinity of the mutant hEGF analog (Fig. 1B).

Mutagenesis and Chemical Modification Studies at Position 45

The charge-conservative mutation, Arg-45→Lys, replaces the guanidinium group of the arginine 45 side-chain with a lysine amine side-chain which, along with the lysine 28 and lysine 48 ε-amino groups and the amino-terminal α-amine, is reactive with the amine-specific protein modification reagent potassium cyanate (KCNO). The positively charged amines of wild-type and Arg-45→Lys hEGF were carbamylated by reaction with an excess of KCNO and the modified proteins purified by reverse-phase HPLC (Fig. 2A). The substitution of arginine 45

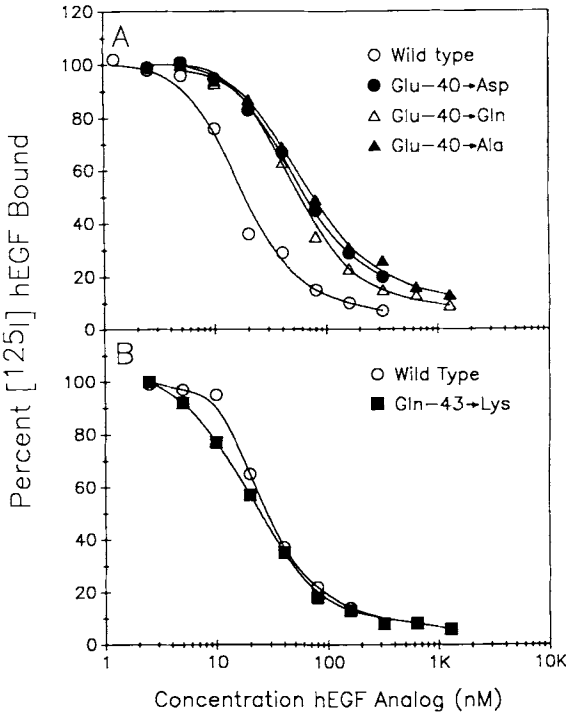

Fig. 1. Displacement of receptor-bound wild-type EGF by competing EGF species. The binding of radioiodinated wild-type hEGF (10 nM) was measured in the presence of an increasing concentration of wild-type or mutant hEGF analog.

with lysine did not affect the chromatographic properties of the growth factor peptide, as untreated wild-type and Arg-45→Lys hEGF proteins had nearly indistinguishable elution profiles. However, the presence of the additional reactive ε-amine of lysine 45 in the Arg-45→Lys mutant was distinguishable from the unreactive arginine 45 of wild-type hEGF by a 1.5 min difference in retention time between the wild-type and Arg-45→Lys hEGF proteins following treatment with potassium cyanate. The effect of Arg-45→Lys mutation and/or neutralization of EGF amine groups on the ability of the growth factor to displace wild-type ^{125}I-hEGF is shown in the displacement curves for the cyanate-treated and untreated wild-type and mutant hEGF analogues (Fig. 2B). Radioiodinated wild-type hEGF was displaced by comparable concentrations of wild-type hEGF and the altered hEGF proteins, indicating similar receptor affinities.

Effects of Substitutions of Aspartate 46

The aspartate 46 residue, although conserved to a lesser degree than arginine 41 or leucine 47, is conserved to a greater extent than other elec-

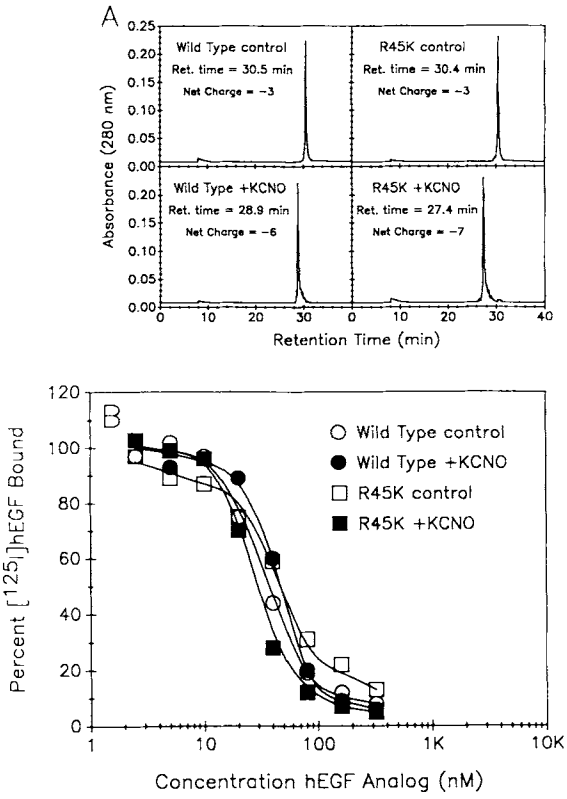

Fig. 2. **A:** Purification of untreated or potassium-cyanate-treated wild-type and mutant (Arg-41→Lys) hEGF by reverse phase HPLC. The retention time is given for each protein sample. **B:** Competition binding curves of ^{125}I-hEGF (10 nM) vs. the untreated or the potassium-cyanate-treated wild-type or mutant Arg-45→Lys hEGF species.

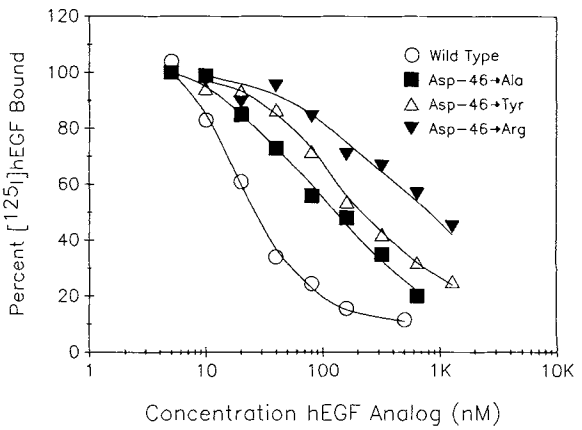

Fig. 3. Radioreceptor competition binding curves of wild-type and position 46 mutant hEGF species. Displacement of receptor-bound wild-type ^{125}I-hEGF by competing hEGF species was measured by the addition of increasing concentrations of wild-type, Asp-46→Ala, Asp-46→Arg, and Asp-46→Tyr hEGF analogs. Radioiodinated wild-type EGF (10 nM) was mixed with the indicated concentration of the various EGF species and incubated with membrane-bound EGF receptor as described in Materials and Methods.

trostatic residues of the carboxy-terminal domain. Elimination of the electrostatic charge of aspartate 46 by replacement with alanine and tyrosine resulted in a decrease in affinity to 23 and 14 percent, respectively (Fig. 3). Placing an arginine side-chain at position 46 introduces an electrostatic charge opposite in polarity to the native acidic aspartate 46. This substitution introduces a residue of similar charge to the neighboring arginine 45, and decreases the relative affinity to 4 percent.

DISCUSSION

The locations of electrostatic residues particularly within the carboxy-terminal domain of the EGF molecule [17–19] suggest that these amino acid side-chains are exposed and accessible to the solvent environment (see Fig. 4). Therefore, the electrostatic and polar side-chains are expected to be substantially hydrated in solution and would be unlikely to participate in attractive electrostatic interactions with surface charges on the receptor. However, the electrostatic and polar amino acids examined here may be important for the stability of protein-protein association involving close range electrostatic interactions and hydrogen bonding within a solvent-excluding receptor binding "pocket." In addition, electrostatic, polar, and non-polar residues might all have a role in the immobilization of solvent water molecules which if liberated upon receptor-ligand association would contribute to an entropy-dependent formation of a thermodynamically stable receptor-ligand complex. The juxtaposition of amino acids having complementary electrostatic charge is found twice in the carboxy-terminal domain of hEGF and the potential intramolecular ion pairing of the acidic Glu-40 with the basic Arg-41 and similarly the pairing of Arg-45 with Asp-46, upon association with the receptor, could facilitate the mobilization of bound water molecules promoting entropy-dependent complex formation. Four residues in the carboxy-terminal domain of hEGF, bearing electrostatic or polar side-chains and having the potential for ionic or hydrogen bond interactions with either neighboring residues on the ligand, with complementary groups on the receptor, or with the aqueous solvent, were altered by site-directed mutagenesis and/or chemical modification to examine their ability to par-

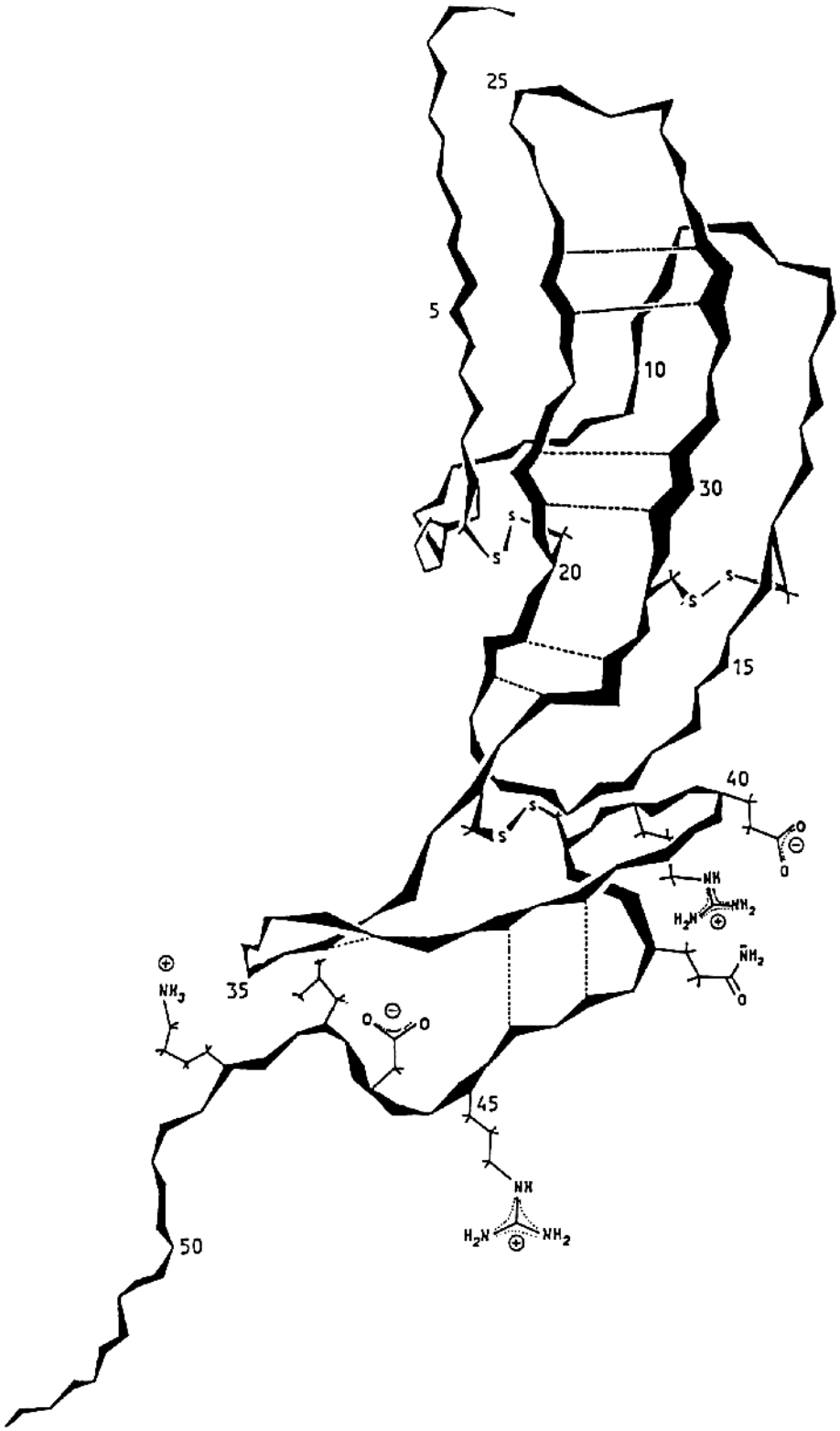

Fig. 4. Location of amino acid residues in the carboxy-terminal domain of hEGF. This model is shown to illustrate the location of amino acid residues glutamate 40, arginine 41, cysteine 42, glutamine 43, arginine 45, aspartate 46, leucine 47, and lysine 48. The model is based on the reported NMR solution structure, and does not represent molecular dimensions.

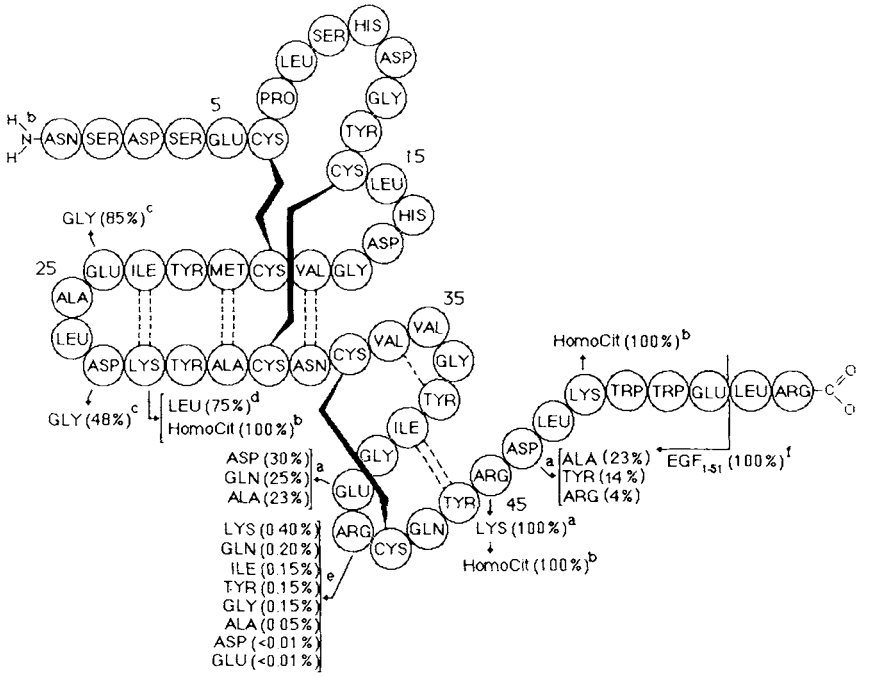

Fig. 5. Summary of the effects of mutations involving electrostatic residues of hEGF. The amino acid substitutions generated at each of the targeted sites are indicated as well as the relative receptor binding affinity for each mutant protein. [a]Mutants studied in this report (see Results). [b]All primary amines in the EGF molecule including the amino-terminal α-amine, lysine 28, lysine 48, and lysine 45 are modified simultaneously in the reaction with potassium cyanate. [c]See [2]. [d]See [1]. [e]See [9]. [f]EGF_{1-51} is a naturally occurring truncated form of recombinant hEGF lacking the last two carboxy-terminal residues (including arginine 53) and appearing as a separate peak during HPLC purification of hEGF. Its receptor binding affinity is identical to that of intact hEGF (data not shown).

ticipate in interactions contributing to EGF receptor-ligand association.

The results presented in this report demonstrate that the electrostatic properties of Glu-40 and Arg-45 are not required for receptor-ligand binding. The results also demonstrate that entropy-enhancing intramolecular ionic interactions of Glu-40 and Arg-45 with neighboring residues Arg-41 and Asp-46, respectively, or with complementary sites on the receptor are not required for EGF-receptor association. The effect of chemical modification of native EGF amino groups, namely, the amino-terminal α-amine, lysine 28, and lysine 48, observed following reaction of wild-type hEGF with potassium cyanate, demonstrates that the electrostatic charge of these residues also is not required for high affinity receptor-ligand binding. Gln-43, located in close proximity to the critical residue Arg-41, was targeted for mutagenesis to investigate the potential participation of the polar glutamine side-chain in either intramolecular or intermolecular hydrogen bonding. A positively charged lysine amine was introduced in place of the neutral Gln-43. Although the substituted lysine bears an electrostatic charge repelled by its positively charged Arg-41 neighbor and has hydrogen bonding character markedly different from the glutamine side-chain, no effect on receptor affinity was observed.

The residue aspartate 46 is conserved almost completely throughout the EGF-like family of growth factors and its role in the binding of the related transforming growth factor–alpha to the EGF receptor has been previously examined using site-directed mutagenesis [20,21]. These studies have reported conflicting results on the effect of the same mutation at this site (Asp-47→Ala) with relative affinity values varying from less than 5 percent [20] to greater than 100 percent [21]. The mutation of Asp-46→Ala in hEGF resulted in a measurable decrease in receptor binding affinity to 23 percent, relative to wild type.

Our laboratory has now examined the role of a majority of the electrostatic residues in hEGF and the results are summarized in Figure 5. The results indicate that, other than Arg-41 and Asp-46, there appears to be no requirement for either specific electrostatic residues or for the

overall electrostatic character of the EGF molecule for its association with the EGF receptor. It is suggested that the electrostatic residues Arg-41 and Asp-46 participate in short-range electrostatic interactions or hydrogen bonding to residues within a sequestered ligand binding site on the receptor's extracellular domain. The function of electrostatic residues, other than Arg-41 and Asp-46, appears to be in either establishing or reinforcing an EGF conformation in which critical non-polar side-chains on the ligand are exposed to the aqueous solvent environment allowing those non-polar residues to contribute to "hydrophobic" bonding with the receptor.

The ability to recover EGF protein from *E. coli* requires that the molecule be processed and folded into the native EGF structure during expression of the recombinant EGF gene product [2]. Deviation from the normal EGF folding motif results in protein molecules which are either significantly altered in their chromatographic behavior or are trapped and/or degraded within the cell. In practice, the elution profile obtained during purification of EGF proteins using reverse-phase HPLC usually permits identification of non-native EGF proteins. The EGF analogues generated in this study exhibited chromatographic elution patterns consistent with the "normal" elution profile of native EGF and were purified as a single homogeneous protein peak. Minor differences in wild-type EGF conformation and dynamics have been observed following single-site mutation, as detected by NMR [1,4,6,8]. However, any structural differences are considered to be generally localized and affect only the site of mutation [unpublished observations, 22,23].

We have observed that severe decreases can occur in the production of some mutant hEGF proteins containing certain amino acid substitutions. In this study, for example, expression of altered recombinant *hEGF* genes in *E. coli* generated mutant hEGF proteins Glu-40→Asp, Glu-40→Gln, and Glu-40→Ala. However, in the expression of the mutant Glu-40→Arg, which has two positively charged arginine residues adjacent to each other, no protein exhibiting native hEGF could be detected during purification. Similarly, while the Gln-43→Lys mutant protein was isolated, Gln-43→Glu and Gln-43→Leu proteins were not detected. Furthermore, although mutant Asp-46→Ala, Asp-46→Tyr, and Asp-46→Arg proteins were isolated in modest amounts, relative to wild-type protein, no Asp-46→Val protein was recovered. We have observed similar decreases in protein production in mutations at other sites in the carboxy-terminal domain, for example, Tyr-37→Gly and Arg-41→Gly, as well as in mutations outside the carboxy-terminal domain, notably the production of mutant proteins Asp-27→Gly and Tyr-29→Gly. The observed decreases in production of certain mutant hEGF proteins is likely due to changes in the stability of either the native hEGF structure or of one or more of the essential folding intermediates, thereby leading to degradation of the misfolded or unfolded protein. The sensitivity of certain sites to mutations affecting protein production does not appear to correlate directly with effects on receptor affinity; some side-chain substitutions generated at sensitive sites can result in extremely low protein yields, although the recovered proteins retain substantial receptor affinity. Certain side-chain substitutions at sites targeted in this report, for example, Glu-40→Ala and Gln-43→Lys, can produce mutant hEGF analogues with relatively high receptor affinity despite significantly altering the chemical character at these sites, suggesting that Glu-40 and Gln-43 may not be required to participate in direct receptor-ligand interactions. On the other hand, other equally severe side-chain altering substitutions at these sites, for example, Glu-40→Arg and Gln-43→Glu, drastically reduce the yield of the mutant proteins, indicating that Glu-40 and Gln-43 possibly contribute to the establishment and/or stability of the functional native hEGF structure.

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REFERENCES

1. Campion SR, Matsunami RK, Engler DA, Niyogi SK: Biochemistry 29:9988–9993, 1990.
2. Engler DA, Matsunami RK, Campion SR, Stringer CD, Stevens A, Niyogi SK: J Biol Chem 263:12384–12390, 1988.
3. Ray P, Moy FJ, Montelione GT, Liu J-F, Narang SA, Scheraga HA, Wu R: Biochemistry 27:7289–7295, 1988.

4. Moy FJ, Sheraga HA, Liu J, Wu R, Montelione GT: Proc Natl Acad Sci USA 86:9836–9840, 1989.
5. Dudgeon TJ, Cooke RM, Baron M, Campbell ID, Edwards RM, Fallon, A: FEBS Lett 261:392–396, 1990.
6. Matsunami RK, Yette ML, Stevens A, Niyogi SK: J Cell Biochem 46:242–249, 1991.
7. Matsunami RK, Campion SR, Niyogi SK, Stevens A: FEBS Lett 264:105–108, 1990.
8. Engler DA, Montelione GT, Niyogi SK: FEBS Lett 271: 47–50, 1990.
9. Engler DA, Campion SR, Hauser MR, Cook JS, Niyogi SK: J Biol Chem 267:2274–2281, 1992.
10. Simpson RJ, Smith JA, Moritz RL, O'Hare MJ, Rudland PS, Morrison JR, Lloyd CJ, Grego B, Burgess AW, Nice EC: Eur J Biochem 153:629–637, 1985.
11. Shoyab M, Plowman GD, McDonald VL, Bradley JG, Todaro GJ: Science 242:1074–1076, 1989.
12. Campbell ID, Cooke RM, Baron M, Harvey TS, Tappin MJ: "Progress in Growth Factor Research," Oxford, UK:Pergamon Press, pp 13–22, Vol I, 1989.
13. Sanger F, Nicklen S, Coulson AR: Proc Natl Acad Sci USA 74:5463–5467, 1977.
14. Akiyama T, Kadooka T, Ogawara H: Biochem Biophys Res Commun 131:422–448, 1985.
15. Carpenter G: Methods Enzymol 109:107–108, 1985.
16. Hunter WM, Greenwood FC: Nature 194:495–496, 1962.
17. Cooke RM, Wilkinson AJ, Baron M, Pastore A, Tappin MJ, Campbell ID, Gregory H, Sheard B: Nature 327:339–341, 1987.
18. Montelione GT, Wuthrich K, Nice EC, Burgess AW, Scheraga HA: Proc Natl Acad Sci USA 84:5226–5230, 1987.
19. Kohda D, Go N, Hayashi K, Inagaki F: J Biochem 103:741–743, 1988.
20. Defeo-Jones D, Tai JY, Wegrzyn RJ, Vuocolo GA, Baker AE, Payne LS, Garsky VM, Oliff A, Rieman MW: Mol Cell Biol 8:2999–3007, 1988.
21. Lazar E, Watanabe S, Dalton S, Sporn MB: Mol Cell Biol 8:1247–1252, 1988.
22. Campion SR, Niyogi SK: Cumulative effects of double-site mutations of human epidermal growth factor on receptor binding. Submitted for publication.
23. Campion SR, Biamonti C, Montelione GT, Niyogi SK: Site-directed mutagenesis of asparagine 32 in the epidermal growth factor hinge region: Evaluation by receptor binding, 1D NMR, and second-site mutation. Manuscript in preparation.