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Receptor Recognition by Histidine 16 of Human Epidermal Growth Factor via Hydrogen-Bond Donor/Acceptor Interactions

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Abstract Human epidermal growth factor (hEGF) and human transforming growth factor α (hTGFα) are prototypical of structurally related polypeptide mitogens which interact with the epidermal growth factor receptor (EGFR). Several determinants of receptor recognition that specify function have been proposed on the basis of structural criteria. This study evaluates the role of one such candidate, H16 of hEGF, by site-specific mutagenesis. When assayed for receptor tyrosine kinase stimulation using $(Glu_4,Tyr_1)_n$ as the exogenous substrate in vitro, the relative agonist activities of position 16 mutants range from 14–263% of wild-type hEGF. The rank order of potency was found to correlate with the relative receptor binding affinities of the mutants, which range from 7–272% of wild-type, as determined by radioreceptor competition assays. The mitogenic activity of the H16 mutants is similar to that of wild-type hEGF as determined by clonogenic assays using rat tracheal epithelial cells. While the colony forming efficiencies do not reflect significant differences in growth rate or survival characteristics in the presence of the hEGF variants, it is reduced to 1.6% in control cultures which lack EGF in the medium. The results show that H16 of hEGF, although not essential for mitogenic activity, optimizes receptor recognition by hydrogen-bond donor/acceptor interactions and may share this feature with H18 of hTGFα. J. Cell. Biochem. 72:16–24, 1999.

Key words: cell proliferation; ligand receptor interaction; protein engineering; rational design; tyrosine kinase

Epidermal growth factor (EGF) is a 6-kDa polypeptide mitogen which activates the EGF receptor (EGFR) tyrosine kinase, a 170-kDa transmembrane glycoprotein expressed in epithelial and mesenchymal cell types [Carpenter and Cohen, 1990]. The cellular response to EGF is mediated by reversible phosphorylation of endogenous substrates which interact in complex biochemical arrays to raise intracellular pH, Ca^{2+} levels and enhance the rates of glycolysis, DNA, RNA, and protein syntheses. Physiological effects observed include growth, proliferation, or differentiation [reviewed in Carpenter and Wahl, 1990; Ullrich and Schlessinger, 1990; van der Geer et al., 1994].

Deregulated expression of transforming growth factor α (TGFα), which is homologous to EGF, contributes to neoplastic transformation [Sporn and Roberts, 1985, 1992; Matsui et al., 1990]. The constitutively active v-erbB oncoprotein, a retroviral homologue of the EGFR which lacks a ligand-binding region and C-terminal autophosphorylation sites, signals aberrantly in transformed cells [McManus et al., 1995]. EGF is involved in pathophysiological states such as autosomal recessive polycystic kidney disease [Orellana et al., 1995], Rieger syndrome [Slavkin, 1993], X-linked ectodermal dysplasia [Blecher et al., 1990], and has also found use in the treatment of burns [Brown et al., 1986; King et al., 1988], corneal traumas [Gospodarowicz et al., 1979; Carter et al., 1988], and gastric ulcers [Schultz et al., 1991]. These findings thus underscore the clinical importance of studying EGF-EGFR interactions.

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Although high-resolution crystal structures for EGF, TGFα, or the ligand-receptor complex are unavailable, nuclear magnetic resonance (NMR) spectroscopy and structure-function analyses indicate that human EGF (hEGF) residues involved in receptor recognition and activation are dispersed throughout its tertiary structure and map to one face of the molecule. They include the crucial guanidinium moiety of R41 and the hydrophobic side-chains of Y13, L15, I23, L26, and L47 [reviewed in Campion and Niyogi, 1994; Groenen et al., 1994; Tadaki and Niyogi, 1996].

The interface (comprising residues 13–16, 37, and 41–43) between the N- and C-terminal domains of hEGF is structurally stabilized by numerous hydrogen bonding interactions [Hommel et al., 1992]. Since there is evidence for significant conformational flexibility of the corresponding region in human TGFα (hTGFα), it has been postulated that this region may contribute substantially to the free energy of receptor binding [Li and Montelione, 1995]. H16 of hEGF is thus implicated in receptor recognition. Sequence alignments and pH titration studies show that the corresponding residue in hTGFα, H18, has a pK_a value of 7.6 [Tappin et al., 1989]. The pK_a value of H16 of hEGF is 7.2 [Murray et al., 1996], which indicates that it is solvent-exposed, ~50% is protonated at physiological pH and the residue is capable of direct interaction with the receptor protein.

Previous studies have suggested differential contributions of H16 in hEGF and H18 in hTGFα to EGFR binding. Partially purified *Pseudomonas* exotoxin A-hEGF chimeras in which H16 was replaced with Q display reduced receptor binding affinity (19% of wild-type) and cytotoxic activity [Shiah et al., 1992]. In another report, a highly purified H18K hTGFα mutant was shown to retain 22% relative binding affinity and 7% relative mitogenic potency [Defeo-Jones et al., 1989]. This suggests a stringent requirement for H since semi-conservative changes to either Q or K result in loss of biological activity. In contrast, partially purified H18A and H18E mutants of hTGFα exhibit relative binding affinities of 30% and 84%; the H18E mutant is also more potent than wild-type in a soft-agar transformation assay [Feild et al., 1992]. These equivocal results prompted a re-examination of the role of H16 in hEGF structure-function. The L, N, Q, R, and Y site-specific mutants were expressed as recombinant proteins in *E. coli* and characterized with regard to their native conformation, binding affinity, agonist activity, and ability to promote clonal growth of cells in culture. Though not essential for biological activity, H16 of hEGF optimizes receptor recognition by hydrogen bond donor/acceptor interactions and may share this feature with H18 of hTGFα.

MATERIALS AND METHODS

Oligonucleotide-Directed Site-Specific Mutagenesis

Deoxyoligonucleotides containing the desired mutations were synthesized in a Milligen/Biosearch Cyclone Plus DNA synthesizer utilizing phosphoramidite chemistry. The sequences of the oligonucleotides with the mutated codons underlined and the mismatches in boldface are as follows: H16L, 5′-CCTGC**T**CGACGGTGTTTGCATG-3′; H16N, 5′-CCTG**A**ACGACGGTGTTTGCATG; H16Q, 5′-CCTGCA**A**GACGGTGTTTGCATG-3′; H16R, 5′-CCTGC**GA**GACGGTGTTTGCATG-3′; H16Y, 5′-CCTGT**T**ACGACGGTGTTTGCATG-3′. Site-directed hEGF mutants were generated by a polymerase chain reaction strategy [Helmsley et al., 1989]. Double stranded DNA of the expression vector, pEGF1 (2.9 kb), containing the hEGF insert was used as template. Each deoxyoligonucleotide primer bearing the mutation was utilized in a "back to back" configuration with the wild-type reverse primer 5′-CAGTACCCGTCGTGAGACAG-3′. A thermostable DNA polymerase isolated from *Pyrococcus furiosus* (*pfu* polymerase, Stratagene, La Jolla, CA) was used to incorporate the mutant primer and amplify the *hEGF* gene-containing plasmid. Reactions were carried out in standard buffer (20 mM Tris-Cl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton- X-100, and 10 μg/ml BSA) supplied by the manufacturer. Final concentrations of reagents present in a 100-μl reaction mixture were: 1 pmol each of mutant and reverse primers, 0.25 mM dNTPs, 0.01 pmol template DNA, and 5 units of *pfu* polymerase. Conditions for the first PCR cycle were as follows: denaturation at 94°C for 3 min, annealing at 40°C for 1 min, and primer extension at 72°C for 12 min. Twenty-five successive cycles were performed under the same conditions except that the denaturation time was reduced to 1 min. The linear amplification products were resolved on a 1%

agarose gel and isolated by electroelution. The DNA samples were treated with Klenow polymerase (New England BioLabs, Beverly, MA) to form blunted ends and then circularized by incubation overnight at 14°C with T4 DNA ligase (New England BioLabs). Transformation of *E. coli* JM107 was according to the procedure of Hanahan [1985]. All engineered mutations were confirmed by sequencing [Sanger et al., 1977] the entire *hEGF* gene.

Expression and Purification of Recombinant hEGF Proteins

Overnight cultures of *E. coli* JM107 cells harboring the pEGF1 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 prewarmed medium. After 3 h (OD_{600} = 0.5–0.8) chloramphenicol was added to a final concentration of 5 μg/ml. When the OD_{600} of the cultures reached 1.2–1.4, 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 protein sequestered in the periplasm was isolated from the cell pellet by resuspension in ice-cold buffer (1 M Tris-Cl, 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 $(NH_4)_2SO_4$ 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 dialyzed against 25 mM sodium phosphate, pH 7.2. The wild-type or mutant hEGF analogue 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% CH_3CN for 15 min followed by a 15–34% CH_3CN 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.

Native Gel Electrophoresis and Immunoblotting of hEGF Proteins

Each hEGF mutant protein was examined by nondiscontinuous gel electrophoresis in the absence of any detergent/denaturing agent. Samples were resolved in 10% polyacrylamide gels buffered with 75 mM Tris-phosphate, pH 7.0, the same buffer being used for electrophoresis at a constant 200 V for 30 min at 4°C. Protein samples were subsequently transferred to Immobilon-P membranes (Millipore, Inc., Bedford, MA) in the same buffer at a constant 20 V for 16 h at 4°C. Blots were probed with an anti-hEGF monoclonal antibody [$IgG1_k$] (Upstate Biotechnology, Inc., Lake Placid, NY) at 1:2,000 dilution. This high-affinity, neutralizing antibody is specific for hEGF and does not cross-react with mEGF or hTGFα. Secondary goat anti-mouse polyclonal antibody conjugated to horseradish peroxidase (Transduction Labs, Lexington, KY) was used at 1:2,000 dilution as per manufacturer's protocol. Enhanced chemiluminescence (Amersham, Inc., Piscataway, NJ) was used for detection purposes.

Radioreceptor Competition Assay

Membrane-bound hEGF receptors were isolated from A431 (human epidermoid carcinoma) cells according to Akiyama et al. [1985] with modifications described by Campion et al. [1990]. Specific binding of hEGF proteins to the EGF receptor was determined by a radioreceptor competition assay [Carpenter, 1985]. Purified membrane fractions from A431 cells were resuspended in buffer (20 mM HEPES, pH 7.4, and 10 mM PMSF) and the total membrane protein content was determined by the Bio-Rad Protein Assay. ^{125}I-hEGF was prepared by the chloramine-T method [Hunter and Greenwood, 1962] to an average specific activity of ~150,000 cpm/pmol hEGF. Two μg of the receptor preparation was incubated with 30 nM wild-type ^{125}I-hEGF in a mixture containing 20 mM HEPES, pH 7.4, and 0.1% BSA, and varying concentrations of unlabeled competing hEGF species in a total reaction volume of 100 μl. Incubation was conducted at 37°C for 30 min. 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 was quantitated by liquid scintillation spectrometry.

Receptor Tyrosine Kinase Stimulation Assay

The ability of wild-type and mutant hEGF ligands to stimulate the protein-tyrosine kinase activity of the EGF receptor was deter-

mined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic polypeptide substrate $(Glu_4,Tyr_1)_n$ (Sigma Chemical Co., St. Louis, MO) as a function of growth factor concentration. Solubilized and partially purified EGF receptors from A431 cell membranes [Akiyama et al., 1985; Campion et al., 1990] were preincubated with increasing concentrations of 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 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 DTT, 250 mM $(NH_4)_2SO_4$, 100 µM Na_3VO_4, 5% glycerol, 0.05% Triton X-100 and ~1 µg of total membrane protein. The kinase reaction was initiated by the addition of [γ-^{32}P]ATP (1.35 Ci/mmol) and $(Glu_4,Tyr_1)_n$ substrates to final concentrations of 75 µM and 0.5 mg/ml, respectively, in a final reaction volume of 100 µl. After incubation at room temperature for 10 min, the reaction was stopped by the addition of 200 µl 5% TCA containing 10 mM sodium pyrophosphate. The acid-insoluble material was collected on 25-mm Millipore HAWP filters which were 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.

Cell Culture and Clonogenicity Assay

C18 is one of a series of epithelial cell lines established following exposure of cultured tracheal explants to 12-O-tetradecanoyl-phorbol-13-acetate [Steele et al., 1978]. These lines can repopulate denuded tracheal grafts [Terzaghi et al., 1978] to form mucociliary epithelia similar to that seen in normal tracheas and are nontumorigenic when inoculated into immunosuppressed hosts. Cell stocks were maintained at 37°C in a humidified atmosphere of 5% CO_2, as proliferating cultures in Ham's F-12 medium (GIBCO, Gaithersberg, MD) supplemented with 2% fetal bovine serum (Hyclone, Logan, UT), 0.1 µg/ml hydrocortisone (Sigma), 1 µg/ml insulin (Sigma), 50 µg/ml gentamycin (GIBCO), and 1 ng/ml EGF (complete medium). For clonogenicity assays, single-cell suspensions were plated in six-well culture dishes (Costar; VWR, West Chester, PA) at a density of 5×10^2 cells per well. The growth medium of experimental cultures contained hEGF variants at the desired concentrations while control samples lacked EGF. After a period of 5–8 days, the dishes were rinsed thoroughly with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, fixed in methanol, stained with Giemsa, and visualized under a dissecting light microscope. The percentage cloning efficiency was calculated from the ratio of discrete colonies scored (criterion:32–50 cells/colony) to the number of cells seeded per well. The assay permits a distinction between alterations in growth rate (colony size) and survival (colony number) in a given population. Cultures used for these experiments were between passage numbers 26–47 and the cloning efficiency was always compared with sample populations obtained from the same passage.

RESULTS

Purification, Electrophoresis, and Immunoblotting of hEGF Mutants

The yields of hEGF mutant proteins obtained after purification by reversed-phase HPLC ranged from 30–75 µg/l. Appropriate fractions were examined by native gel electrophoresis and immunoblotting with a neutralizing anti-hEGF monoclonal antibody. The results (see Fig. 1) indicate that the H16L, H16N, H16Q,

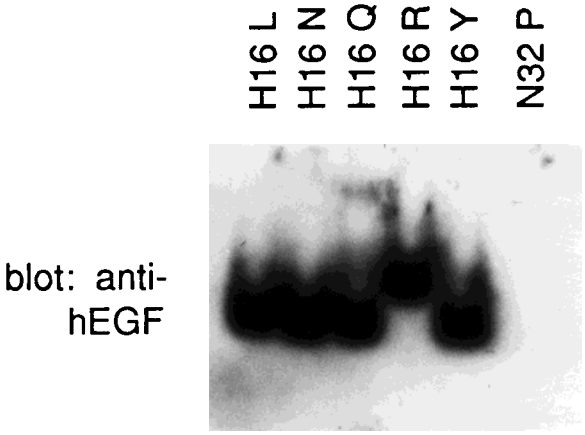

Fig. 1. Native gel electrophoresis and immunoblotting of H16 hEGF mutant proteins with a neutralizing anti-hEGF monoclonal antibody.

and H16Y proteins comigrate since they possess the same net charge of −4. The altered electrophoretic mobility of the H16R mutant derives from a net charge of −3. Antibody specificity is demonstrated by the absence of signal in the control lane which is loaded with the N32P hEGF mutant; structural alterations in the native N32P mutant [Campion et al., 1993] preclude antibody recognition. The immunoassay also confirms the identity of the mutants as hEGF molecules with no major structural perturbations compared to wild-type hEGF.

Determination of Relative Binding Affinities of hEGF Mutants

Specific binding of each position 16 mutant was assessed with respect to its ability to compete with ^{125}I-labeled wild-type hEGF bound to EGF receptors in membrane fractions of A431 cells. The competition curves, presented in Figure 2, permit the estimation of IC_{50} values which indicate the concentration of competing species required for inhibition of binding by 50%. The ratio of wild-type and mutant IC_{50} values was used as an index of the relative affinity of the receptor for each mutant.

Binding data (see Table 1) for the H16 mutants of hEGF show that their relative receptor affinities range from 7–272% of wild-type hEGF. Substitution of the polar H16 side-chain with a bulky, hydrophobic residue (H16L) decreases the affinity to 13% of wild-type. Interestingly, replacement with an amphipathic tyrosyl moiety also decreases the relative binding affinity of the H16Y mutant to a modest 32%. This suggests a role for the aromaticity and/or hydrogen-bonding potential of H16 in receptor binding. The introduction of a positively charged guanidinium moiety reduces the binding affinity of the H16R variant to 7% of wild-type hEGF. Interactions necessary for optimal receptor recognition may be compromised by the delocalized charge and decreased flexibility of the bulky arginine side-chain at this site. Among this series of mutant proteins, the isosteric N and Q substitutions exhibit elevated receptor affinities (150 and 272%) compared to wild-type. This indicates that neither aromaticity nor charge but hydrogen-bonding interactions via the imidazole nitrogen(s) of H16 are important for receptor binding.

TABLE I. Biochemical Properties of H16 hEGF Mutants[a]

	Radioreceptor competition assay		Tyrosine kinase stimulation assay	
hEGF species	IC_{50} (nM)	Relative binding affinity (%)[b]	EC_{50} (nM)	Relative agonist activity (%)[c]
Wild-type	30	100	50	100
H16N	20	150	33	152
H16Q	11	272	19	263
H16Y	95	32	127	39
H16R	224	7	200	25
H16L	420	13	350	14

[a]Data are the representative of average values from at least two independent determinations. See text under Results for determination of IC_{50} and EC_{50} values.
[b]Relative binding affinity = IC_{50} (wild-type)/IC_{50} (mutant) × 100%.
[c]Relative agonist activity = EC_{50} (wild-type)/EC_{50} (mutant) × 100%.

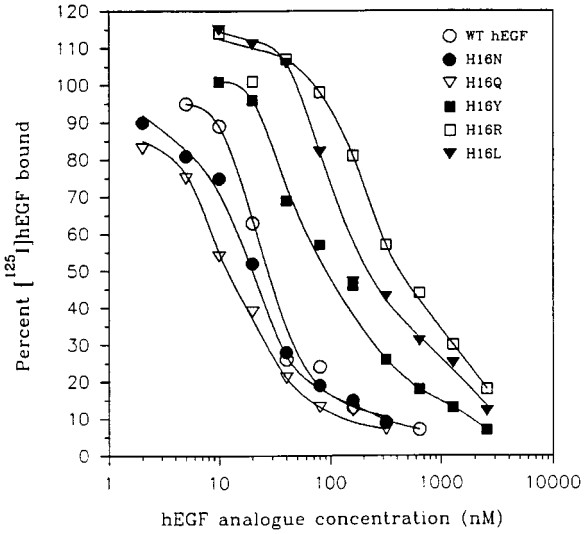

Fig. 2. Radioreceptor competition binding curves for H16 hEGF mutants. Varying concentrations of each mutant were incubated at 37°C for 30 min with ^{125}I-labeled wild-type hEGF (~150, 000 cpm/pmol) and EGF receptor-containing membrane fraction (20 μg/ml total protein) in 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin. All curves shown represent the best fit through the average data points of duplicate experiments.

Determination of Relative Agonist Activities of hEGF Mutants

Using the synthetic polymer $(Glu_4,Tyr_1)_n$ as the exogenous substrate, ligand-dependent activation of the EGFR was measured as a function of ligand concentration. Protein concentrations required for half-maximal stimulation (EC_{50}) were determined from the dose-response

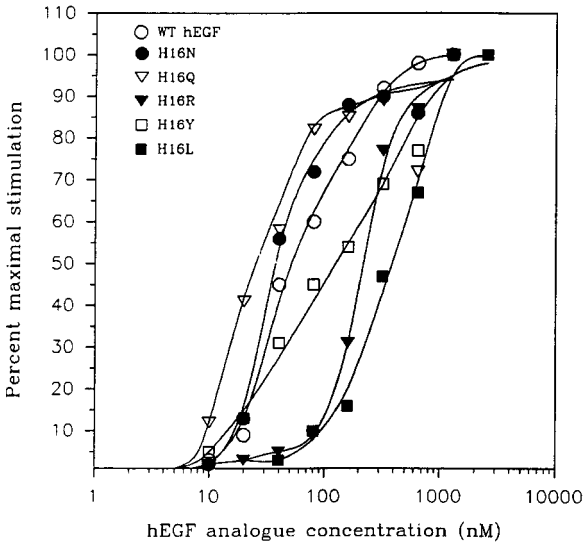

Fig. 3. Receptor tyrosine kinase stimulation curves for H16 hEGF mutants. The rate of EGF-dependent phosphorylation of exogenously added $(Glu_4,Tyr_1)_n$ substrate by partially purified EGF receptor was measured as described in Materials and Methods. All curves depicted represent the best fit through the average data points of duplicate experiments.

curves presented in Figure 3. The ratio of the EC_{50} value of wild-type compared with that obtained for each mutant was used to assess the relative agonist activity of each mutant under the kinase assay conditions.

Activity profiles of the H16 mutants show that their EC_{50} values range from 14–263% of wild-type hEGF (see Table 1). In general, the rank order of potency correlates with the relative receptor affinity of the mutants. The reduced potency of the H16L mutant (14%) reveals the deleterious effects of hydrophobicity at this site. While the H16R and H16Y variants exhibit moderate activity (25 and 39%), the enhanced potency of the H16N and H16Q analogues (139 and 263%) shows that they bind even the detergent-solubilized receptor with great tenacity. The difference in agonist activity and relative affinity of the H16R mutant may be a reflection of the assay conditions which differ in receptor microenvironment, ionic strength, temperature and incubation time.

Clonogenic Efficiencies in the Presence of hEGF Mutants

The mitogenic activity of H16 mutants of hEGF was evaluated in a clonogenic assay with C18 cells which are EGF-dependent for growth. Table 2 shows that H16N, H16Q, H16Y, and wild-type hEGF stimulate colony formation with similar efficiencies at doses of 0.9 and 2.7 ng/ml (0.15–0.44 nM).

Only a small number (< 0.01%) of colonies appear more elongated and less adherent than cells of normal epithelial morphology, which may reflect the clonal variation in the population. No appreciable differences in growth rates and colony survival were discernible. Cells stimulated with 0.9 ng/ml H16L or H16R form ~10–15% fewer colonies compared to wild-type. When the dose is increased to 2.7 ng/ml, to compensate for their reduced potency, both H16L and H16R are similar to wild-type hEGF in their activity. No significant morphological difference or increase in colony diameter was observed in cultures stimulated with any of the growth factor variants characterized in this study. Hence, we conclude that the clonogenic efficiency observed in the presence of hEGF variants is due to cell survival, as reflected by colony number.

DISCUSSION

Biological responses to EGF and TGFα are not always identical [Derynck, 1992] and some studies have suggested that the EGFR discriminates between bound EGF and bound TGFα [Lax et al., 1988; Richter et al., 1995; van de Poll et al., 1995; Puddicombe et al., 1996; Summerfield et al., 1996]. Furthermore, pH sensitivity of the ligand-receptor interaction dictates intracellular trafficking (endocytosis/degradation/recycling) of the EGF/TGFα superfamily of ligands [Ebner and Derynck, 1991; French et al., 1995] and may therefore influence long term responses to growth factor stimulation [Reddy et al., 1996]. The H16 hEGF mutants were examined in a clonogenic assay to determine whether H16 was essential for mitogenic activity.

Colony forming efficiency is a direct, albeit empirical, measure of the proportion of clonogenic cells in a given population and can be used to distinguish growth rate (colony size) from survival (colony number) [Wilson, 1992]. Table 2 shows that saturating concentrations of the H16 variants promote growth of C18 cells as replicating subcultures at clonal density. No major differences in growth rate or survival characteristics are discernible in cultures that have been stimulated with the hEGF variants. However, the colony forming efficiency is drastically reduced to 1.6% in control cultures which lack EGF. This demonstrates that the cells are

TABLE II. Clonogenic Efficiencies in the Presence of H16 hEGF Mutants[a]

Dose	(−) EGF control	Wild-type hEGF	H16L	H16N	H16Q	H16R	H16Y
0	1.6%	—	—	—	—	—	—
0.9 ng/ml	—	32%	14%	38.4%	34.1%	20.2%	43%
2.7 ng/ml	—	28.8%	29.9%	28.6%	34.2%	32.5%	41.7%

[a]The clonogenic efficiency was calculated from the ratio of discrete colonies scored (criterion: 32–50 cells/colony) to the number of cells seeded per well as described in Materials and Methods. Data represent average values from at least two independent determinations.

EGF-dependent for growth stimulation. When the mitogenic potencies of the H16 mutants are compared to that of wild-type hEGF, it appears that survival may be governed by kinetically controlled events not easily correlated with receptor binding affinities determined at equilibrium. Similar conclusions have been reached in studies where the metabolic activity/viability of cell populations in response to genetically engineered EGF or TGFα has been assessed by ^{3}H-TdR [Engler et al., 1991] and ^{125}I-UdR [Puddicombe et al., 1996] uptake into DNA.

The H16Y mutant displays a higher clonogenic efficiency, despite having 30–40% of wild-type receptor affinity and agonist activity. Because of the long assay period (5–8 days), this increased clonogenicity may be partly due to diminished receptor downregulation and/or decreased ligand depletion, kinetic factors that can play a major role in cell proliferation [Reddy et al., 1996]. However, other factors such as new receptor synthesis and/or synergy with components of the growth medium (serum, insulin, hydrocortisone), cell-cell communication, etc., may also play a significant role during this assay period, which is considerably longer than that (3 days) of Reddy et al. [1996] in their endocytic trafficking experiments. A firm conclusion has to await further studies.

The effect of growth rate on survival is difficult to address in quantitative terms since true enhancement/impairment of cell survival should also reflect an enhanced/depressed rate of precursor incorporation into DNA, RNA, or protein after several population doubling times. In contrast to assays in 'cell-free' systems or short-term assays that do not include a recovery period, the colony forming efficiency assay is long enough (5–8 days) to permit potential clonogens to be scored as macrocolonies. This may aid characterization of ligands with the potential to alter growth rates in the context of physiologically responsive cell types.

Site-directed mutagenesis of H16 does not indicate an intrinsic structural requirement for the imidazole moiety in hEGF structure-function. Unlike the H16R mutant, H16Q and H16N exhibit increased relative binding affinities and agonist activities in vitro (Table 1). At physiological pH, the R side-chain acts only as hydrogen-bond donor in contrast to the Q and N side-chains which may engage in hydrogen-bond donor/acceptor interactions and thereby compensate for normal H16 function. This interpretation is consistent with NMR analyses of hEGF solution structure, which predict the following hydrogen-bonding interactions: V34-H16(CO), H16(H^{δ})-Y44(CO), and Cδ/Cϵ protons of H16 with the Cϵ protons of Y37 [Hommel et al., 1991, 1992].

The role of H16 of hEGF in receptor-ligand interaction is revealed by the following: (i) The pK_a value for H16 in hEGF is similar to that of H18 in hTGFα suggesting *common* modes of EGFR recognition. (ii) The binding data indicate the requirement for a polar, isosteric side-chain at position 16 of hEGF that functions as both hydrogen-bond donor and acceptor. The reduced binding affinity of the H18K hTGFα mutant [Defeo-Jones et al., 1989] suggests that H18 in hTGFα may play a similar role. (iii) As the H16 mutants are similar to wild-type hEGF in their native conformation (Fig. 1), the observed loss or gain of function arises from the differential ability of these mutants to alter contacts that promote optimal receptor recognition.

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