Modification of a receptor-binding surface of epidermal growth factor (EGF): analogs with enhanced receptor affinity at low pH or at neutrality

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Six mutants of human epidermal growth factor (EGF), which carry single point substitutions within a surface patch proposed to juxtapose the bound receptor, were prepared and characterized for receptor affinity and mitogenicity. Receptor affinities relative to EGF are G12Q > H16D > Y13W > Q43A ≈ H16A ≈ EGF >> L15A. Notably, the reduced receptor affinity of mutant L15A indicates that Leu15 probably contributes substantially to receptor binding whereas unaltered receptor affinities observed for analogs H16A and Q43A indicate that neither His16 nor Gln43 contributes significantly to this interaction. On the other hand, the observed enhanced receptor affinities of analogs G12Q, Y13W and H16D highlight surface loci where additional productive receptor-binding contacts can be introduced. Interestingly, at acidic pH analog H16A reveals substantially greater receptor affinity than that of EGF, a property which may offer enhanced therapeutic utility in acidic environments in vivo.

Keywords: EGF/epidermal growth factor/pH dependence/protein engineering/site-directed mutagenesis/urogastrone

Introduction

Epidermal growth factor (EGF) is a 6.2 kDa protein of 53 amino acids with three internal disulfide bridges which, upon binding the extracellular domain of the EGF receptor, induces a conformational change of the receptor (Greenfield et al., 1989) and receptor aggregation (Yarden and Schlessinger, 1987). Such aggregation in turn triggers an intrinsic tyrosine protein kinase activity within the receptor’s cytoplasmic domain (Carpenter et al., 1978; Ushiro and Cohen, 1980), which mediates DNA synthesis and cell division as well as other events (as reviewed by Carpenter and Cohen, 1979; Carpenter, 1987; Yarden, 1988; Ullrich and Schlessinger, 1990; Boonstra et al., 1995). Though X-ray diffraction analysis has yet to afford a highly resolved crystal structure of EGF (Guenther et al., 1990; Lax et al., 1991), NMR studies have yielded solution structures (Cooke et al., 1987; Montelione et al., 1987; Kohda and Inagaki, 1988; Kohda et al., 1988; Hommel et al., 1992; Montelione et al., 1992; Kohda and Inagaki, 1992). Furthermore, a number of structure–function studies utilizing site-directed mutagenesis as well as chemical modification techniques have focused on the role of individual residues in mediation of these biological responses (for reviews see Campion and Niyogi, 1994; Groenen et al., 1994). Other studies have evaluated chimeras of EGF and transforming growth factor-α (TGFα) (Kramer et al., 1994; Richter et al., 1995; van de Poll et al., 1995) or have focused on properties of truncated linear segments of EGF as well as those of polymeric constructs (Komoriya et al., 1984; Simpson et al., 1985; Heath and Merrifield, 1986; Nelson et al., 1991). Notably, the related growth factor, TGFβ, also binds and signals via the EGF receptor, possesses both sequence and structural homology with EGF (Campbell et al., 1989; Kohda et al., 1989; Montelione et al., 1989; Campbell et al., 1990; Hommel et al., 1992; Moy et al., 1993), and has been similarly analyzed by mutagenesis (Defeo-Jones et al., 1988; Lazar et al., 1988; Campbell et al., 1989; Lazar et al., 1989; Defeo-Jones et al., 1991; Harvey et al., 1991; Schultz and Twardzik, 1991). Structural and functional analysis of both EGF and TGFβ indicates that high affinity binding requires important contributions from residues located along most of the length of these factors’ primary structures. For EGF in particular, a surface patch defined by residues Tyr13/Leu15/His16 together with Arg41/Gln43/Leu47 has been proposed to play a key functional role (Campbell et al., 1989). Moreover, a number of analogs have been prepared which partially inhibit receptor-mediated signal transduction at high concentration (Matsumoto et al., 1990), usually analogs have mediated the signal with magnitudes proportional to their affinities for receptor, suggesting then that signaling tends to be tightly coupled to the binding event and that successful construction of potent antagonists will be challenging.

Though other proteins which bind the EGF receptor have also been discovered, extensive structure–function relationships have not yet been reported. For example, amphiregulin, which is 30% homologous to human EGF, has been shown to bind the EGF receptor with high affinity (Shoyab et al., 1989) as has its isoform, Schwannoma-derived growth factor (Eppstein et al., 1985). Other structurally related ligands such as heparin-binding EGF-like growth factor (Higashiyama, et al., 1991), epiregulin (Toyoda et al., 1995) and betacellulin (Shing et al., 1993) also bind the EGF receptor. Furthermore, of three pox virus proteins with homology to EGF (Eppstein et al., 1985; Chang et al., 1987; Lin et al., 1988; Lin et al., 1990; Lin et al., 1991), at least two have now been shown to bind this receptor, suggesting that infectivity and/or tumorigenicity of these agents are so mediated. Finally, EGF-like structural motifs occur within various other proteins such as heregulin/Neu differentiation factor (a ligand for the EGF-receptor-related ErbB-3 and ErbB-4 receptors), CRIPTO, enzymes of the coagulation and fibrinolytic cascades, the low density
lipoprotein receptor, the latent TGF-\(\beta\)-1-binding protein, and domains within EGF’s own unprocessed leader sequence.

In order to assist in the design of EGF antagonists which may offer utility in antitumor therapy (Bishop, 1987; Ulrich and Schlessinger, 1990; Aaronson, 1991; Ennis et al., 1991; Modjtahedi et al., 1993; Salomon et al., 1995; Davies and Chamberlin, 1996) and the design of EGF agonists which may facilitate wound healing (Read and George-Nascimento, 1990; Bennett and Schultz, 1993; Calabro et al., 1995), we have chosen to probe the functional relevance of surface residues Gly12, Tyr13, Leu15, His16 and Gln43 in receptor binding as well as mitogenicity, because these residues are members of, or are adjacent to, the above mentioned surface patch proposed to juxtapose the bound receptor (Campbell et al., 1989). Their relative positions may be seen in a series of molecular models presented elsewhere (Groenen et al., 1994). Interestingly, in so probing this patch, some analogs of potential therapeutic utility have also been prepared which, unlike EGF, maintain affinity for the receptor at reduced pH or which have enhanced receptor affinity near neutrality.

Materials and methods

Construction of analog expression vectors

A gene encoding human EGF was constructed according to published procedures (Mullenbach et al., 1986) as shown in Figure 1 from 10 synthetic deoxyoligonucleotides, but where complementary pairs of oligonucleotides were first annealed separately before gene assembly. This gene segment was then cloned into bacterial expression vector pPHSOD1LacIq (Getzoff et al., 1992) which had been predigested with NheI and SalI to produce the EGF expression vector, pEGF, as shown in Figure 2. Plasmid pEGF-flag, an expression vector for EGF equipped with the N-terminal affinity label, flagTM (Hopp et al., 1988) was then also constructed by cloning a synthetic cassette, composed of 5'-AGC TTT AGC GGA CTA CAA AGA CGA GGA CTA GGA TCG C-3' (coding) and 5'-CAT TCA CTG TCA GAA TTC TTA TCG TCA TCG TCT TAG TCC GCT AA-3' (noncoding), into pEGF predigested with HindIII and BsmI.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Coding</th>
<th>Noncoding</th>
<th>Altered nucleotides</th>
<th>Vector prep.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G12Q</td>
<td>10–165</td>
<td>108–169</td>
<td>124–6(CAG)</td>
<td>BsmI/XbaI²</td>
</tr>
<tr>
<td>L15S</td>
<td>110–165</td>
<td>108–169</td>
<td>133–5(GCA),165(G)</td>
<td>BsmI/XbaI²</td>
</tr>
<tr>
<td>H16A</td>
<td>110–165</td>
<td>108–169</td>
<td>135–8(CCG),165(G)</td>
<td>BsmI/XbaI²</td>
</tr>
<tr>
<td>H16D</td>
<td>110–165</td>
<td>108–169</td>
<td>136(G),165(G)</td>
<td>BsmI/XbaI²</td>
</tr>
<tr>
<td>Q43A</td>
<td>166–225</td>
<td>170–229</td>
<td>217–9(GCA)</td>
<td>XbaI/BglII</td>
</tr>
</tbody>
</table>

Expression vectors for all point mutants were derived from synthetic cassettes and parent vector pEGF or pEGF-flag as summarized in Table I using standard cloning techniques. Expression of analogs JM107 cells were transformed with pEGF, pEGF-flag or these parent plasmids carrying point mutations. A 1:100 dilution of a saturated culture of transformants (25 mg/ml ampicillin in L-broth) was then agitated at 350 r.p.m. and 37°C for 3 h. According to an expression amplification protocol devised and suggested by David Engler (Oak Ridge National Laboratory), chloramphenicol was then added to a final concentration of 5 mg/ml and shaking was continued for 2 h. Finally, isopropyl-
d-thiogaloctopyranoside was added to a final concentration of 0.1 mM and shaking at reduced speed (250 r.p.m.) was maintained for 12 h.

### Purification of analogs

Bacterial cultures (1 liter) were centrifuged (3500 r.p.m., 30 min) and pellets were very gently resuspended in 100 ml ice-cold high salt buffer (1.0 M Tris–HCl, 2 mM EDTA, pH 9.0) and incubated in an ice bath (20 min). Resulting periplasmic lysates were then clarified by centrifugation (3500 r.p.m., 30 min, 4°C). For EGF-flag, 50 ml of the clarified periplasmic lysate were dialyzed against 1.0 M CaCl₂ in PBS (0.01 M NaH₂PO₄ and 0.15 M NaCl titrated to pH 7.4 with NaOH) (2×1.0 liter, 4°C) and reclarified by centrifugation (25 000 g, 45 min). This supernatant was then applied at room temperature to a 1.0 ml antiflag™ monoclonal antibody (IgG₂b) affinity chromatography column (International Biotechnology, Inc.), the column was washed with 4×1.0 ml aliquots of 1.0 M CaCl₂ in PBS, and then (as described by the supplier) protein was eluted in 1.0 ml fractions with 2.0 mM EDTA in PBS where an initial 30 min pause after the first 1.0 ml entered the column, was followed by 10 min pauses between subsequent eluted 1.0 ml fractions. On the other hand, to those clarified periplasmic lysates containing analogs G12Q-flag, Y13W-flag, L15A, H16A, Q43A and H16D-flag was added ammonium sulfate to 60% of saturation and the resulting suspensions were agitated on ice for 1 h before centrifugation (40 000 g, 15 min). The resulting pellets were then dissolved in 25 mM ammonium acetate, pH 5.8, and the solutions were dialyzed against the same buffer 3 times before centrifugation (40 000 g, 15 min) to remove insoluble contaminants. These clarified supernatants were then concentrated by pressure dialysis on an Amicon YM-2 stir cell membrane and isocratically fractionated in 25 mM ammonium acetate, pH 5.8, on a 100–200 mesh P-10 gel filtration column (Bio-Rad Laboratories). Fractions revealing EGF immunoreactivity were combined and concentrated by pressure dialysis using Amicon YM-2 stir cell membranes. These concentrates (as well as the above EGF-flag purified by affinity chromatography) were finally subjected to reverse phase HPLC on a Vydac analytical C4 column equilibrated with 0.1% trifluoroacetic acid in 20% acetonitrile. Five minutes after sample injection, analogs were eluted with a linear acetonitrile gradient from 20 to 50% over 55 min and those purified analogs, recovered between 30 and 35 min, were dialyzed against 0.1 M acetic acid and concentrated on an Amicon YM-2 stir cell membrane. Control rEGF (full length recombinant epidermal growth factor) was expressed in Saccharomyces cerevisiae as described and characterized elsewhere (Brake et al., 1984; George-Nascimento et al., 1988). Composition, purity and concentration of rEGF and all analogs were established by amino acid analysis. In each case purity was confirmed by obtaining a homogeneous elution pattern upon resubmission of proteins to reverse phase HPLC (described above).

### Receptor binding assay

Affinity of each analog for the EGF receptor was determined by measuring its competition with 125I-rEGF for binding to paraformaldehyde-fixed A431 cells, a system wherein ligand and receptor internalization processes are thus inhibited (Gill et al., 1984). Pure rEGF was used as a standard and radio-labelings were performed using Iodogen (Salacinski et al., 1981). A431 cells were grown to confluence in 96-well plates (Dynatech Removacells) and then fixed on the day of the assay with 3% paraformaldehyde in PBS. rEGF and its analogs were serially diluted with 1.0 nM 125I-rEGF in PBS containing 0.1% BSA. Incubations were then carried out at 37°C for 2 h before each well was washed 3 times with PBS containing 0.1% BSA. Finally, wells were snapped apart and counted directly in an LKB gamma counter.

### Receptor-affinity as a function of pH

pH was varied in the assay binding buffer (0.1% BSA in PBS) by adjustment with either 1.0 N HCl or 1.0 N NaOH. The conductivity of each solution was determined and found to be similar for each sample. 0.20 ml of 1.0 nM 125I-rEGF or 125I-H16A in each of the different pH-adjusted buffers was then added in triplicate to paraformaldehyde-fixed A431 cells in 96-well plates. Plates were incubated (2 h, 37°C) and then the wells were washed 3 times with 0.1% BSA in PBS, detached and counted in a gamma counter. In parallel experiments, but in the presence of excess unlabeled rEGF, nonspecific binding was similarly determined and subtracted from plotted results.

### Biological assays

Mitogenicity of analogs was determined by measuring [3H]thymidine incorporation into DNA of human foreskin fibroblasts as described elsewhere (George-Nascimento et al., 1988) and modified as follows: cells were plated at a density of 1×10⁴ cells/well and analogs were diluted in Dulbecco’s modified Eagle’s medium containing 1% BSA. Background incorporation was usually in the range 10 000–20 000 c.p.m./well whereas maximum incorporation was approximately 100 000 c.p.m./well.

### Proton NMR spectroscopy

Purified protein samples were dialyzed against distilled water at pH 6.5 and lyophilized. The samples were then dissolved in 2H₂O (99.99% deuterated, Cambridge, Isotopes), adjusted to pH 7.0 with dilute NaOH, transferred into 5 mm NMR tubes, and then placed in a water bath at 30°C for 24 h to allow exchange of all but the hydrogen-bonded and buried amide protons with deuterons. The samples were again lyophilized, and the proteins were then redissolved in 2H₂O at pH 7.0 ± 0.1 at a concentration of ~1 mg/ml. 1D NMR spectra were recorded at a temperature of 30°C on a Varian Unity 500 MHz NMR spectrometer over a 2 hr period for each sample.

### Results

#### Preparation of EGF analogs

A chemically synthesized gene coding human EGF was assembled as shown in Figure 1. Additional restriction sites were included by altering the wild type nucleotide sequence with appropriate silent mutations in order to facilitate subsequent cassette mutagenesis. Expression plasmids pEGF and pEGF-flag shown in Figure 2 were then utilized to produce recombinant EGF and EGF-flag (carrying an N-terminal affinity label to facilitate purification) via secretion from Escherichia coli (Getzoff et al., 1992) at ~4 and 2 mg/l of culture, respectively, as judged from Western blots. Expression vectors encoding EGF analogs were then constructed from these parent plasmids by cassette mutagenesis as summarized in Table I. These analogs were similarly expressed at 0.5–4 mg/l. They were then purified as described in Materials and methods and were judged to be essentially pure by reverse phase HPLC and amino acid analysis.
Characterization of analogs

Relative affinities of mutants for the human EGF receptor displayed on paraformaldehyde-fixed A431 cells are presented in Tables II and III and have been calculated from IC_{50} (ligand concentration yielding half-maximal inhibition of 125I-EGF binding). These IC_{50}s were derived from data shown in Figure 3 in which test analogs were competed with 125I-rEGF for binding to fixed A431 cells. Mitogenic activities, on the other hand, were established from data presented in Figure 4 wherein the ligand concentration inducing half maximal [3H]thymidine incorporation into DNA of foreskin fibroblasts was determined. As above, relative activities as a percentage of rEGF’s activity were then calculated from these values as presented in Tables II and III.

As noted above, some analogs were equipped with an N-terminal epitope tag which was used to facilitate purification using an antibody affinity column (Hopp et al., 1988). Accordingly, EGF-flag was examined in order to assess its impact on receptor affinity and mitogenicity. As noted in Table II, the tag’s effect is modest in both receptor affinity and mitogenicity assays as was anticipated from results of others which indicated that such a solvated N-terminal modification would be remote from the receptor/ligand interface as well as from those residues replaced in this study.

As shown in Tables II and III, where the impact of the various point substitutions are quantified, it is apparent within experimental error that substitutions His16→Ala and Gln43→Ala have little or no effect upon receptor affinity nor mitogenicity at neutral pH. Interestingly though, substitutions Gly12→Gln, Tyr13→Trp and His16→Asp each confer some enhanced receptor affinity and mitogenicity. On the other hand, a rather dramatic reduction in mitogenicity as well as receptor affinity accompanies substitution Leu15→Ala. To determine whether dramatic structural changes may have accompanied this substitution which, in turn, might have severely hindered receptor binding, 1D NMR spectra of rEGF and L15A were obtained as shown in Figure 5. Comparison of the aromatic and down field Hα regions of the spectra indicate, however, that while some structural perturbations accompany the substitution, the overall tertiary structure of the analog is similar to that of rEGF at pH 7.0 (see Discussion).

Finally, in order to further explore the functional relevance of His16, we determined the receptor affinity of mutant H16A at various pHs. As shown in Figure 6, the amount of 125I-H16A which binds fixed A431 cells over a range of pHs was determined as a percentage of 125I-rEGF which binds fixed cells at neutrality. Nonspecific binding contributions were subtracted where appropriate (see Materials and methods). Notably, these results have revealed a shift in the titration profile of this analog of approximately 1.0 pH units to lower pHs which has interesting implications (see Discussion).

Discussion

Surface scanning of EGF

We have endeavored to elucidate the functional role of five surface residues of human EGF which define one face of this

Table II. Activities of analogs versus EGF

<table>
<thead>
<tr>
<th>Analog</th>
<th>Experimental rationale</th>
<th>Receptor affinity</th>
<th>Mitogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>rEGF</td>
<td>control</td>
<td>100 ± 3.0</td>
<td>100</td>
</tr>
<tr>
<td>L15A</td>
<td>Ala scanning</td>
<td>0.91 ± 0.10</td>
<td>2.8</td>
</tr>
<tr>
<td>Q43A</td>
<td>Ala scanning</td>
<td>130 ± 23</td>
<td>51</td>
</tr>
<tr>
<td>H16A</td>
<td>Ala scan and pH effects</td>
<td>99 ± 13</td>
<td>57</td>
</tr>
<tr>
<td>EGF-flag</td>
<td>impact of tag</td>
<td>42 ± 6.9</td>
<td>67</td>
</tr>
</tbody>
</table>

*Relative affinities are presented as the ratio of the reciprocal of the IC_{50} ± SEM of the EGF mutant versus that of rEGF×100.

Table III. Activities of flagged analogs versus EGF-flag

<table>
<thead>
<tr>
<th>Analog</th>
<th>Experimental rationale</th>
<th>Receptor affinity</th>
<th>Mitogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF-flag</td>
<td>control (tagged)</td>
<td>100 ± 14</td>
<td>100</td>
</tr>
<tr>
<td>G12Q-flag</td>
<td>as in TGFα</td>
<td>460 ± 28</td>
<td>470*</td>
</tr>
<tr>
<td>Y13W-flag</td>
<td>volume constraints</td>
<td>218 ± 11</td>
<td>520*</td>
</tr>
<tr>
<td>H16D-flag</td>
<td>affinity enhancement</td>
<td>36 ± 33</td>
<td>ND</td>
</tr>
</tbody>
</table>

*All analogs are equipped with an N-terminal affinity label, i.e., flag^{TM} (Hopp et al., 1988).

Fig. 3. Receptor-binding of analogs versus concentration. The binding of 125I-rEGF to paraformaldehyde-fixed A431 cells was measured in the presence of increasing concentrations of ligand. (A) Analogs L15A, H16A and Q43A. (B) Analogs EGF-flag, G12Q-flag and Y13W-flag. (C) Analog H16D-flag with standard, rEGF. Data are expressed as mean CPM ± SEM.
Fig. 4. Mitogenic activity of analogs. Analogs were tested for mitogenic activity by monitoring [3H]thymidine incorporation into DNA using quiescent cultures of human foreskin fibroblasts. (A) Analogs L15A, H16A and Q43A with control rEGF. (B) Affinity-labeled analogs G12Q-flag and Y13W-flag with controls rEGF and EGF-flag.

Fig. 5. Proton NMR spectra of rEGF (bottom) and L15A (top). Comparison of the aromatic and down field H^2 regions were obtained at 500 MHz, 30°C and pH 7.0 in H_2O.

ligand. NMR solution structures of human and mouse EGF have shown that amino acids Gly12, Tyr13, Leu15, His16 and Gln43 are proximal surface residues (Cooke et al., 1987; Montelione et al., 1987; Kohda and Inagak, 1988; Kohda et al., 1988). Furthermore, as suggested by analysis of patterns of sequence conservation among related growth factors when compared with EGF-like domains of nonmitogenic proteins, of these five residues, highly conserved Tyr13, Leu15, His16
altering binding affinities (Willingham et al., 1984) to inhibit endocytic processes (Gill et al., 1979) so that there is virtually no binding of EGF at pH 5.0 as reported by others as well (Nunez et al., 1993). To test this premise, and perhaps to ascertain whether the biological response in at least these five cases is tightly constrained on Tables II and III.

Accordingly, human EGF analogs G12Q, Y13W, L15A, H16A and Q43A (or their affinity-labeled derivatives) were expressed and their affinities for the EGF receptor on parafomaldehyde-fixed A431 cells were determined and compared with appropriate controls. Cells were fixed in this manner in order to inhibit endocytic processes (Gill et al., 1984) without altering binding affinities (Willingham et al., 1979) so that binding events would not be confused by potential changes in ligand and receptor trafficking pathways. Mitogenic assays were also performed on viable cells. Results from these approaches have then allowed the formulation of some conclusions: first, for each analog so examined, the mitogenic response was observed to be essentially proportional to the analog’s affinity for receptor (within the rather broad experimental error inherent in the mitogenic assay), thus indicating that the biological response in at least these five cases is tightly coupled to the receptor-binding event and that none of these analogs can therefore be utilized as an EGF antagonist.

Second, analysis of analog L15A, in which a 30- to 100-fold reduction in mitogenicity and receptor affinity is observed, respectively, suggests that Leu15 may mediate the biological response by contributing very substantially to binding. This notion is further supported by NMR analysis of this analog at pH 7.0 which reveals no global conformational changes from that of rEGF, thus indicating that the observed effects of this substitution, indeed, likely originate from the replacement per se, rather than any associated global changes on the surface of the ligand. We conclude then that Leu15 is probably of critical importance to the function of EGF. Such results are consistent with the work of others on L15A as well (Shin et al., 1994), though its activity was only evaluated in mitogenic assays and an evaluation of its tertiary structure was not performed.

Third, substitutions of His16 or Gln43 by alanine, on the other hand, apparently have negligible impact on receptor affinity or on mitogenicity, thus indicating that neither residue contributes significantly to ligand binding nor to triggering receptor kinase activation despite their location within the putative receptor-binding patch (Campbell et al., 1989).

Fourth, because others had shown that residue Tyr13 is juxtaposed with the bound receptor (Campion et al., 1993; Taiaki and Niyogi, 1993; Shin et al., 1994) and because our computer-aided tertiary structural examination revealed that this locus could probably accommodate the larger aromatic residue, tryptophan, we therefore prepared mutant Y13W. Interestingly, we observe here that not only is this accommodation possible, but that such a substitution enhances both receptor affinity and mitogenicity probably more than 2-fold, presumably by introducing a supplemental binding contact with the receptor. In a preliminary report (i.e., an abstract) others have reported that such a mutation confers marginal enhancement of receptor affinity (Taiaki and Niyogi, 1994). Notably, still others have shown that substitution of Tyr13 by leucine also increased receptor affinity marginally (Hommel et al., 1991) or perhaps not at all (Taiaki and Niyogi, 1993).

Fifth, we similarly observe that analog G12Q (a substitution identical to that found in TGFX) exhibits approximately a 5-fold increase in receptor affinity and mitogenicity. Because an additional binding contact then likely occurs in TGFX which we have now shown can also be added productively to EGF, it seems likely that TGFX in fact makes at least one different receptor contact than does EGF, while still maintaining an identical overall spatial relationship with the receptor. This finding then also demonstrates that EGF’s Gly12 is, in fact, at the ligand/receptor interface.

Receptor affinity versus pH

In order to further investigate the functional importance of titratable residues located within the putative binding patch, we have compared the receptor affinities of H16A and EGF as a function of pH using parafomaldehyde-fixed cells. It is evident from these studies, as shown in Figure 6, that H16A at acidic pH possesses considerably enhanced affinity for the receptor when compared with that of EGF at such pHs. Note, for example, that there is virtually no binding of EGF at pH 5.0 as reported by others as well (Nunez et al., 1993), whereas H16A at pH 5.0 still binds at 30% of maximal potential binding. At low pH we hypothesize the formation of a positively charged imidazolium ion at His15 which disrupts receptor binding via repulsive interactions with an opposing positive charge on the receptor, thus favoring dissociation (Nunez et al., 1993). To test this premise, and perhaps to enhance binding by introduction of an additional intermolecular

![Fig. 6. Receptor affinities of rEGF and H16A as a function of pH. Paraformaldehyde-fixed A431 cells were treated with 125I-rEGF or 125I-H16A, then washed and radio-counted. Counts which bound are reported as a percentage of those of 125I-rEGF which bound at pH 7.5.](image-url)
salt bridge, we therefore introduced a negative charge at this locus which could oppose the suspected positive charge on the receptor. To this end we prepared analog H16D-flag and observed that it does, indeed, exhibit nearly a 4-fold enhancement in receptor affinity at neutral pH. Interestingly, others have noted a loss of activity when uncharged glutamine is placed at this locus (Shiah et al., 1992), thus further suggesting that a salt bridge may, in fact, be important for the enhanced affinity of H16D-flag. Of course then it should also prove interesting to determine whether H16D maintains its enhanced receptor affinity at reduced pH as well, as this analog could then supersed H16A as a more active ligand under acidic conditions. In any case though, it should be noted that this affinity-enhancement design strategy may not necessarily be applicable to other members of the EGF-related family, because the unmodified ligand may not respond to acidic environments like EGF and/or may not be similarly modulated by substitutions at the ligand’s homologous histidine due to the presence of additional titratable histidines (e.g., TGFα with three more histidines) and/or formation of a different relative ligand/receptor binding geometry.

In conclusion, we have identified a residue in EGF that is likely required for tight receptor binding, i.e., Leu15. Furthermore, we have identified three surface loci where enhancement of receptor-binding is afforded by appropriate substitutions, i.e., at Gly12, Tyr13 and His16. Perhaps in enhancement of receptor-binding is afforded by appropriate substitutions at the ligand’s homologous histidine due to the ability of the ligand to establish a salt bridge with an appropriate receptor residue. In any case though, it should be noted that this affinity-enhancement design strategy may not necessarily be applicable to other members of the EGF-related family, because the unmodified ligand may not respond to acidic environments like EGF and/or may not be similarly modulated by substitutions at the ligand’s homologous histidine due to the presence of additional titratable histidines (e.g., TGFα with three more histidines) and/or formation of a different relative ligand/receptor binding geometry.

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