Journal of Cellular Biochemistry 46:242–249 (1991)

Mutational Analysis of Leucine 47 in Human Epidermal Growth Factor

Risë K. Matsunami, Margaret L. Yette, Audrey Stevens, and Salil K. Niyogi

The Protein Engineering and Molecular Mutagenesis Program, (M.L.Y., A.S., S.K.N.) and The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, (R.K.M.), Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077

Abstract Seven site-specific mutants (including changes to other hydrophobic, charged, and heterocyclic amino acids) of leucine 47 of human epidermal growth factor (EGF) were generated by protein engineering and characterized for their activity in three assays: radioreceptor competition binding in membrane fractions, the stimulation of the EGF receptor's tyrosine kinase activity, and the stimulation of thymidine uptake in tissue culture cells. $K_{½}$ (concentration required for half maximum response) values for each of the mutants are reported in the three assays. The results show that the native leucine residue is quite important for EGF activity. Substitutions are tolerated to different degrees, depending upon hydrophobicity and size of the side chain. Substitution with ionic residues led to the most drastic reduction in activity. One-dimensional nuclear magnetic resonance spectroscopy, at physiological pH, of several of the mutants did not detect any major structural perturbations which would account for the loss of activity. The results suggest that the side chain of leucine 47, because of its charge neutrality, size, and hydrophobicity, is highly important, although not absolutely essential for the interaction of EGF with its receptor. A striking finding was the lower (compared with wild type) V_{max} values of the mutants in the tyrosine kinase reaction, but these low V_{max} mutants, in cell culture experiments, were able to stimulate at high concentrations a growth response equivalent to wild type EGF.

Key words: hEGF, site-directed mutagenesis, receptor affinity, receptor kinase, mitogenesis, NMR

EGF is a 6 kDa (53 amino acid residues) polypeptide with three internal disulfide bonds. High-affinity binding to its specific cell surface receptor (the EGF receptor) activates the receptor's protein-tyrosine kinase activity, thereby unleashing a cascade of biochemical events leading to the stimulation of DNA synthesis and cell proliferation.

Several amino acids in the EGF molecule are highly conserved through different species and in EGF-like molecules known to interact with the EGF receptor (TGF-α, VGF, SFGF, MGF, etc.,—see [1,2] for reviews) signifying their possible importance for growth factor activity. The leucine residue at position 47 is one of these highly conserved residues and was shown to be necessary for EGF activity, first in carboxy-terminal deletion studies [3,4], and later through mutagenesis studies with hEGF [5,6], and mEGF [7,8]. Results from these studies have suggested that the leucine residue may be "essential" for biological activity. In the present study we have further examined Leu-47 of hEGF through a series of seven mutations (isoleucine, alanine, glycine, proline, histidine, aspartic acid, and arginine) to test the type of residue, i.e., hydrophobic, heterocyclic, or charged, that might be tolerated at this position, and an analysis has been done of different parameters of interaction of the mutant protein with the EGF receptor.

Abbreviations used: EGF, epidermal growth factor; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; TGF-α, transforming growth factor alpha; hTGF-α, human transforming growth factor alpha; VGF, vaccinia growth factor; SFGF, shope fibroma growth factor; MGF, myxoma growth factor; MEM, minimal essential medium; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; BSA, bovine serum albumin; TCA, trichloroacetic acid; 1D, one dimensional; 2D, two dimensional; TGF-β, transforming growth factor beta.

Received December 17, 1990; accepted February 28, 1991.

Address reprint requests to Salil K. Niyogi, Biology Division, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, TN 37831-8077.

MATERIALS AND METHODS

Materials

Materials have already been described [5].

Oligonucleotide-Directed Mutagenesis

EGF mutants were generated by oligonucleotide-directed mutagenesis using protocols described earlier [5,9]. The *hEGF* gene used as a template was cloned into M13mp19 as described earlier [5]. All engineered mutations as well as the absence of any undesired genetic alterations were confirmed by sequencing (10) the entire *hEGF* coding region.

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 [5]. It was found that the addition of 1 to 5 μg/ml of chloramphenicol to the growth medium during the induction phase greatly increased EGF yields (Engler, D.A., Ph.D. thesis); therefore, this was used routinely. Purification was carried out as previously described [5].

Membrane Preparation

Membrane-bound EGF receptors were prepared using a modification of the procedure described by Akiyama et al. [11]. Confluent A431 (human epidermoid carcinoma) cell cultures (grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) were washed twice with ice-cold PBS and removed from the surface of the plates using a rubber policeman. The cell suspension was kept on ice, the cells were allowed to settle, and the buffer was discarded. The cells were rinsed with several volumes of hypotonic KMP buffer (20 mM Pipes, pH 7.2, 1 mM $MgCl_2$, and 5 mM KCl), placed on ice, allowed to settle, and the buffer again was discarded. The cells were allowed to swell in 4 volumes of KMP buffer and homogenized by 25–30 strokes in a glass Dounce homogenizer. The cell lysate was centrifuged at 15,000*g* for 5 min at 4°C; the pellet was resuspended in KMP buffer, centrifuged, and the supernatants combined. Cell lysis and fractionation were monitored by microscopic examination. The combined supernatant was centrifuged at 100,000*g* for 30 min at 4°C and the membrane pellet was resuspended in 20 mM Hepes, pH 7.4, containing 0.1% BSA, for use in the receptor binding assays.

Soluble Receptor Isolation

A431 cell membranes were resuspended in solubilization buffer (40 mM Hepes, pH 7.2, 0.5 M NaCl, 10% glycerol (v/v), 1 mM EGTA, and 10 μg/ml leupeptin) and the receptor solubilized by addition of Triton-X-100 to a concentration of 1% (v/v). The membrane suspension was kept at room temperature for 20 min followed by centrifugation for 30 min at 100,000*g*. The soluble fraction was loaded onto a wheat germ agglutinin-agarose column, equilibrated in solubilization buffer containing 0.05% Triton-X-100, and the EGF receptor eluted with 0.3 M N-acetylglucosamine. Active receptor fractions were stored at −80°C for use in the tyrosine kinase stimulation assays.

Radioreceptor Competition Binding Assay

The binding of EGF to it's receptor was measured using the radioreceptor competition method described by Carpenter [12] and is described briefly below. [^{125}I]hEGF was prepared by the chloramine-T method [13] to an average specific activity of ~150,000 cpm/pmol. The receptor-containing A431 membrane preparation (approximately 0.2 μg/ml total membrane protein) was incubated with [^{125}I]EGF in a mixture containing 20 mM Hepes, pH 7.4, 0.1% BSA, and the unlabelled competing hEGF species. The mixtures were allowed to reach equilibrium, after which the receptor-bound [^{125}I]EGF was collected on cellulose acetate filters (Millipore GVWP). Unbound ligand was removed by washing filters with 20 mM Hepes, pH 7.4, containing 0.1% BSA. Filters were dried and the radioactivity quantitated by liquid scintillation spectrometry.

Receptor Tyrosine Kinase Stimulation Assay

The rate of EGF receptor-catalyzed incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic polypeptide substrate $(Glu_4, Tyr_1)_n$ (Sigma Chemical Co.) was measured as a function of growth factor concentration. Solubilized and partially purified EGF receptors, prepared as described above from A431 cell membranes, were preincubated with increasing concentrations of wild type or mutant EGF protein for 15 min at room temperature under conditions similar to those described by Koland and Cerione [14]. With 0.25

M $(NH_4)_2SO_4$ and 0.05% Triton-X-100 in the assay mixture, the receptor kinase activity was strongly EGF-dependent, as observed by Koland and Cerione [14]. The tyrosine kinase reaction was initiated by the addition of [γ-^{32}P]ATP (0.5 Ci/mmol) and $(Glu_4, Tyr_1)_n$ substrates to final concentrations of 75 μM and 0.5 mg/ml, respectively, in a final volume of 0.1 ml. The complete reaction mixture was incubated at room temperature for 10 min and the reaction stopped by addition of 1 ml of 5% TCA containing 10 mM sodium pyrophosphate. The acid-insoluble material was collected on 25 mm Millipore HAWP filters, washed extensively with the same solvent, dried, and the incorporated radioactivity quantitated by liquid scintillation spectrometry. Greater than 98% of the acid-insoluble radioactivity was found to be incorporated into the exogenously added $(Glu_4, Tyr_1)_n$ substrate under these conditions.

Thymidine Uptake Assay

Stimulation of mitosis was measured by the uptake of [^{3}H]thymidine in a BALB/c 3T3 fibroblast cell line, A31, obtained from the laboratory of Dr. John Cook (Biology Division, Oak Ridge National Laboratory). The procedure followed was a modification of one described by Carpenter and Cohen [15] and was developed by Dr. Melinda Hauser (Biology Division, Oak Ridge National Laboratory). Cells were grown and maintained in MEM Alpha medium (Hazleton Biological Co.) supplemented with 10% fetal calf serum (Gibco), penicillin (100 U/ml), streptomycin (1000 Meq/ml), and 2 mM glutamine. Cells were divided into 6-well, 35 mm dishes and allowed to grow to confluence, after which time they were starved for 5–7 days with no additional feedings. Purified hEGF proteins were added at different concentrations and the cells were incubated for 20–22 h, at which time [^{3}H]thymidine was added (specific activity 0.4 Ci/mmol) to a final concentration of 5 μM. After incubation at 37°C for an additional 4 h, the medium was siphoned off and the cells were washed two times with one ml of PBS. Cells were rinsed with 0.025% trypsin containing 0.02% EDTA and incubated at 37°C to allow the cells to detach from the dishes. Cells were resuspended in 2 ml of ice-cold PBS. One milliliter (or less) was diluted with 10 ml of Isoton II (Coulter Diagnostics) and the number of cells determined using a Coulter counter. One milliliter was filtered on Whatman GF/C glass filter disks and washed with 10% TCA containing 0.01 M sodium pyrophosphate. The filters were dried, placed in a liquid scintillant, and counted. The amount of ^{3}H label per cell was calculated, and the values as a percent of maximum stimulation were determined.

NMR Studies

Samples for proton NMR spectra were prepared by first dialyzing the purified proteins extensively against water and lyophilizing to dryness. Samples were resuspended in D_2O to a final concentration of approximately 1.5 mM and the pH was adjusted to 7.18 ± 0.02, with no correction on the pH reading for isotope effects. All labile protons were allowed to exchange for deuterons for at least 3 h at 22°C before spectral analysis. NMR spectra were acquired at 30°C on a Varian VXR 500 (500 MHz) spectrometer. 1D spectra were collected using the pulsed Fourier mode with 512 scans per spectrum, and with pre-irradiation of the residual solvent resonance.

RESULTS

Table 1 shows the oligonucleotide primers that were synthesized in order to produce the mutant replicative form DNAs. All oligonucleotides were 18 bases long with one or two mispairings, and all destroyed the *Afl*II restriction site; this provided a convenient assay, besides differential hybridization, for screening mutants (cf. reference 5). The desired mutations as well as the absence of any other genetic alterations were confirmed by sequencing [10] the entire hEGF coding region.

TABLE I. Features of Position 47 Mutagenic Oligonucleotides*

Mutation	Sequence
Wild Type Sequence (Leu)	5′-TAC CGT GAC CTT AAG TGG-3′
Gly	5′-TAC CGT GAC **GG**T AAG TGG-3′
Ala	5′-TAC GCT GAC **GC**T AAG TGG-3′
Ile	5′-TAC CGT GAC **A**TT AAG TGG-3′
Pro	5′-TAC CGT GAC **C**CT AAG TGG-3′
Asp	5′-TAC CGT GAC **GA**T AAG TGG-3′
Arg	5′-TAC CGT GAC **CG**T AAG TGG-3′
His[a]	5′-TAC CGT GAC C**A**T AAG TGG-3′

*Mismatches are underlined and boldface.
[a]Described earlier [5].

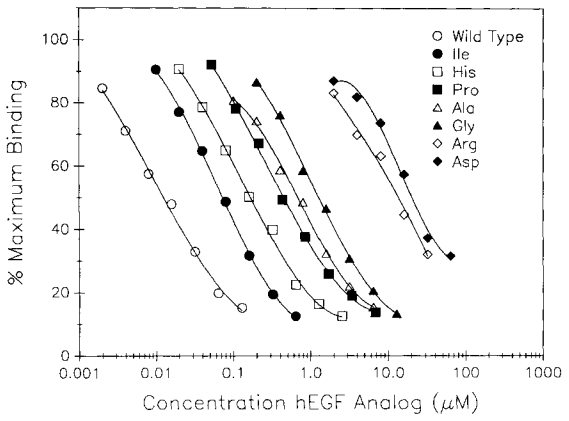

Fig. 1. Radioreceptor competition binding curves of ^{125}I-labeled hEGF vs. wild type and mutant EGFs.

Radioreceptor Competition Binding Assay

The hEGF mutants were assayed for their ability to compete with ^{125}I-labeled wild type hEGF in binding to the EGF receptor. Binding curves are shown in Figure 1 and are plotted as hEGF analog concentration vs. the percentage of maximum binding, with the maximum wild type [^{125}I]hEGF binding in the absence of cold hEGF protein representing 100%. The curves presented are an average of two or more experiments. The IC_{50} value, or the concentration of protein needed to compete with 50% of the labeled EGF, is reported in Table 2 for each mutant, as well as its relative receptor affinity compared with wild type. The most conservative change to an isoleucine lowered binding to 16.9% that of wild type. As the side chain is shortened to an alanine or glycine, the binding was further diminished to 1.8% or 0.9%, respectively. The charged amino acids, arginine and aspartic acid, were the least tolerated mutations, leading to receptor affinities of less than 0.1% of wild type. Replacement with heterocyclic residues, proline and histidine, reduced receptor binding to 2.3% and 7.2%, respectively.

Stimulation of Receptor Tyrosine Kinase Activity

Five of the mutants were assayed for their ability to stimulate the EGF receptor's protein-tyrosine kinase activity, as measured by the amount of ^{32}P label (from [γ-^{32}P] ATP) incorporated into the exogenously added $(Glu_4,Tyr_1)_n$ substrate. Figure 2 shows the phosphorylation curves with wild type hEGF and the Leu-47 mutants plotted as concentration of hEGF protein vs. ^{32}P incorporated. A striking observation is that the maximum velocity of the reactions obtained with the mutants is lower than that with wild type hEGF. This is a phenomenon we have observed with many, though not all, of lower binding mutants produced in our laboratory, and a more detailed study is described in Matsunami et al. [16]. Table 2 lists the V_{max} values of the mutants and how they compare with wild type. The aspartic acid and arginine mutants could not be assayed in this manner due to their extremely low receptor affinities;

TABLE II. Biochemical Properties of Leucine 47 hEGF Mutants

	Radioreceptor competition assay		Tyrosine kinase stimulation assay				Tissue culture [^{3}H]thymidine uptake	
Residue at position 47 of hEGF	IC_{50} μM	Relative Receptor Affinity[a]	EC_{50} μM	Relative receptor affinity[b]	V_{max} cpm × 10^{-3}	Relative stimulation[c]	ED_{50} nM	Relative mitogenic activity[d]
Wild-type (Leu)	0.012	100	0.1	100	137	100	0.19	100
Ile	0.072	16.9	0.62	16.1	68	50	0.43	44.2
His	0.17	7.2	1.1	9.1	60	44	1.87	10.2
Pro	0.44	2.3	2.9	3.4	60	44	0.84	22.6
Ala	0.69	1.8	3.4	2.9	28	20	0.76	25.0
Gly	1.3	0.94	3.7	2.7	15	11	2.55	7.4
Asp	13.8	0.09	nd[e]	nd	nd	nd	47.0	0.4
Arg	20.0	0.06	nd	nd	nd	nd	6.1	3.1

[a]Relative receptor affinity = IC_{50}(wild type)/IC_{50}(mutant) × 100%. Values were derived from Figure 1.
[b]Relative receptor affinity = EC_{50}(wild type)/EC_{50}(mutant) × 100%. Values were derived from Figure 3.
[c]Relative stimulation = V_{max}(mutant)/V_{max}(wild type) × 100%. Values were derived from Figure 2.
[d]Relative mitogenic activity = ED_{50}(wild type)/ED_{50}(mutant) × 100%. Values were derived from Figure 4.
[e]nd = not determined.

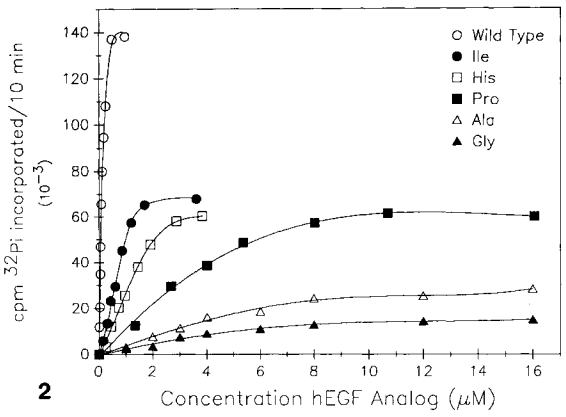

Fig. 2. Dose-response curves for stimulation of EGF receptor tyrosine kinase activity by wild type and mutant EGFs.

the kinase reaction was unreliable at protein concentrations above 15–20 μM.

To determine the EC_{50} value (the concentration of mutant protein that gives 50% maximum stimulation of the kinase reaction), the phosphorylation curves were plotted as percent of maximum stimulation and are shown in Figure 3. The EC_{50} values, as well as the relative binding affinities of the various hEGF analogs, are shown in Table 2. The order in which the mutants stimulate the tyrosine kinase activity follows the same order as found in the radioreceptor binding assay, and their relative binding affinities are also similar, differing by less than 2%.

Stimulation of Thymidine Uptake in Cell Culture

All seven mutants were assayed for their ability to stimulate mitosis in a fibroblast cell line as measured by [^{3}H]thymidine incorporation. The cells incorporated [^{3}H]thymidine in response to EGF in a concentration-dependent manner, as shown by the curves in Figure 4. The results plotted are an average of 3 or more experiments. The effective dose, ED_{50}, or the concentration needed for half maximal stimulation of thymidine uptake for wild type and each mutant are shown in Table 2, as is their relative percentage compared with wild type. The mutants tended to follow the trend seen in the binding and phosphorylation assays with isoleucine being the best at 44%, and aspartic acid and arginine the worst at 0.4% and 3.1%, respectively. All the mutants, in general, tended to be more active in their response (as a percentage of wild type) in this assay as compared to the in vitro receptor binding and phosphorylation assays.

NMR Spectra of Proteins

NMR spectra for wild type and three mutants, isoleucine, alanine and glycine, were recorded, and plots of the downfield region (from 5.0–8.5 ppm) of these spectra, which show the resonances for the protons from aromatic residues, are shown in Figure 5. The spectra are arranged from bottom to top in the order of best binding to worst binding hEGF proteins. These data suggest that there are no major structural perturbations between wild type and the mutant proteins, but some slight changes are seen in the peaks around 6.6 ppm and at 7.4 ppm. The spectra tend to look less like "wild type" as the binding affinities decrease, but still contain most of the wild type structure. Assignments for the peaks are not yet determined.

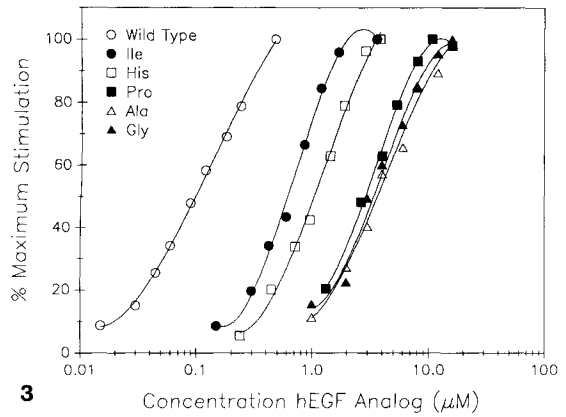

Fig. 3. Stimulation of the EGF receptor tyrosine kinase activity plotted as a percent of maximum stimulation.

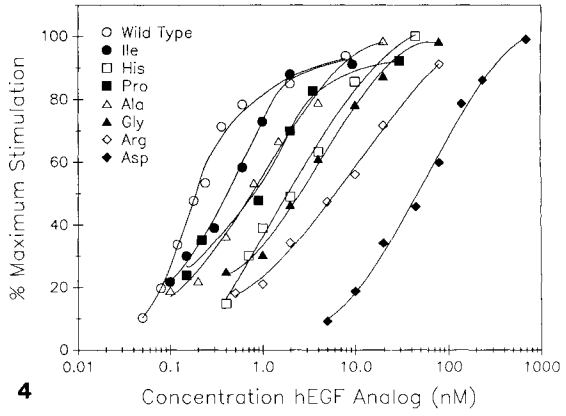

Fig. 4. Dose-response curves for the uptake of [^{3}H]thymidine in cell culture.

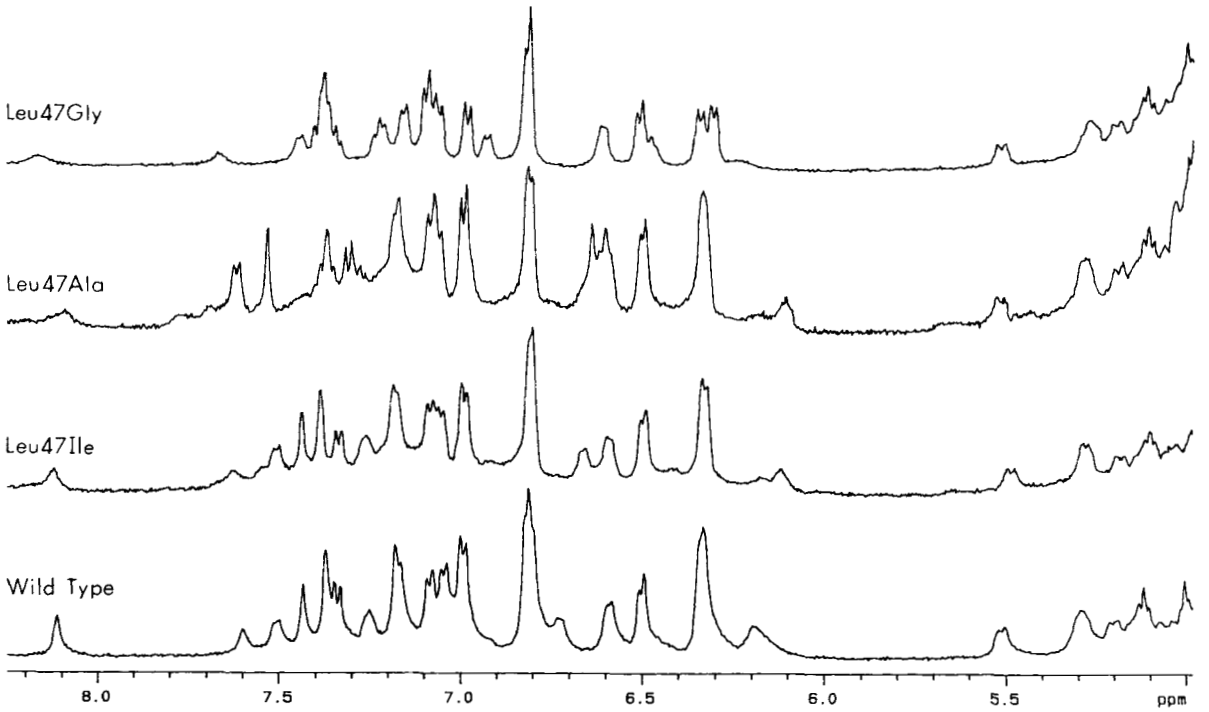

Fig. 5. Comparison of the downfield resonances of 1D ^{1}H-NMR spectra of wild type and leucine 47 mutants. All spectra represent the average of 512 scans on a Varian VXR500 spectrometer under the conditions described in the text.

DISCUSSION

The results of the three assays, namely, receptor binding, stimulation of receptor tyrosine kinase activity, and stimulation of mitosis, suggest that the leucine residue at position 47 of hEGF is necessary for full receptor affinity and activation of other biological responses, but the residue is not absolutely needed for activity, since other mutations are tolerated. The change to a charged residue (namely, aspartic acid or arginine) is the least tolerated, however, indicating that there may be some type of hydrophobic rather than ionic interaction between EGF and its receptor in this region of the ligand.

In order to determine whether or not the lower receptor affinities of the EGF mutants were due to structural changes in the molecule, 1D proton NMR analysis was performed on the isoleucine, alanine, and glycine variants. These spectral analyses were done at physiological pH. It is possible, by comparing peak resonances in the 1D spectra of mutants and wild type, to determine if there are any gross structural changes in the molecule, as demonstrated by Moy et al. [8], who showed that the 1D spectrum is a reliable indicator of structural changes that are seen in followup analysis using 2D NMR spectroscopy. The reason for choosing isoleucine, alanine, and glycine mutants for NMR analysis was to ascertain whether or not any loss of structural integrity of the molecule occurred due to chain shortening and loss of hydrophobicity and whether or not this could be correlated with the loss of activity. This does not appear to be the case. It should be noted that the overall structure is still well preserved and that structural perturbations are not the cause for lower biological activity. Rather, the loss of activity is due to alteration, namely, the diminution of the hydrophobicity, of the sidechain at position 47.

The lower V_{max} values of the mutants, as compared with wild type, in the phosphorylation assay did not appear to have any effect on the stimulation of mitosis under the assay conditions used here, but this may not mean that the V_{max} values are of no significance. The low V_{max} of the mutants may affect the kinetics of the mitotic response or it may produce a change in one or more of the EGF-stimulated pathways. Although time courses were not run, other investigators have reported that cells need to be exposed at least eight hours to EGF before they are committed to stimulation of mitosis

[15,17,18]. The mutants with the lower V_{max} values may require more time than wild type to commit to mitogenesis, and preliminary work in this laboratory with other low V_{max} mutants indicate that this may be the case (Melinda Hauser, personal communication). It has been reported that only a small percentage of the EGF receptors need to be occupied for full biological effect [19,20]; thus, the binding of some of the mutant ligands could be sufficient to trigger mitogenesis. Studies of the low V_{max} mutants in whole cell phosphorylation reactions show some quantitative differences in the phosphorylation of some proteins (Melinda Hauser, unpublished results), signifying the possible importance of the full kinase response in other cellular pathways.

Site-specific mutagenesis studies of mEGF in which serine and valine variants were made at position 47, were performed by Ray et al. [7]. The receptor affinities, based on radioreceptor competition and receptor autophosphorylation assays were 20–33% for the valine and 6–14% for the serine mutant, as compared with wild type. In a follow-up paper, Moy et al. [8] determined the 1D and 2D NMR spectra of the serine mutant, and results indicated no significant structural change in this mutant as compared to wild type.

Dudgeon et al. [6] very recently reported on the mutagenesis of the Leu-47 site in hEGF. Using a yeast expression system, they purified proteins that were 52 amino acids in length, lacking residue #53. Four variants were created, changing the leucine to an alanine, glutamic acid, aspartic acid, and valine. Results from the competition binding assay were 14% of wild type for the valine mutant, and the other 3 mutants had about 2% of wild type activity. These results agree with the results from the mutagenesis studies presented here, including two of the same changes—alanine and aspartic acid. Dudgeon et al. also studied the 1D NMR spectra of their four variants (although these studies were done at the non-physiological pH 3.0) and found, in agreement with our results, that there were only minor differences in the spectra. They therefore assumed that the overall structure was still intact.

The corresponding leucine residue in TGF-α (Leu-48) was mutated to an isoleucine, alanine, and methionine in a yeast expression system, and these variants were reported as having low to no activity in both competition binding assays and the ability to stimulate the formation of colonies in soft agar in the presence of TGF-β [21]. Although this conclusion generally supports the results obtained with Leu-47 in hEGF and mEGF, the data upon which they were based are questionable. The TGF-α used was not highly purified and in the quantification of the protein it was assumed that all the protein detected was properly folded, which may not have been the case.

The importance of the leucine 47 residue for the biological activity of EGF and EGF-like molecules is now well established both by the present study and work of others. The fact that it is highly conserved lends credence to its importance. It is apparent that the leucine residue itself, possibly because of its size and hydrophobic character, is necessary for full EGF activity, since even rather conservative changes (to a valine or an isoleucine) decrease the activity to less than one third that of wild type. Thus, it appears that Leu-47, although highly important, is not absolutely essential. Recent studies with an EGF-like molecule, amphiregulin, further support this tenet. Amphiregulin, an 84-amino acid glycoprotein, is a bifunctional cell growth modulator [22]. The carboxy-terminal half of amphiregulin (residues 46–84) bears a 38% homology to hEGF with the correct alignment of cysteines, but its sequence ends at residue 45 of hEGF, thus the leucine 47 residue is missing. Despite this truncation, amphiregulin is able to bind to the EGF receptor, although with 10% of the affinity of EGF.

Recent studies in our laboratory have indicated a nearly absolute requirement for the guanidinium group of Arg-41 of hEGF in receptor binding [23]. Other residues found to be functionally important are two hydrophobic residues in the amino terminal domain, namely, Ile-23 and Leu-26 [24], further suggesting the importance of hydrophobic EGF-receptor interactions. In addition, mutations at positions 23 and 26, similar to those at position 47, produced hEGF analogs with low V_{max} values in the receptor tyrosine kinase reaction. These mutants, similar to the mutants at position 47, inhibited the stimulation by wild type hEGF of the receptor tyrosine kinase activity [16]. These results indicate a partial "uncoupling" of EGF binding and receptor tyrosine kinase stimulation. It is the latter activity that triggers the cascade of biochemical events leading to cell proliferation. Our results suggest that the activated receptor-

growth factor complexes formed with the low V_{max} mutants have a conformation somewhat altered from the complex formed with wild-type EGF. Studies of the structure of the complexes formed between these EGF analogs and the receptor may provide evidence of altered receptor conformation. The biological consequences of the partial uncoupling between receptor binding and receptor activation are currently under study.

ACKNOWLEDGMENTS

We thank Dr. G. T. Montelione, Rutgers University, Piscataway, N.J., for collection, interpretation, and valuable discussion of NMR data. The research was sponsored by the Office of Health and Environmental Research, United States Department of Energy under contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc., and was supported by U.S. Public Health Service grant CA 50735 (to S.K.N.). R.K.M. was supported by National Cancer Institute predoctoral training grant CA 09104 to the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences.

REFERENCES

1. Carpenter G, Wahl MI: Handb Exp Pharmacol 95 (vol 1):69–171, 1990.
2. Rees DJG, Jones IM, Handford PA, Walter SJ, Esnouf MP, Smith KJ, Brownlee GG: EMBO J 7:2053–2061, 1988.
3. Burgess AW, Lloyd CJ, Smith S, Stanley E, Walker F, Fabri L, Simpson RJ, Nice EC: Biochemistry 27:4977–4985, 1988.
4. Gregory H, Thomas CE, Young JA, Willshire IR, Garner A: Regul Pept 22:217–226, 1988.
5. Engler DA, Matsunami RK, Campion SR, Stringer CD, Stevens A, Niyogi SK: J Biol Chem 263:12384–12390, 1988.
6. Dudgeon TJ, Cooke RM, Baron M, Campbell ID, Edwards RM, Fallon A: FEBS Lett 261:392–396, 1990.
7. Ray P, Moy FJ, Montelione GT, Liu J, Narang SA, Scheraga HA, Wu R: Biochemistry 27:7289–7295, 1988.
8. Moy FJ, Sheraga HA, Liu J, Wu R, Montelione GT: Proc Natl Acad Sci USA 86:9836–9840, 1989.
9. Niyogi SK, Foote RS, Mural RJ, Larimer FW, Mitra S, Soper TS, Machanoff R, Hartman FC: J Biol Chem 261:10087–10092, 1986.
10. Sanger F, Nicklen S, Coulson AR: Proc Natl Acad Sci USA 74:5463–5467, 1977.
11. Akiyama T, Kadooka T, Ogawara H: Biochem Biophys Res Commun 131:442–448, 1985.
12. Carpenter G, Methods Enzymol 109:107–108, 1985.
13. Hunter WM, Greenwood FC: Nature 194:495–496, 1962.
14. Koland JG, Cerione RE: J Biol Chem 263:2230–2237, 1988.
15. Carpenter G, Cohen S: J Cell Physiol 88:227–238, 1975.
16. Matsunami RK, Campion SR, Niyogi SK, Stevens A: FEBS Lett 264:105–108, 1990.
17. Carpenter G, Cohen S: Annu Rev Biochem 48:193–216, 1978.
18. Aharonov A, Pruss RM, Herschman HR: J Biol Chem 253:3970–3977, 1978.
19. Wiley HS, Walsh BJ, Lund KA: J Biol Chem 264:18912–18920, 1989.
20. Ruddon RW: In "Cancer Biology." New York: Oxford University Press, 1987, pp 244–273.
21. Lazar E, Watanabe S, Dalton S, Sporn MB: Mol Cell Biol 8:1247–1252, 1988.
22. Shoyab M, Plowman GD, McDonald VL, Bradley JG, Todaro GJ: Science 242:1074–1076, 1989.
23. Engler DA, Montelione GT, Niyogi SK: FEBS Lett 271: 47–50, 1990.
24. Campion SR, Matsunami RK, Engler DA, Niyogi SK: Biochemistry 29:9988–9993, 1990.