Human epidermal growth factor

Distinct roles of tyrosine 37 and arginine 41 in receptor binding as determined by site-directed mutagenesis and nuclear magnetic resonance spectroscopy

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Site-directed mutagenesis was employed to examine the function of two highly conserved residues, Tyr-37 and Arg-41, of human EGF (hEGF) in receptor binding. Both a conservative change to phenylalanine and a semi-conservative change to histidine at position 37 yield proteins with receptor affinity similar to wild-type hEGF. A non-conservative change to alanine results in a molecule with about 40% of the receptor affinity, indicating that an aromatic residue is not essential at this position. Both conservative (to lysine) and non-conservative (to alanine) substitutions at position 41 drastically reduced receptor binding to $<0.5\%$ of the wild-type activity. 1D-NMR data indicate that the replacement of Arg-41 by lysine does not significantly alter the native protein conformation. Thus, Arg-41 may be directly involved in ligand–receptor interaction, whereas the side chain of Tyr-37, although possibly important structurally, is not essential for receptor binding.

Epidermal growth factor; Protein engineering; Receptor affinity; Structure by NMR; Rational drug design

1. INTRODUCTION

EGF is a 6-kDa (53 residues) polypeptide with three internal disulfide bonds. Upon binding to its specific cell surface receptor, EGF elicits multiple molecular and cellular responses which ultimately lead to increased DNA synthesis and cell proliferation (see [1–3] for recent reviews).

Recently, there has been increased interest in identifying residues in EGF that function in receptor recognition and binding (see [3] for a review). In previous reports from our laboratory ([4,5]; Campion, S.R., Matsunami, R.K., Engler, D.A. and Niyogi, S.K., manuscript submitted for publication) we described site-directed mutagenesis studies of hEGF aimed at identifying the residues involved in receptor recognition, based on functional assays of ligand binding and activation of the protein-tyrosine kinase activity of the EGF receptor. The residues chosen for mutagenesis were based largely on considerations of their species invariance and recently described solution structures of hEGF [6] and mEGF [7–9]. Our studies involved residues in different regions of the EGF molecule and showed the importance of Tyr-22, Ile-23, Leu-26, Tyr-29 and Leu-47 in receptor binding. Residues in the third disulfide loop region distal to the amino terminal end were not included in those studies.

The third loop of EGF is formed as a consequence of the disulfide bridge between Cys-33 and Cys-42 (see Fig. 1) and comprises the most highly conserved region in the EGF-like family of molecules [10]. Of the five residues that are consistently conserved in this loop of those EGF-like species that have actually been shown to bind to the EGF receptor, two are the cysteines that define the loop and a third is the glycine at position 39 that is presumed to be conserved for energetic reasons due to its location in a short left-handed helical conformation [8]. The remaining two residues that are absolutely conserved are Tyr-37 and Arg-41. We have combined structural information, based on NMR measurements, with functional activity of several site-directed mutants at positions 37 and 41 of hEGF to clarify the roles of these residues in receptor binding.

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Abbreviations: EGF, epidermal growth factor; NMR, nuclear magnetic resonance; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; ppm, parts per 10^6; ppb, parts per 10^9; TGF-α, transforming growth factor alpha; hTGF-α, human transforming growth factor alpha

2. MATERIALS AND METHODS

2.1. *Materials*

Materials have already been described [4].

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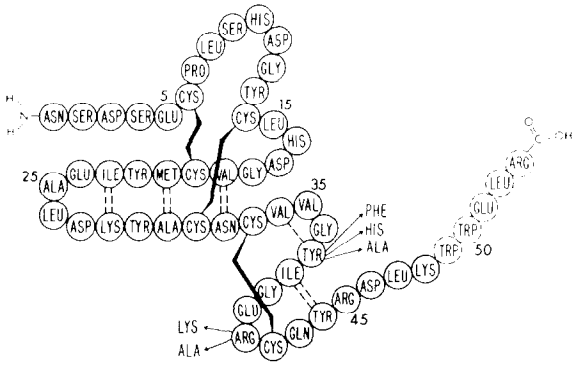

Fig. 1. Schematic representation of secondary structural features of hEGF. *Solid lines* indicate disulfide bridges, and hydrogen bonds are indicated by *dashed lines*. *Dashed circles* indicate residues with no rigid structural characteristics (as determined by NMR). The various single amino acid substitutions performed at each site are also indicated.

2.2. *Oligonucleotide-directed mutagenesis*

EGF mutants were generated by oligonucleotide-directed mutagenesis using protocols described earlier [4,11]. The *hEGF* gene used as a template was cloned into M13mp19 as described earlier [4]. All engineered mutations as well as the absence of any undesired genetic alterations were confirmed by sequencing [12] the entire *hEGF* gene.

2.3. *Expression and purification of wild type and mutant hEGF proteins*

Expression of wild type and mutant proteins was achieved with modifications to the protocol already described [4]. It was found that the addition of 1–5 μg/ml of chloramphenicol to the growth medium during the induction phase greatly increased EGF yields (Engler, D.A., PhD thesis; Engler and Niyogi, manuscript in preparation); therefore this was used routinely. Purification was carried out as previously described [4].

2.4. *EGF receptor binding assay*

Specific binding of EGF proteins to the EGF receptor was measured by radioreceptor competition [13] using purified membrane fractions from the human epidermoid carcinoma cell line, A431. A431 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A431 membranes were isolated as described [14]. [^{125}I]hEGF was prepared by the chloramine-T method [15] to an average specific activity of approx. 150000 cpm/pmol.

2.5. *NMR spectroscopy of proteins*

Purified protein samples were dialyzed against distilled deionized water at pH 6.5 and lyophilized to dryness. Samples were resuspended in D_2O to a concentration of 1 mM and the reading of the pH meter (without correction for isotope effects) adjusted to 7.2 ± 0.1 with 0.1 M NaOH in D_2O. Samples were allowed to exchange all labile protons for deuterons for a minimum of 3 h before measurements were recorded. ^{1}H-NMR spectra were recorded at 30°C on a Varian VXR 500 spectrometer.

3. RESULTS

3.1. *Mutant verification*

Homogeneity of each EGF protein was confirmed by analytical reverse-phase chromatography and amino acid composition analysis. The latter measurements verified the expected amino acid change for each mutant protein.

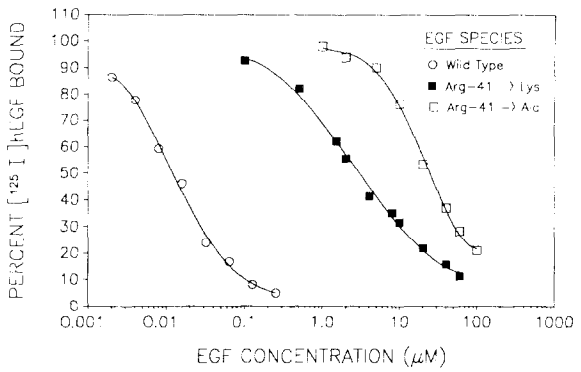

Fig. 2. Competition binding curves of 13 nM [^{125}I]hEGF vs wild-type and arginine-41 analogs.

3.2. *Radioreceptor competition assays*

Fig. 2 shows the competition binding curves for wild-type hEGF in relation to the singly substituted mutants of arginine 41; namely, $Arg^{41} \longrightarrow Lys$ and $Arg^{41} \longrightarrow Ala$. Similarly, Fig. 3 shows the relationship of competition binding curves for wild-type hEGF and the singly substituted mutants of tyrosine 37; namely, $Tyr^{37} \longrightarrow Phe$, $Tyr^{37} \longrightarrow His$ and $Tyr^{37} \longrightarrow Ala$. The concentration of competing species that gave a 50% inhibition of binding of [^{125}I]hEGF to the receptor (IC_{50}) was estimated graphically from the curve for each mutant. This concentration is listed in Table I for each mutant along with its relative receptor affinity as compared to that of wild-type which was set at 100%. The results show that substitutions, even a non-conservative one, at position 37 are tolerated quite well, while both substitutions at position 41 drastically reduce the receptor affinity. However, even the poorest binders could displace [^{125}I]hEGF at sufficiently high concentrations, indicating their specific interactions with the EGF receptor.

3.3. *NMR spectra of proteins*

Aromatic residues are spread throughout the hEGF molecule in its 3-dimensional structure [6] and the

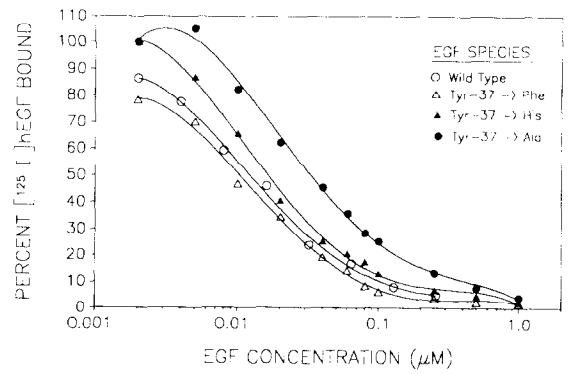

Fig. 3. Competition binding curves of 13 nM [^{125}I]hEGF vs wild-type and tyrosine-37 analogs.

Table I

Comparison of IC_{50} values and relative receptor binding affinities

EGF species	IC_{50} (μM)	Relative binding affinity (% of wild type)
Wild type	13.0×10^{-3}	100
$Tyr^{37} \longrightarrow Phe$	10.3×10^{-3}	126
$Tyr^{37} \longrightarrow His$	17.5×10^{-3}	74
$Tyr^{37} \longrightarrow Ala$	33.0×10^{-3}	39
$Arg^{41} \longrightarrow Lys$	3.0	0.43
$Arg^{41} \longrightarrow Ala$	26.2	0.05

IC_{50} is the concentration of the competitor giving 50% inhibition of binding of ^{125}I-labeled wild-type EGF to the receptor (see Figs 2 and 3). Relative binding affinity = IC_{50}(wild type)/IC_{50}(mutant) × 100%.

resonance frequencies of these aromatic ring protons are sensitive to molecular conformation and solvent accessibility. Accordingly, comparisons of the aromatic resonance frequencies of wild-type and mutant proteins provide a convenient approach for distinguishing correctly from incorrectly folded mutants [16]. The NMR data were obtained for protein solutions at neutral pH. Proteins which exhibit native-like 1D-NMR spectra may still have some localized minor structural perturbations which will be characterized only by more detailed 2D-NMR studies. We have been very careful to avoid interpreting chemical shift changes accompanying mutations involving aromatic ring substitutions, since alterations in aromatic 'ring-current' effects can result in conformation-independent changes in chemical shifts. Thus, no serious attempt was made to interpret changes observed in the NMR spectra of the Tyr-37 mutants (data not shown).

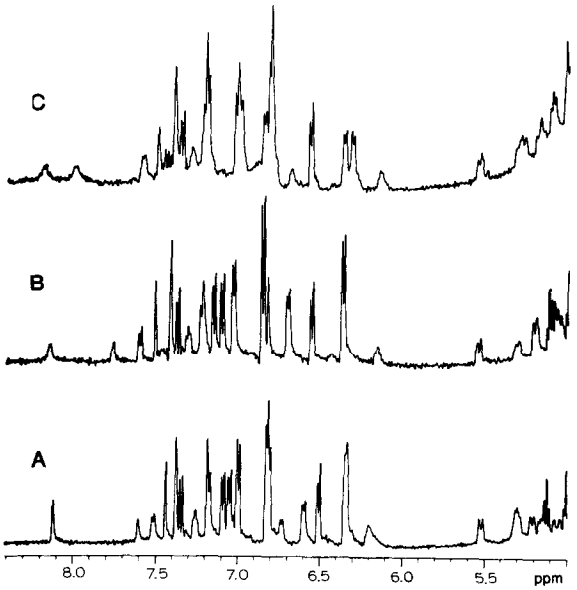

Fig. 4. Comparison of aromatic and downfield $C^{\alpha}H$ spectral regions from 500 MHz 1D ^1H-NMR spectra of (A) wild-type EGF, (B) $Arg^{41} \longrightarrow Lys$ EGF, (C) $Arg^{41} \longrightarrow Ala$ EGF at neutral pH. Conditions were as described in section 2.

For mutants involving Arg-41, the aromatic and downfield $C^{\alpha}H$ regions of the 1D ^1H-NMR spectra, from 5.0 to 8.5 ppm, are used for comparison of structural integrity. These spectra are shown in Fig. 4. The 1D-NMR spectra of the wild-type (Fig. 4A) and the Lys-41 mutant (Fig. 4B) display very similar profiles with very few peaks exhibiting significant chemical shifts (≥50 ppb) indicating no major structural changes in the global chain fold of this particular mutant with respect to the wild-type native conformation. The Ala-41 mutant (Fig. 4C) displays a somewhat altered NMR profile with respect to wild-type in that there are several peaks with small chemical shift differences. Some peak broadening can also be observed in the spectrum of this particular mutant.

4. DISCUSSION

Either a conservative or non-conservative substitution at the arginine 41 site results in an EGF molecule with very poor receptor affinity. 1D ^1H-NMR measurements indicate no major structural alterations in either of the mutants. The qualitative difference in the spectra for the Lys-41 and Ala-41 mutants is consistent with our finding that notwithstanding their extremely poor affinities for the EGF receptor, the Ala-41 mutant has an affinity approximately 8-fold lower than that of the Lys-41 mutant. Our results indicate that the guanidino group of arginine 41 may be involved in receptor interaction.

The tyrosine residue at position 37 seems to play a different role in the EGF molecule. A very conservative change at this site, namely to phenylalanine, produced a mutant that retained the aromatic character of the original phenol ring but lost the polar −OH group. This mutant has a somewhat higher affinity for the EGF receptor than wild-type. When position 37 was mutated to histidine, in which the imidazole ring retains some of the aromatic character and the approximate size of the tyrosine but again lacks the polar −OH group, the binding affinity dropped to 74% of that of wild type. Replacement of the tyrosine side chain with the small hydrophobic alanine side chain only moderately decreased the receptor binding affinity, to about 40% of wild-type. These results indicate that a tyrosine at position 37 is not essential for interaction with the receptor. However, the nature of the aromatic residue at position 37 does influence, to some small degree, the binding affinity for the receptor. Precise characterization of a possible structural role of tyrosine 37 in EGF must await detailed 2D-NMR studies of these mutants.

That tyrosine 37 may play a role in maintaining some structural feature(s) common to all EGF-like species is indicated by the fact that chemical functionality of an aromatic residue at position 37 remains uncompromised even in that group of EGF-like species that contain

EGF-like domains and are thought to adopt similar conformations to EGF, but do not bind to the EGF receptor [3,10]. Chemical functionality of the arginine at position 41, however, is not maintained in all EGF-like species, rather only in those proteins, including TGF-α, that bind to the EGF receptor [3,10]. This supports our observation of the essentiality of Arg-41 of hEGF in receptor binding. It should be noted also that the replacement of the analogous residue (Arg-42) of hTGF-α with lysine reduced receptor binding to $< 1.0\%$ of wild-type [17]. Although NMR data were lacking for the Lys-42 hTGF-α mutant, the disulfide arrangement was shown to be identical to that of wild-type [17].

This similarity between EGF and TGF-α in receptor binding does not completely extend to Tyr-37 (Tyr-38 in hTGF-α). Although a conservative change to phenylalanine at position 38 also produced a mutant hTGF-α with a somewhat higher receptor binding affinity, the Ala-38 hTGF-α mutant (unlike our Ala-37 hEGF mutant) was only approx. 3% as active as wild-type [18]. Lazar et al. [19] also postulated the essentiality of an aromatic residue at position 38 of TGF-α, using partially purified mutant analogs, but reported a His-38 mutant with negligible activity. Thus, although an aromatic residue at position 38 *may* be essential for the biological activity of TGF-α, tyrosine 37 in hEGF is not essential for receptor binding.

EGF and TGF-α may recognize and/or bind to the EGF receptor in a dissimilar manner as indicated by recent studies [20] showing that a monoclonal antibody to the EGF receptor had only small effects on the binding of EGF to the EGF receptor but had large effects on the binding of TGF-α to the EGF receptor. Differences between these two *different* ligands in their mode of binding to their common receptor, as exemplified by the different roles of Tyr-37 (in EGF) and Tyr-38 (in TGF-α), as discussed above, may account for the differences in the ultimate biological responses elicited by these distinct growth factors [21].

The present study suggests an essential role of Arg-41, and/or atoms nearby in the 3-dimensional structure, in interactions with the EGF receptor. Similar studies suggest that Leu-47, and/or residues nearby in the 3-dimensional structure, also play some role in interactions of mEGF [16] and hEGF ([22]; Matsunami, R.K., Stevens, A., Montelione, G.T. and Niyogi, S.K., manuscript in preparation) with the receptor. Examinations of the solution structures of both hEGF and mEGF reveal that these two side chains (Arg-41 and Leu-47) are located on one face of the protein molecule. Other side chains contributing to this face include Tyr-13, Leu-15, His-16, Gln-43, Tyr-44, Arg-45 and Asp-46 (Montelione, G.T., unpublished results). Structure-function studies, in progress, are aimed at characterizing the roles these spatially adjacent residues play in forming the receptor recognition site of EGF.

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