The functional importance of Leu15 of human epidermal growth factor in receptor binding and activation

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The biological importance of Leu15 of epidermal growth factor (EGF) is suggested by its conservation through evolution, its critical location in the domain-domain interface of EGF and its close proximity to Arg41, a residue that is crucial for receptor binding and activation. Mutagenesis of Leu15 of human EGF (hEGF) was employed to examine the role of this residue in the ligand–receptor interaction. The relative receptor affinities of the hEGF variants, as determined by radioreceptor competition assays, varied depending on the amino acid substitution. The L15F, L15W and L15V hEGF analogues had receptor affinities 45, 26 and 18% respectively of wild type hEGF. The L15A and L15R analogues displayed receptor affinities of only 2.4 and 1.6% relative to wild type hEGF. No binding of the L15E analogue was detected. The relative agonist activities, as measured by receptor tyrosine kinase stimulation assays, generally followed a similar trend. The L15F, L15W and L15V analogues stimulated the receptor kinase to a level (Vmax) similar to that for wild type hEGF. A striking difference was observed between the L15A and L15R variants; although having similar binding affinities, the L15A mutant activated the receptor to only ~5% of the wild type Vmax in contrast to 53% for the L15R mutant. 1H-NMR analysis of the L15R and L15A mutants showed only minor structural alterations that were not sufficient to account for the dramatic losses in binding and agonist activities. The results indicate that both the size and hydrophobicity of the γ-branched aliphatic side chain of Leu15 of hEGF are important in the formation of a catalytically active ligand–receptor complex.

Keywords: Leu15 of human EGF/mutagenesis/NMR analysis/partial agonist/receptor binding and activation

Introduction

Epidermal growth factor (EGF) is a 6 kDa polypeptide mitogen composed of 53 amino acid residues and three intramolecular disulfide bonds. High-affinity binding of EGF to the EGF receptor, a 170 kDa transmembrane glycoprotein, stimulates the latter's intrinsic protein tyrosine kinase activity (Ushiro and Cohen, 1980). This in turn leads to receptor autophosphorylation, as well as phosphorylation of endogenous cytoplasmic substrates, via phosphotyrosine-binding SH2 domains. Adaptors couple the signal generated at the membrane to effector proteins downstream and thereby trigger a cascade of biochemical reactions leading ultimately to cell growth, proliferation and differentiation (for reviews, see Carpenter and Cohen, 1990; Ulrich and Schlessinger, 1990; Cadena and Gill, 1992; Fantl et al., 1993; van der Geer et al., 1994).

The solution structure of EGF has been determined by 2-D NMR (Cooke et al., 1987, 1990; Montelione et al., 1987, 1992; Kohda et al., 1988; Hommel et al., 1992; Kohda and Inagaki, 1992). The EGF protein folds into a conformation with two slightly overlapping structural motifs, the N-terminal segment comprising residues 1–35 and the C-terminal segment corresponding to residues 30–53 (see Figure 1). Structure–function studies indicate that the amino acid residues important for the ligand–receptor interaction (Campion et al., 1993a; reviews by Campion and Niyogi, 1994) and Groenen et al. (1994) are located in both the N- and C-terminal segments of EGF. Molecular models based on NMR data indicate that the key receptor-binding residues of human EGF (hEGF) are positioned to form a solvent-exposed triad—(i) Tyr13 and Arg41, (ii) Ile23 and Leu26 and (iii) Leu47—on one face of

Fig. 1. Representation of the hEGF solution structure showing the relative position of Leu15 as well as the positions of other residues found to be important in the EGF–receptor interaction. The computer model of EGF was generated from the NMR coordinates provided by Dr. G.T. Montelione (Rutgers University) using MOLSCRIPT (Kraulis, 1991).

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the EGF molecule (see Figure 1). The solvent-exposed Leu15 residue, also on the same face of hEGF, is closely associated with Arg41 which is a crucial component (Engler et al., 1992) of the first member of the triad (Figure 1).

The hydrophobicity at position 15 in hEGF is phylogenetically conserved among the ligands of the EGF family that bind to the EGF receptor. The residue at this position is leucine for EGF and phenylalanine (Phe17) in the case of transforming growth factor alpha (TGFα). High-resolution analyses of the 3-D structures, derived from 2-D NMR data, of hEGF (Hommel et al., 1992) and mouse EGF (Kohda and Inagaki, 1992; Montelione et al., 1992) [and also human TGFα (Miy et al., 1993)] predict that the critical Arg41 lies in a hydrophobic pocket, which includes Leu15 and the important Tyr13, near the domain–domain interface of hEGF (Figure 1).

To evaluate critically the possible role of Leu15 in hEGF structure–function, site-directed mutagenesis was employed [preliminary results of these studies were reported earlier (Nandagopal et al., 1994)] to replace this residue with amino acids having a variety of side chain functional groups. The receptor-binding affinity and agonist activity of mutant hEGF analogues were determined by radioreceptor competition and receptor tyrosine kinase stimulation assays respectively. Selected mutants were evaluated by NMR spectroscopy for possible structural perturbations introduced by specific amino acid substitution. These comprehensive studies help define the molecular architecture of the putative receptor-binding epitope in the domain–domain interface of hEGF.

Materials and methods

Oligonucleotide-directed site-specific mutagenesis

Synthetic deoxyoligonucleotides containing the desired mutations were synthesized in a Milligen/Biosearch Cyclone Plus DNA synthesizer utilizing phosphoramidite chemistry (Sinha et al., 1984). The sequences of the oligonucleotides with the mutated codons underlined and the mismatches in bold face are as follows: L15A, 5′-CTCGCGCCACCG-AAGGGGTTTGTG-3′; L15E, 5′-CTCGCGAGCAGCGACGGGTTTGTG-3′; L15F, 5′-CTCGCTTCCACGAGGTTTGTG-3′; L15R, 5′-CTCGCGACCGAGGTTTGTG-3′; L15V, 5′-CTCGCTTGACGCAAGGTGTTTGTG-3′; and L15W, 5′-CTGCACGCGACGGGTTTGTG-3′.

Site-directed mutagenesis by a PCR strategy (Helmsley et al., 1989) was utilized to generate hEGF analogues with high efficiency (Tadaki and Niyogi, 1993). Double-stranded DNA of the expression vector, pEGFl, containing the hEGF gene. Overnight cultures of E.coli JM107 cells harbouring the pEGFl plasmids were grown at 37°C to mid-log phase in LB medium containing 25 μg/ml ampicillin and then diluted 100-fold into the same pre-warmed medium. After 3 h (OD600 = 0.5–0.8) chloramphenicol was added to a final concentration of 5 μg/ml. When the OD600 of the cultures reached 1.2–1.4, the expression of protein was induced by the addition of isopropylthiogalactoside to a final concentration of 1 mM. The cultures were allowed to grow until EGF production was maximal, typically 12 h later. The hEGF sequestered in the periplasm was isolated from the cell pellet by resuspension in ice-cold buffer (1 M Tris–HCl, pH 9.0, 2 mM EDTA) and incubation on ice for 20 min. Proteins in the Tris–EDTA fractions were precipitated by the gradual addition of (NH₄)₂SO₄ to 80% saturation with stirring at 4°C for 1 h. After centrifugation at 39 000 g for 30 min, the pellets were resuspended in and dialysed against 25 mM sodium phosphate, pH 7.2. The wild type or mutant hEGF protein was first separated by gel filtration chromatography on a Sephadex G-75 column (1×90 cm) using 25 mM sodium phosphate, pH 7.2. The fractions containing the hEGF protein were pooled and loaded onto a reversed-phase HPLC column (Vydac 218TPS, 4.6×250 mm). Elution conditions, using a Waters Model 840 HPLC system, consisted of an isocratic wash of 15% CH₃CN for 15 min followed by a 15–34% CH₃CN gradient in 10 mM sodium phosphate, pH 7.2, increasing linearly at a rate of 1% per min. The fractions containing each hEGF protein were pooled, then lyophilized and stored at −80°C.

Radioreceptor competition assay

Membrane-bound EGF receptors were isolated from A431 (human epidermoid carcinoma) cells according to Akiyama et al. (1985) with modifications described by Campion et al. (1990). The specific binding of hEGF proteins to the EGF receptor was determined by a radioreceptor competition assay (Carpenter, 1985). The membrane fractions were resuspended in buffer (20 mM HEPES, pH 7.4 and 10 mM PMSF) and the total membrane protein content was determined by the BioRad Protein Assay. [125]IhEGF was prepared by the chloramine-T method (Hunter and Greenwood, 1962) to an average specific activity of ~150 000 c.p.m./pmol hEGF. Two micrograms of the receptor preparation were incubated with 30 nM wild type [125]IhEGF in a mixture containing 20 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethane-sulfonic acid) (HEPES), pH 7.4 and 0.1% bovine serum albumin (BSA) and varying concentrations of unlabelled competing hEGF species in a total reaction volume of 100 μl. The incubation was conducted at 37°C for 30 min. Receptor-bound [125]IhEGF 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 ability of the wild type and mutant hEGF proteins to stimulate the protein tyrosine kinase activity of the EGF receptor was determined by measuring the incorporation of \(^{32}\)P from \([\gamma^{32}\text{P}]\text{ATP}\) into the synthetic polypeptide substrate (Glu, Tyr), (Sigma Chemical Co.) as a function of the growth factor concentration. Solubilized and partially purified EGF receptors from A431 cell membranes (Akiyama et al., 1985) were pre-incubated with increasing concentrations of the 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 Koland and Cerione (1988). Pre-incubation in the absence of the hEGF protein 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 dithiothreitol (DTT), 250 mM (NH\(_4\))\(_2\)SO\(_4\), 100 \(\mu\)M Na\(_2\)VO\(_4\), 5% glycerol, 0.05% Triton X-100 and 1 \(\mu\)g of total membrane protein. The kinase reaction was initiated by the addition of \([\gamma^{32}\text{P}]\text{ATP}\) (1.35 Ci/mmol) and (Glu, Tyr),\(_n\) substrates to final concentrations of 75 \(\mu\)M and 0.5 mg/ml respectively, in a final reaction volume of 100 \(\mu\)l. After incubation at room temperature for 10 min, the reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate. The acid-insoluble material was then washed extensively with 5% TCA containing 10 mM Na pyrophosphate. Filters were then dried and the incorporated radioactivity was determined by liquid scintillation spectrometry. The radioactivity incorporated in the absence of hEGF was subtracted from hEGF-stimulated values. The kinase activities reported here include the incorporation of \(^{32}\)P into both the polypeptide substrate and the receptor. The contribution of the latter, as determined by assaying the receptor in the absence of the polypeptide substrate, was found to be less than 2% of the total activity.

**NMR spectroscopy**

Purified protein samples were prepared for NMR experiments by repeated lyophilization and dissolution in \(^2\)H\(_2\)O to a concentration of 0.96 mM in a final volume of 500 \(\mu\)l. All NMR spectra were obtained on a wide-bore Bruker AMX 400 MHz spectrometer at 27°C. Samples were titrated first to pH 3.4 and then pH 7.2 (without correction for the isotope effect); spectral data were obtained at both acidic and neutral pH. 1-D data sets were collected over a spectral width of 4807 Hz with an acquisition time of 3.4 s. Quadrature phase detection and a 90° observation pulse (6.7-7 \(\mu\)s) were used. There were 32 000 data points of 64-128 transients collected. The residual \(^2\)H\(_2\)O signal was saturated with a low-power pulse during the relaxation delay of 3.0 s. Data were processed with 0.5-1 Hz line broadening before Fourier transformation. Chemical shifts were referenced to 2,2-dimethyl-2-sila-pentane-5-sulfonate (DSS).

**Results**

**Purification and electrophoresis of hEGF analogues**

The yields of purified hEGF mutant proteins varied depending on the nature of the amino acid substitution. L15F, L15V and L15R proteins were obtained in modest amounts (~100 \(\mu\)g/l) while a larger yield (~450 \(\mu\)g/l) was obtained with the L15A mutant. The introduction of a strongly absorbing (at 280 nm) tryptophan residue into the hEGF molecule increased its extinction coefficient. This was taken into consideration while estimating the yields for the L15W mutant (~200 \(\mu\)g/l). Very low yields of the L15E mutant (10 \(\mu\)g/l) were obtained using the same purification protocol. Variability in the yields of mutant hEGF protein may be due to differences in the processing of these proteins, cleavage of the alkaline phosphatase signal peptide being necessary for secretion into the periplasmic space of *E.coli*. Alternatively, the stability of the native hEGF structure or of essential intermediates in the folding pathway may be affected. The absence of polymerization, of mispairing of sulfhydryls and of gross conformational changes from the wild type hEGF was suggested by similarities observed in reversed-phase HPLC elution profiles (data not shown).

Appropriate fractions, containing each mutant hEGF protein obtained after purification by reversed-phase HPLC, were examined by native gel electrophoresis. The results confirmed the homogeneity of the hEGF mutant proteins used in the present study (data not shown). The observed electrophoretic mobilities agreed with the mutations introduced. For example, the altered migration pattern of the L15R or L15E mutant could be ascribed to the additional positive or negative charge introduced into the hEGF molecule.

**Determination of the relative affinities of hEGF analogues by radioreceptor competition assay**

The competition binding curves depicted in Figure 2 were generated by measuring the amount of receptor-bound \([^{125}\text{I}]\text{hEGF}\) remaining on the filters, after equilibrium binding in the presence of increasing concentrations of competing hEGF species. The concentration of the wild type or mutant hEGF protein required for 50% displacement of wild type \([^{125}\text{I}]\text{hEGF}\) (IC\(_{50}\) was thus determined graphically for each hEGF variant. The ratio of the wild type to mutant IC\(_{50}\) values
The L15E mutant could not be biochemically characterized in the wild type V\(^{\text{hEGF}}\) at saturating concentrations of the ligand. Significant response in terms of kinase stimulation (53\% of the activity profile for the L15R mutant which still elicits an EC\(_{50}\) value for this variant. This is in marked contrast to the relatives of the hEGF species present in the assay. Relative maximal stimulation = V\(_{\text{max}}\) (mutant)/V\(_{\text{max}}\) (wild type) \times 100\%. See the Results for a description of the determination of the IC\(_{50}\) and EC\(_{50}\) values.

**Table I. Biochemical properties of Leu15 mutants**

<table>
<thead>
<tr>
<th>hEGF species</th>
<th>Relative receptor affinity(a)</th>
<th>Relative agonist activity(b)</th>
<th>V(_{\text{max}}) (c.p.m.(\times 10^{-3}))</th>
<th>Relative maximal stimulation(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>100</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>L15F</td>
<td>45</td>
<td>27</td>
<td>123</td>
<td>103</td>
</tr>
<tr>
<td>L15W</td>
<td>26</td>
<td>22</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>L15V</td>
<td>18</td>
<td>12</td>
<td>119</td>
<td>100</td>
</tr>
<tr>
<td>L15A</td>
<td>2.4</td>
<td>\text{nd}</td>
<td>5.6</td>
<td>4.6</td>
</tr>
<tr>
<td>L15R</td>
<td>1.6</td>
<td>4.0</td>
<td>64</td>
<td>53</td>
</tr>
<tr>
<td>L15E</td>
<td>\text{nd}</td>
<td>\text{nd}</td>
<td>\text{nd}</td>
<td>\text{nd}</td>
</tr>
</tbody>
</table>

\(a\)Relative receptor affinity = IC\(_{50}\) (wild type)/IC\(_{50}\) (mutant) \times 100\%.

\(b\)Relative agonist activity = EC\(_{50}\) (wild-type)/EC\(_{50}\) (mutant) \times 100\%.

\(c\)Relative maximal stimulation = V\(_{\text{max}}\) (mutant)/V\(_{\text{max}}\) (wild type) \times 100\%.

Aromatic residues are spread throughout the hEGF molecule in its 3-D structure and the resonance frequencies and intensities of these aromatic ring protons are sensitive to molecular conformation and solvent accessibility. Therefore, comparisons of these parameters between the wild-type and mutant proteins provide a convenient approach for distinguishing correctly provided an index of the relative receptor affinity of each hEGF mutant.

As shown in Table I, the relative binding affinities of the hEGF analogues ranged from 45 to 1.6\% of the wild type hEGF. The most conservative change to a valine lowered the binding to 18\%, whereas when the side chain was shortened to an alanine, the binding was diminished to 2.4\%. The charged amino acid substitutions, L15R and L15E, were the least tolerated at this site. While the receptor affinity of the L15R ligand was found to be 1.6\% of the wild type, no appreciable binding was detected in the case of the L15E mutant. Replacement of leucine with tryptophan or phenylalanine, which are both bulky and hydrophobic, resulted in relative binding affinities that were 26 and 45\% respectively.

**Determination of the relative agonist activities of hEGF analogues by the receptor kinase stimulation assay**

The mitogenic effect of EGF is mediated through the stimulation of the receptor's intrinsic tyrosine kinase activity and this agonist activity is in turn the initiating event for the signal transduction cascade. The kinase stimulation curves, depicted in Figure 3(A) and (B), represent the velocity of the EGF-dependent kinase reaction measured as a function of the concentration of the hEGF species present in the assay. The protein concentrations required to elicit half-maximal stimulation of the protein tyrosine kinase activity of the receptor (EC\(_{50}\)) were determined for wild type hEGF and the mutants from Figure 3(B). The ratio of the EC\(_{50}\) value of the wild type compared with that for each mutant was used to assess the relative agonist activities of the hEGF variants. The data presented in Table I show that, in general, the order in which the L15F, L15W, L15V and L15A mutants stimulate the tyrosine kinase activity follows the same order as observed in the radioreceptor competition assay.

At saturating ligand concentrations, the maximal levels of kinase stimulation achieved by the L15F, L15W and L15V variants were comparable to the level (V\(_{\text{max}}\)) reached by the wild type hEGF. However, saturating concentrations of the L15A analogue activated the receptor to a lower V\(_{\text{max}}\) (4.6\% relative to the wild type hEGF) and precluded estimation of an EC\(_{50}\) value for this variant. This is in marked contrast to the activity profile for the L15R mutant which still elicits a significant response in terms of kinase stimulation (53\% of the wild type V\(_{\text{max}}\)) at saturating concentrations of the ligand. The L15E mutant could not be biochemically characterized in this assay due to its very low affinity for the receptor; the kinase reaction was found to be unreliable at protein concentrations >15–20 \(\mu\)M. The minor differences observed in the relative receptor affinities and agonist activities for some variants in the two assays may be a reflection of the assay conditions which differ in receptor microenvironment, ionic strength, temperature and incubation time.

**NMR spectra of selected hEGF analogues**

NMR analysis was utilized to evaluate potential structural changes in hEGF resulting from site-directed mutagenesis. Aromatic residues are spread throughout the hEGF molecule in its 3-D structure and the resonance frequencies and intensities of these aromatic ring protons are sensitive to molecular conformation and solvent accessibility. Therefore, comparisons of these parameters between the wild type and mutant proteins provide a convenient approach for distinguishing correctly
Role of Leu15 of hEGF in receptor interaction

Table II. Listing of proton resonances exhibiting minor changes ($\Delta \delta \leq 75$ p.p.b.) in chemical shifts between the wild type and mutant hEGF molecules at pH 3.4

<table>
<thead>
<tr>
<th>Wild-type hEGF</th>
<th>L15A</th>
<th>L15R</th>
</tr>
</thead>
<tbody>
<tr>
<td>W50C7H/L26HN</td>
<td>W50C7H/L26HN</td>
<td>W50C5H</td>
</tr>
<tr>
<td>W50C5H</td>
<td>W50C5H</td>
<td>W50C5H</td>
</tr>
<tr>
<td>W49C5H</td>
<td>W49C5H</td>
<td>W49C5H</td>
</tr>
<tr>
<td>W49C2H</td>
<td>W49C2H</td>
<td>W49C2H</td>
</tr>
<tr>
<td>Y378H/Y13eH</td>
<td>Y375H/Y13eH</td>
<td>Y138H</td>
</tr>
<tr>
<td>Y138H</td>
<td>Y138H</td>
<td>Y138H</td>
</tr>
</tbody>
</table>

Proton resonances listed together have degenerate chemical shifts under these conditions and could not be distinguished in the 1-D NMR spectrum.

The multiple entries for W49 and W50 reflect the fact that these residues have several aromatic resonances which can be identified in the 1-D NMR spectra of hEGF.

Fig. 4. Aromatic region of the 1H-NMR spectra of the wild type and mutant hEGF proteins at (A) pH 7.2 and (B) pH 3.4. The samples were prepared in 2H2O to a final concentration of ~0.05 mM. The spectra were acquired at 27°C over a spectral width of 4807 Hz; 64-128 transients were collected.

As most chemical shifts of the wild type and mutant hEGF proteins are very similar at pH 3.4 (Figure 5), it was possible to extend resonance assignments from the wild type hEGF to the mutant proteins. With the exception of minor shifts of the W49C2H, Y13eH/Y378H (~6.8 p.p.m. region) and the W50C4H/W49C7H/W49C6H (~7.3-7.4 p.p.m. region) resonances in the case of the L15A variant, other minor perturbations that could be characterized were very similar in the spectra of both mutants (see Table II). The spectral shifts that were common to both the L15R and L15A mutants, as compared to the wild type hEGF, involved residues dispersed throughout these molecules. Therefore, the structural differences between the L15R and L15A mutants are relatively minor and are not localized in the 3-D structures of these mutant proteins. Spectral data obtained at pH 7.2 (Figure 4A) reveal that unlike L15R, the L15A mutant protein displays a somewhat 'altered' NMR profile when compared to the wild type hEGF. There are several peaks with small chemical shift differences; some peak broadening can also be observed in the spectrum of this variant (see Discussion). We were careful not to assign the few resonances with comparatively large chemical shifts (>75 p.p.b.) and changes in lineshape or intensity relative to the corresponding resonances in the wild type hEGF.

Discussion

The importance of the Leu15 residue of hEGF in receptor interaction is revealed by the following:

(i) The binding data indicate that both the geometry of the amino acid side chain and hydrophobic character of the residue at position 15 in hEGF are important in receptor binding.
(ii) Although the L15A and L15R mutants have comparable relative receptor affinities, the low $V_{max}$ phenotype observed with regard to the L15A mutant indicates that it is a partial agonist and suggests a partial uncoupling between receptor binding and activation. The kinase data thus implicate a functional role for Leu15 of native hEGF in receptor activation.
(iii) 1-D NMR analyses of the L15A and L15R analogues do not indicate the presence of gross structural perturbations that could possibly explain their drastically reduced biochemical activity compared to the wild type hEGF. From the present study it is clear that Leu15 joins a select group of residues in the EGF molecule (see Figure 1) that are critically important in receptor binding and activation.

Site-directed mutagenesis (in this laboratory) of Leu15 in the N-terminal domain (this study) and that of Leu47 in the C-terminal domain (Matsunami et al., 1991) indicates a stringent requirement for a $\beta$-branched 'leucine-type' residue at these positions in the hEGF molecule. Substitution with a $\gamma$-branched amino acid residue resulted in a relative binding affinity of only 18% (L15V) or 17% (L47I) compared to the wild type hEGF. The L15I mutant protein could not be tested.
in the present studies because it was not produced in our expression system. Replacement with a methyl group (Ala for Leu) at either position further decreased the relative binding affinity to 2.4% (L15A) and 1.8% (L47A). The reduced binding affinity of the L15A mutant described in this study is consistent with the observation made by Shin et al. (1994), who reported that the chemically synthesized L15A hEGF analogue had a drastically reduced mitogenic potency. The moderate loss of receptor affinity seen with L15F and L15W mutants (the present study) could be partially due to steric crowding: these hEGF variants may not fit properly into the ligand-binding ‘pocket’ of the EGF receptor, even though the mutations may not greatly disturb interactions within the hEGF molecule itself. Feild et al. (1992) showed that replacement of the corresponding residue (Phe17) in hTGFα, with tyrosine decreased the receptor affinity to ~8% of the wild type, although the colony-forming ability was not impaired. No comparable data are available with respect to the L47 site of hEGF.

Dose–response curves for kinase stimulation indicate that the L15A (present study) and L47A (Matsunami et al., 1991) mutants exhibit a low $V_{\text{max}}$ mutant phenotype. The I23T and L26G hEGF variants also belong to the class of low $V_{\text{max}}$ mutants (Matsunami et al., 1990). In contrast, the L26A mutant stimulates the receptor to the wild type $V_{\text{max}}$ (D.K. Tadaki and S.K. Niyogi, unpublished results). Clearly, subtle differences do exist in the individual contributions of these hydrophobic residues towards the formation of a receptor-binding epitope. Properties of the low $V_{\text{max}}$ phenotype indicate a partial uncoupling between EGF binding and receptor kinase stimulation. Time-course and dose–response curves with L15A mutant, as compared to the wild type hEGF, also indicate decreased tyrosine phosphorylation of the EGF receptor and other cellular proteins in cultured mammalian cells (K. Nandagopal and S.K. Niyogi, unpublished results). Studies of the mechanism(s) underlying the impaired receptor activation and signal transduction in response to the L15A and other low $V_{\text{max}}$ mutants are in progress.

The 1-D NMR spectra of the L15A and L15R mutant proteins obtained at pH 3.4 are similar to the spectrum of the
wild type hEGF with very few peaks exhibiting significant chemical shifts (>75 p.p.b.) indicating no major structural changes in the global chain fold of these mutants with respect to the wild type hEGF conformation. In interpreting the spectral data obtained at neutral pH, however, it is important to note that chemical shifts are extremely sensitive to even minor conformational changes. These changes may be due to the introduction or replacement of charged functional groups (e.g. L15R). If the size of the side chain is decreased significantly (e.g. L15A), altered packing interactions could give rise to differences in the internal flexibility of the protein with resultant changes in chemical shifts. The relationships between the chemical shifts and conformational changes cannot be easily interpreted in structural terms (Redfield and Dobson, 1990).

The 3-D structure of EGF has been reported to be largely insensitive to the sequence of mutations (see Figure 1), with Arg41 decreased the receptor affinity to only 7% of the wild type receptor (Clackson and Wells, 1995). Interestingly, the amino group of Arg41 in position 15 mutants of hEGF lend credence to Leu15 being a central hydrophobic region in the human EGF receptor along with Tyrl3 (Tadaki and Niyogi, 1993). Ile23 (Campion et al., 1990), Ile26 (Campion et al., 1990, 1993a) and Leu47 (Moy et al., 1989; Dudgeon et al., 1990; Matsunami et al., 1991) by promoting hydrophobic interactions. The hydrophobic contribution of a given residue to the conformational free energy of a protein is proportional to the difference between the standard state accessibility and the mean solvent accessibility. In effect, this measures the area buried by the residue upon folding (Rose et al., 1985). Qualitative considerations of such thermodynamic parameters reveal that subtle variations in the side chain length have profound effects on the mean fractional area loss and AG°, the Nozaki–Tanford free energy of transfer from an aqueous to a non-aqueous environment (Rose et al., 1985). We had previously studied the nature of the physiochemical forces responsible for the formation of the highly stable hEGF–receptor complex and found it to be the result of an entropy-driven change in the free energy characteristic of hydrophobic protein–protein interactions (S.R. Campion and S.K. Niyogi, unpublished results). Interestingly enough, a central hydrophobic region in the human growth hormone–receptor complex accounts for more than three-quarters of the binding free energy required for optimal receptor–ligand interactions (Clackson and Wells, 1995).

The amino group of Leu15 in the N-terminal domain is in close proximity to the carbonyl group of the critical Arg41 in the C-terminal domain. The non-conserved substitution of arginine at position 15 may either interfere with the interdomain hydrogen bonding capacity of Arg41 or affect the pKa of the Arg41 partner on the receptor by altering the local charge distribution. This could lead to a drastically decreased receptor affinity. Interestingly, the substitution of Tyr13, the other important member in the domain–domain interface of hEGF (see Figure 1), with Arg decreased the receptor affinity to only 5.5% of the wild type hEGF (Tadaki and Niyogi, 1993). It can also be pointed out that the negative charge in the L15E mutant may neutralize the positive charge of Arg41, thereby preventing Arg41 from binding complementary residues on the extracellular surface of the EGF receptor.

We propose that Leu15 of hEGF, in concert with Tyr13, provides the crucial guanidinium moiety of Arg41 (Engler et al., 1992) with a unique orientation in a hydrophobic cleft and this configuration is conducive to the formation of resonance-stabilized guanidinium-carboxylate ion pairs necessary for high-affinity binding to the EGF receptor. Alternatively, planar stacking interactions (see Flocco and Mowbray, 1994 and references therein) between Arg41 and an aromatic side chain in the receptor protein can be proposed. Confirmation of these predictions awaits resolution of co-crystal structures of the active receptor–ligand complex.

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