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**STRUCTURE-FUNCTION ANALYSIS OF THE HIGHLY CONSERVED LEUCINE 47
RESIDUE OF HUMAN EPIDERMAL GROWTH FACTOR AND STUDIES
OF THE RECEPTOR'S PHOSPHORYLATION ACTIVITY**

A Dissertation

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DEDICATION

This thesis is dedicated to my children Alexander and Sabrina, and to my husband,
David, without whose support this dissertation would not be possible.

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ABSTRACT

The gene for human epidermal growth factor was synthesized and placed behind the gene coding for the signal sequence of *E. coli* alkaline phosphatase. The chimeric gene was then placed in an expression vector behind the *tac* promoter and under the control of the *lac* repressor/operator and transformed into *E. coli* JM107. This construct allows for the expression of human epidermal growth factor and secretion of the protein into the periplasmic space of *E. coli*. Protein of correct length, and properly folded was isolated and found to be fully active in several biological assays: binding to the EGF receptor, stimulation of the receptor's tyrosine kinase, and stimulation of thymidine uptake in tissue culture cells.

Site-directed mutagenesis was carried out at two conserved sites within the EGF molecule. The proline residue at position 7 was changed to a threonine and was found to have about 50% of the wild type activity in receptor binding and in tyrosine kinase stimulation. The leucine residue at position 47 was changed to an isoleucine, histidine, proline, alanine, glycine, aspartic acid, and arginine. The activities for these mutants in the receptor binding assays ranged from 17% to .06% of wild type with the order from best to worst as listed above. Results from the tyrosine kinase stimulation were similar to the binding results. Stimulation of thymidine uptake ranged from 44% to 0.4% of wild type, in approximately the same order. Three of the leucine mutants, isoleucine, alanine, and glycine, were examined for their structural integrity using nuclear magnetic resonance (NMR) spectroscopy. One dimensional proton NMR showed little structural perturbations in these mutants.

The EGF receptor's tyrosine kinase activity was investigated more fully. Several

tyrosine-containing synthetic polymers were tested for their ability to act as substrates for the EGF receptor tyrosine kinase. Polymers poor in tyrosine were found to be good substrates, while polymers rich in tyrosine were found to inhibit the phosphorylation of the tyrosine poor polymers. This inhibition appears to be of a mixed type. Tyrosine-rich polymers also inhibited receptor autophosphorylation.

Several mutants of EGF were found to have a lower $V_{\max}$ in the tyrosine kinase phosphorylation assay. These mutants were further examined and were shown to competitively inhibit the wild type protein in its ability to stimulate the receptor's tyrosine kinase activity.

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ABBREVIATIONS

Amino acids = Ala or A, alanine	His or H, histidine
Arg or R, arginine	Ile or I, isoleucine
Asn or N, asparagine	Leu or L, leucine
Asp or D, aspartic acid	Lys or K, lysine
Asx, asparaginy or aspartyl	Met or M, methionine
Cys or C, cysteine	Phe or F, phenylalanine
Gln or Q, glutamine	Pro or P, proline
Glu or E, glutamic acid	Ser or S, serine
Gly or G, glycine	Thr or T, threonine
Glx, glutaminy or glutamyl	Trp or W, tryptophan
	Tyr or Y, tyrosine
	Val or V, valine

ATP = adenosine 5'-triphosphate

BSA = bovine serum albumin

cDNA = copy deoxyribonucleic acid

cpm = counts per minute

DNA = deoxyribonucleic acid

DTT = dithiothreitol

E. coli = *Escherichia coli*

EDTA = ethylenediaminetetraacetic acid

EGF = epidermal growth factor; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; rEGF, rat epidermal growth factor

EGTA = ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid)

hEGF = gene for human epidermal growth factor

HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC = high performance liquid chromatography

IPTG = isopropyl- β -D-thiogalactopyranoside

kD = kilo Dalton

K_m = Michaelis-Menten constant

M_r = relative molecular weight

MGF = myxoma virus growth factor

MHz = mega Hertz

NMR = nuclear magnetic resonance; 1D ^1H -NMR, one-dimensional proton nuclear magnetic resonance; 2D NMR, two-dimensional nuclear magnetic resonance

NOE = nuclear Overhauser effect

PBS = phosphate buffered saline

PCV = packed cell volume

phoA signal = alkaline phosphatase signal peptide

phoA signal = gene for alkaline phosphatase signal peptide

PIPES = piperazine-N,N'-bis[2-ethanesulfonic acid]

PMSF = phenylmethylsulfonyl fluoride

RF = replicative form

SDS = sodium dodecyl sulfate

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFGF = Shope fibroma virus growth factor

ss = single stranded

TCA = trichloroacetic acid

T_d = temperature of dissociation

TGF- α = transforming growth factor alpha

Tris = Tris(hydroxymethyl)aminomethane

VGF = vaccinia virus growth factor

$V_{\max}$ = maximum velocity

CHAPTER 1

EPIDERMAL GROWTH FACTOR AND ITS RECEPTOR

Review of the Literature

The regulation of cell growth is controlled by a variety of pathways within the cell, each interacting in some way with the other to create a balanced environment conducive for normal cell growth and maintenance. One of these pathways through which cell growth is regulated is that mediated by growth factors. Growth factors are defined as low molecular weight proteins, 6000-30,000 daltons, each interacting with high affinity and specificity to a cell surface receptor which in turn activates a second messenger, usually through an endogenous protein kinase activity, that will eventually alter cell growth. Growth factors differ from hormones in that they do not have to be released into the bloodstream in an endocrine fashion, but can act by either autocrine, paracrine, or endocrine mechanisms, depending upon the cell type and the growth factor produced. Epidermal growth factor (EGF) is one member of this growth factor family.

EGF, a 53 amino acid molecule containing three disulfide bonds, binds to its specific cell surface receptor, activating the receptor's kinase domain which phosphorylates itself as well as other protein molecules in the cell on tyrosine residues. Ultimately the binding of EGF can lead to increased DNA synthesis and cell growth. EGF was first discovered by Stanley Cohen in 1960 as a contaminant in a nerve growth factor extract isolated from mouse submaxillary glands (1). Injection of this extract into newborn mice caused early incisor eruption and precocious eyelid opening. Histologic examination of tissue samples from these mice showed a thickening of the epidermal layer of the skin and increased keratinization (2), thus it was called epidermal growth factor.

In 1975 a protein was isolated from human urine that had the same properties as mouse EGF and was thus thought to be the human equivalent to mouse EGF (3). At the same time, Gregory (4) had isolated and purified, also from human urine, a protein that inhibited gastric acid secretion and promoted proliferation and epithelialization of gastric mucosa. He called this substance β -urogastrone. Amino acid composition comparisons of the three proteins, mEGF, human urinary EGF and β -urogastrone, showed a close homology; thus, Gregory postulated "urogastrone and human epidermal growth factor are one and the same".

The primary sequence of EGF was determined by Savage *et al.* (5) and the three disulfide bonds were placed pairing the first and third cysteines, the second and fourth cysteines and the fifth and sixth cysteines, thus creating three looped regions designated A, B and C (see Fig. 1-1 for a structural diagram of EGF). This structural motif of EGF, the pairing of the cysteines, became the standard for identifying a variety of other proteins containing "EGF-like" units; some of these proteins bind to the EGF receptor and some do not (for review see Ref. 6,7). The general pattern of cysteine distribution in the sequences of these molecules is $CX_{4-14}CX_{3-8}CX_{4-14}CXCX_{8-14}C$, where X represents any non-cysteine residue. Of the different proteins that have this motif, most of them fall into three categories: i) proteases or protease cofactors, ii) developmental proteins, and iii) proteins that interact between cells and the extracellular matrix. The presence of this motif in a variety of protein molecules may be due to its structural stability and/or may be a feature needed for mediating protein-protein interactions.

The gene for the hEGF ligand was isolated by Bell *et al.* by cDNA cloning and is found on chromosome four. The gene codes for a 1200 amino acid precursor in which only 53 residues are present in the mature ligand. A linear diagram of the precursor is

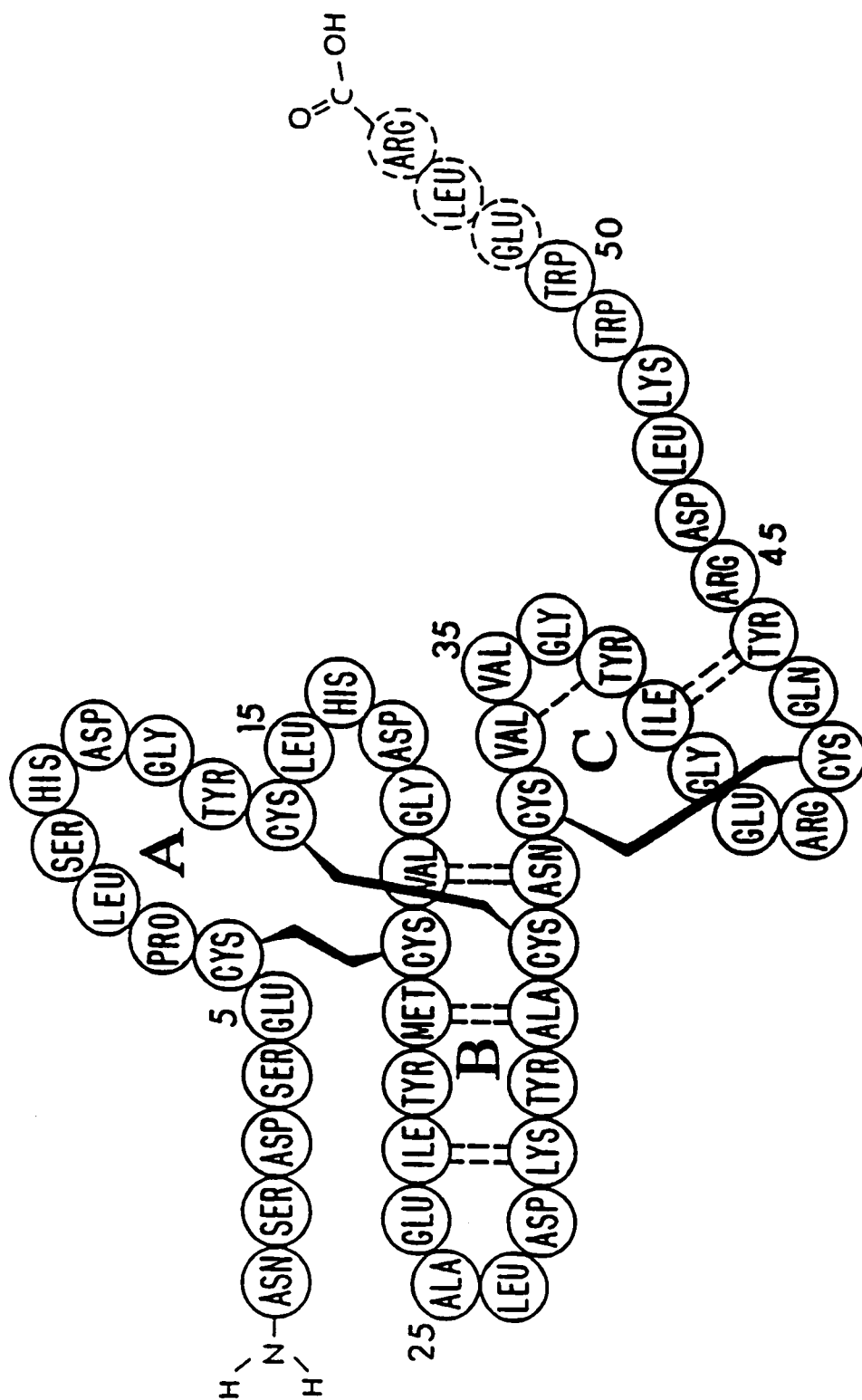


Figure 1-1. Schematic representation of secondary structural features of hEGF. Solid lines indicate disulfide bridges, and hydrogen bonds are indicated by dashed lines. Residues 51-53 are added for completeness; however, dashed circles indicate that no rigid secondary structural characteristics have been assigned to these residues.

shown in Fig. 1-2. Besides the mature EGF, which is found from amino acids 971-1023, there are eight other EGF like sequences, numbered one through eight in the figure, none of which has been isolated as an EGF-like molecule. The EGF precursor contains features, such as sequences for possible glycosylation sites, a signal sequence and a membrane spanning region, that may indicate its possible role as a receptor (9).

There have been a number of proteins isolated that resemble EGF in their sequence homology, disulfide bond arrangement, and their ability to bind to the EGF receptor. These molecules and sequences of EGFs isolated from different sources are shown in Fig. 1-3. One of these, transforming growth factor alpha (TGF- α), was first isolated from a retrovirus-transformed murine fibroblast cell line (10,11) and has since been isolated from other tumor cells as well as normal cells. Its mode of action is similar to EGF, and in most cases can replace EGF biologically, but there are distinct quantitative differences in how TGF- α can stimulate specific responses in the cell which are not yet understood (12,,13,14). A series of proteins from the pox virus family were also found to be EGF-like (6), including vaccinia growth factor (VGF), Shope fibroma growth factor (SFGF), and myxoma growth factor (MGF). Amphiregulin, another growth regulating protein, was isolated from the media of phorbol ester-treated tumor cells and it also proved to be EGF-like (15).

Recently the solution structure for mouse EGF (16,17), rat EGF (18), human EGF (19), and human TGF- α (20) have been determined using nuclear magnetic resonance spectroscopy, and all spectra show a similar polypeptide backbone structure. A representative diagram of hEGF is shown in Fig. 1-4. The N-terminal domain, amino acids 1-33, is characterized by a double stranded, anti-parallel β -sheet linking residues 19-23 and 28-32. Residues 2-4 occasionally interact with 21-23 to form a triple-stranded

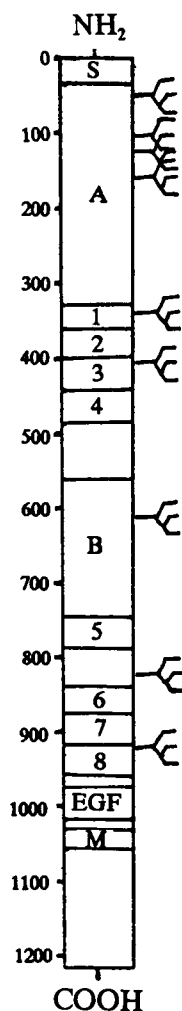


Figure 1-2. **Linear diagram for the peptide coding portion of mRNA for hEGF.** Amino acid residue numbers are placed on the left of the diagram. *Branched symbols* indicate potential glycosylation sites. *Numbers 1-8* indicate those regions possessing an EGF-like sequence. Regions *A* and *B* have homology with each other. The putative positions of the signal- and membrane-spanning regions are designated with an *S* and *M*, respectively. (Modified from reference #6.

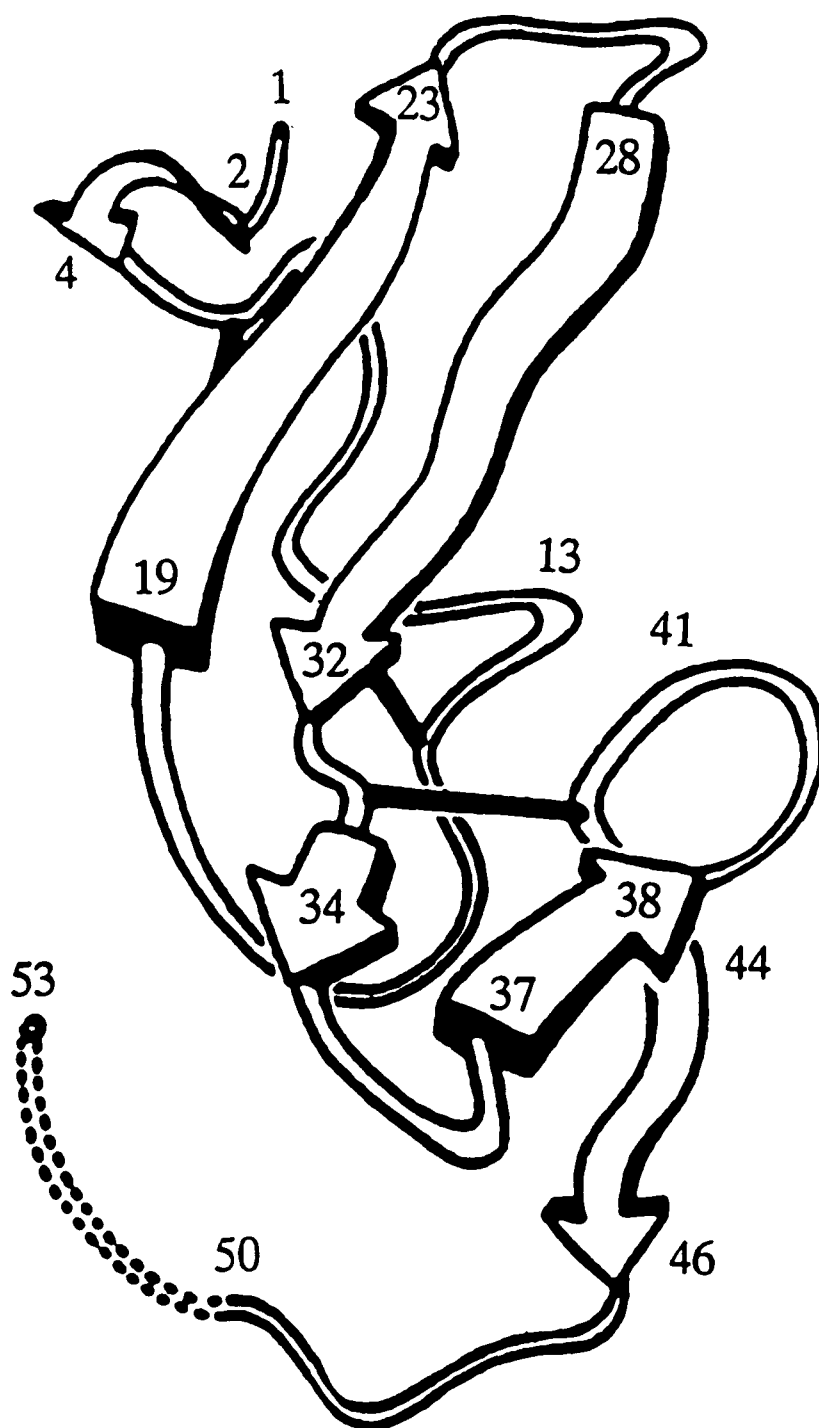


Figure 1-4. Solution structure ribbon diagram representing the polypeptide folds of EGF. Structure is derived from nuclear Overhauser effect (NOE) distance constraints. The polypeptide backbone is represented by the *rope* which broadens into *arrows* which represent the location and direction of the β -sheet strands. The *broken rope* indicates the COOH-terminal tail; residues 51-53, which lack NOE tertiary determinants. (Taken from reference #17)

sheet. A β -turn with residues 25-27 separates the β -sheet strands. Cysteines 6 and 20, and 14 and 31 form disulfide bonds which stabilize the structure. The C-terminal domain residues 34-53, includes the Cys33-Cys42 disulfide bond. Residues 34-37 form another β -turn followed by a short segment of anti-parallel β -sheet pairing positions 37 and 38 with 44 and 45. The C-terminal tail from 46 to 53 is less well defined, but there are some interactions between residues 45 and 50 with residues 35-37.

EGF is capable of eliciting a variety of physiological and cellular responses. In cell culture, cells grown in the presence of EGF proliferate even after the cultures become confluent, thus exhibiting the loss of density-dependent growth inhibition observed in transformed cells. There is no evidence to suggest that EGF *per se* is associated with either induction, promotion or invasiveness of tumor cells, but TGF- α is associated with proliferative epithelial and tumor cells (14,21,22). A variety of tumors from breast carcinomas, squamous lung cancers, glioblastomas, and immature melanomas have an amplified number of EGF receptors due to amplification or augmented transcription indicating that they may be more responsive to EGF-like growth factors (23,24,25).

Because it can act as a mitogen, EGF has been shown to be therapeutically useful. Its ability to promote healing has been demonstrated for different wound types such as: burns (26,27), corneal epithelia (28,29), diabetic ulcers (30), mid dermal wounds (31), and psoriasis (32). Because it is known to inhibit gastric acid secretion (4), it may also be useful in the treatment of ulcers. Other groups (33) have attached toxins to EGF in an effort to destroy cells that contain EGF receptors, particularly certain tumors that contain a large number of EGF receptors.

The cellular response to EGF includes both early and late events. In cell culture, events observed within 5 minutes after EGF addition are: increases in protein

phosphorylation, ion fluxes, and membrane ruffling. Responses seen between 15 and 60 minutes are: internalization of the receptor-ligand complex, increased amino acid and glucose transport, onset of RNA synthesis, and increased protein synthesis. It is not until at least eight hours of constant exposure to EGF that DNA synthesis is induced followed by cell division (for a complete review see Ref. 34,35). There is a wide variety of cellular responses to EGF that will not be discussed here, but are listed in Table 1-1.

As stated above, EGF interacts with a cell through its binding to its specific cell surface receptor (for reviews see Ref. 6,36,37). This transmembrane receptor is a monomer of M_r 170,000 (1186 amino acids) and is divided into two domains with a single transmembrane region. The entire sequence of the receptor has been determined through cDNA cloning (38). Fig. 1-5 is a linear diagrammatic representation of the structural features of the EGF receptor.

The extracellular domain, from amino acids 1-621, possesses two structural features: a high content of cysteine (approximately 10% compared to a normal abundance of 1%), and 12 possible sequences for N-linked glycosylation. The 51 cysteines are clustered into two regions and a potential for 25 disulfide linkages exists, but the actual number of linkages and how they are paired is unknown. Studies have shown that the region located between the two cysteine-rich regions is the ligand binding site (39). The actual number of N-linked glycosylation sites is unknown, but carbohydrates account for approximately 30,000 daltons of the protein mass. The oligosaccharide chains are further modified by the addition of terminal sugars (40), and these post-translational modifications of the receptor are necessary for ligand binding (41).

The transmembrane region of the receptor is composed of a stretch of 23 hydrophobic residues (amino acids 622 to 644) that are characteristic of membrane

Table 1-1

Biological Effects of Epidermal Growth Factor

In vivo:

- Accelerated proliferation/differentiation
 - of skin tissue
 - of corneal epithelial tissue
 - of lung and trachea epithelia
- Potential of methylcholanthrene carcinogenesis
- Inhibition of gastric acid secretion
- Increased activity of ornithine decarboxylase and accumulation of putrescine
- Formation of fatty liver
- Increase of disulfide group content in skin
- Hepatic hypertrophy and hyperplasia
- Potential of cleft palate

Organ cultures:

- Accelerated proliferation/differentiation
 - of skin tissue
 - of corneal epithelial tissue
 - of mammary gland epithelial tissue
- Induction of ornithine decarboxylase and accumulation of putrescine
- Enhanced protein synthesis, RNA synthesis
- Inhibition of palate fusion

Cell cultures:

- Increased transport
 - of α -aminoisobutyrate
 - of deoxyglucose
 - of K^+
 - Activation of glycolysis
 - Stimulation of macromolecular synthesis
 - of hyaluronic acid
 - of RNA
 - of protein
 - of DNA
 - Enhanced cell multiplication
 - Alteration of membrane properties
 - Stimulated hCG secretion
 - Increased biogenesis of fibronectin
 - Enhanced prostaglandin biosynthesis
 - Alteration of viral growth
 - Increased squame production by keratinocytes
-
-

Adapted from reference #34

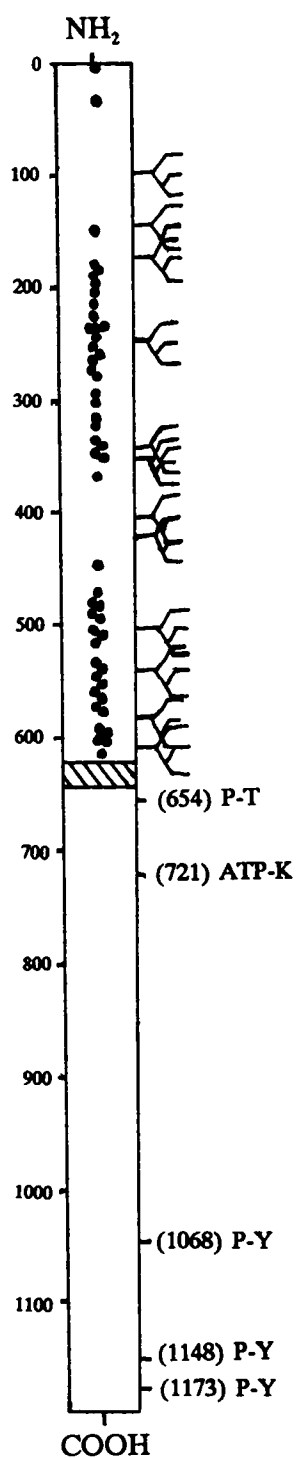


Figure 1-5. Linear diagram depicting features of the EGF receptor. Amino acid residue numbers are placed to the left of the sequence. *Branched symbols* indicate potential glycosylation sites; *filled dots* indicate cysteine residues; *hatched area* represents the transmembrane domain. Numbers in parenthesis designate specific amino acid residues involved in either tyrosine (P-Y) or threonine (P-T) phosphorylation or the lysine residue critical for ATP binding (ATP-K). (Modified from reference #6).

spanning regions. This is followed by a 13-residue sequence of basic residues that likely serves as a stop transfer sequence and is found in all transmembrane growth factor receptors.

The cytoplasmic domain (amino acids 645 to 1186) of the receptor contains the tyrosine kinase activity which includes an ATP binding site as well as several autophosphorylation sites. A common sequence associated with ATP binding in other ATP binding proteins, G-X-G-X-X-G is found starting at amino acid 695, and the lysine at position 721 is thought to provide a salt bridge to β/γ phosphates of ATP in that binding site (42). The mutation of this lysine to methionine (43) or alanine (44) abolished protein tyrosine kinase activity, and subsequently the ability of the mutant receptors to stimulate mitogenesis and other cellular responses elicited by the wild type receptor, thus signifying that the receptor tyrosine kinase activity is necessary for the generation of biological responses to EGF (6,45). Many proteins are known to be phosphorylated by the EGF receptor both *in vivo* and *in vitro* (see references 6,9). This diversity of proteins may explain the fact that EGF elicits a variety of responses.

The EGF receptor shares a close homology to the transforming protein of the *v-erb-B* oncogene (46), and in fact the *v-erb-B* protein is a truncated version of the EGF receptor, lacking most of the extracellular domain and the end portion of the carboxyl domain that contains the autophosphorylation sites. Since the *v-erb-B* is found only in transformed cells, and lacks the C-terminal autophosphorylation sites, it is postulated that it may act as an EGF receptor that is constitutively turned on, thus creating unregulated growth (6).

Whether autophosphorylation of the receptor is necessary for further biological responses was investigated by mutating the known autophosphorylation sites, tyrosine

residues 1068, 1148, and 1173 (45,47), and by deletion of the carboxyl terminal portion of the receptor that contains the autophosphorylation sites (6,45). Results indicate that the receptors devoid of one or more autophosphorylation sites were still able to bind EGF, and elicit mitogenic responses. In some, but not all, mutants, binding affinities were changed; the K_m for an exogenous peptide substrate was decreased, and a decrease in the V_{max} of phosphorylation was seen. The autophosphorylation sites may thus play a role in regulation of how the receptor activity. One theory (48) postulates that the carboxyl tail of the receptor is folded towards the active site and is readily accessible for phosphorylation. Upon phosphorylation, the tail unfolds allowing the binding of other substrates. High concentrations of alternative substrates can compete with the autophosphorylation sites in binding to the active site.

Purpose of Study

The key step in generating an EGF dependent response within a cell is the activation of the EGF receptor tyrosine kinase. In order to understand how the receptor can then propagate the EGF response it is necessary to look at features of the tyrosine kinase, specifically what type of molecules are needed to act as substrates, activators or inhibitors of the reaction. If this can be answered it may help define the type of molecules needed for the transfer of the EGF response to other pathways in the cell, and how this response may then be regulated. In this thesis peptides of known composition will be used to address some of the requirements needed for molecules associating with the EGF receptor kinase.

The first event in generating an EGF-dependent response in a cell is the binding of EGF to its receptor. How binding on the extracellular domain of the receptor can

trigger the activation of the kinase in the cytoplasm is unknown, but must rely on some specific residues in the EGF molecule making contact with the receptor. A way to determine which residues in the ligand are necessary for binding and for eliciting a cellular response is to examine specific features of the molecule through site-directed mutagenesis. Toward that end, a second part of this study involves cloning, expression, and mutagenesis of the EGF ligand. A study of mutant proteins and a comparison of their activities to wild type in the different biological assays--receptor binding, stimulation of the receptor tyrosine kinase, and the stimulation of thymidine incorporation in tissue culture cells--will be presented. These studies will hopefully elucidate how EGF recognizes and binds to its receptor, and which, if any, single amino acids are necessary for maintaining function and/or structure of the EGF molecule.

During the evaluation of the biological activity of the site-specific EGF mutants some of them were seen to have an altered activity in the tyrosine kinase assay. These mutants were unable to stimulate the receptor tyrosine kinase to the same extent as wild type, indicating a possible partial uncoupling of binding and tyrosine kinase activity. The kinetics of the receptor tyrosine kinase reaction stimulated by these low $V_{\max}$ mutants was further examined.

These studies will hopefully bring about a better understanding of signal transduction by the EGF/receptor complex. Ultimately a goal in mutagenesis and biological characterization is to develop an EGF molecule that is either a superagonist or an antagonist of the normal EGF action. A superagonist would be a molecule that could elicit the same effect as EGF, but be able to do it faster or in a smaller quantity, therefore be therapeutically helpful in wound repair. An EGF antagonist would be able to bind to the EGF receptor with the same affinity as the wild type protein, but it would

not elicit a response, or it would decrease a specific response. Such an antagonist could possibly be helpful in the treatment of those cancers that have an elevated number of EGF receptors in that it may turn off, or slow down the uncontrolled cell growth seen in neoplasia.

CHAPTER 2

DIFFERENT TYROSINE CONTAINING POLYMERS CAN ACT AS SUBSTRATES OR INHIBITORS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR KINASE

Introduction

The use of tyrosine-containing random synthetic polypeptides for assaying protein-tyrosine kinase activity was first described by Braun *et al.* (49) and Zick *et al.* (50) and afforded a simple assay that assured incorporation of phosphate exclusively into tyrosine residues. Polymers rich in glutamic acid such as $(\text{Glu}_4\text{Tyr}_1)_n$ were found to be excellent substrates, and further study showed that the substrate specificity of tyrosine kinases could be characterized by comparing their reactivity with copolymers of glutamic acid and tyrosine (51). Zick *et al.* also found that these tyrosine-rich polymers, $(\text{Glu}_1\text{Tyr}_1)_n$, $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$, and $(\text{Lys}_1\text{Tyr}_1)_n$, failed to serve as substrates for the insulin or epidermal growth factor-stimulated receptor kinase as well as for preparations of tyrosine kinase activity stimulated by insulin-like growth factor-I and a phorbol ester. In this study of solubilized EGF receptor kinase from both human placental membranes and A431 cell membranes the three tyrosine-rich polymers described above are shown to be inhibitory to the phosphorylation of tyrosine-poor polymers. The demonstration of and specific features of the inhibition are described in this chapter.

Materials and Methods

Isolation and purification of EGF receptors from human placenta. Fresh human placenta was obtained from local hospitals and kept on ice for transport to the laboratory. The isolation and purification procedure of the EGF receptor was as described by

Harrison and Itin (52): the umbilical cord and connective tissue were removed and the placenta was cut up into smaller pieces and washed several times with a buffer containing 0.05 M Hepes, pH 7.6, 0.25 M sucrose, and 1 mM PMSF. Final weight of placenta was 100-250 grams. Homogenization was done in an equal amount (m/v) of the same buffer in a Sorvall Omni-mixer. Each batch was homogenized four times for 30 seconds with 30 second interim, and the homogenate was centrifuged at 12,000 x g for 15 min. The supernatant was adjusted to 0.1 M NaCl and 0.2 mM $MgCl_2$ and centrifuged at 45,000 x g for 40 min to obtain the microsomal membrane pellet. This pellet was washed three times with 0.03 M Hepes buffer, pH 7.6, containing 1 mM PMSF and resuspended in the same buffer to a protein concentration of approximately 20 mg/ml. Solubilization of receptors was done by adding Triton X-100 to 1% (v/v) and stirring 45 min at room temperature. Centrifugation at 105,000 x g for 90 min at 4°C left the solubilized membrane proteins in the supernatant. This supernatant was frozen in aliquots at -80°C.

A column containing wheat germ agglutinin agarose (U.S. Biochemical Corp.) was then used to purify the EGF receptor in a manner similar to that described (52). Six ml of the receptor preparation was diluted with 12 ml of buffer A (40 mM Hepes buffer (pH 7.4), 10% glycerol, 0.5 M NaCl, 0.5 mM PMSF) and passed four successive times through a 2 ml wheat germ agglutinin agarose column. The column was washed with 20 ml of buffer A containing 0.05% Triton X-100, and the receptor was then eluted with buffer A containing both 0.05% Triton X-100 and 0.3 M N-acetyl glucosamine. Fractions consisting of 2 ml each were collected and assayed for activity. The active fractions were pooled and frozen in aliquots at -80°C.

Isolation and purification of EGF receptors from A431 Cells. A431 cell membranes were isolated according to the procedure of Akiyama *et al.* (53). A431 cells were grown to confluence in roller bottles in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco). Cells were washed two times with cold phosphate buffered saline (PBS), then harvested by scraping. Cells were pooled and the packed cell volume (PCV) determined. After washing twice with cold hypotonic KMP buffer (20 mM Pipes, pH 7.2, 1 mM $MgCl_2$, 5 mM KCl) (x2 PCV), they were resuspended in KMP buffer (x4 PCV) and allowed to swell while sitting on ice. After 30 min the cells were homogenized with 25 strokes of a Dounce homogenizer, the homogenate was centrifuged at 27,000 x g for 5 min at 4°C, and the supernatant saved. The pellet was washed twice by resuspension of the pellet in 5 ml KMP and centrifugation at 27,000 x g for 5 min and the supernatants saved. All supernatants were pooled and centrifuged at 100,000 x g for 30 min at 4°C. The resulting pellet was the membrane fraction and was stored at -80°C

For solubilization, the membrane pellets were resuspended in buffer composed of 20 mM Hepes, pH 7.4, 10% glycerol, 1 mM PMSF, 1 mM EGTA, and 10 μ g/ml leupeptin to a final protein concentration of 5-10 mg/ml. Triton X-100 was added to 1% final concentration, and after stirring for 20 min at room temperature, the solution was centrifuged at 100,000 x g for 30 min. The supernatant was incubated with wheat germ agglutinin agarose that had been previously equilibrated with buffer B (40 mM Hepes, pH 7.4, 0.5 M NaCl, 1 mM PMSF, 10% glycerol, 0.1% Triton X-100), and an additional equal volume of buffer B minus Triton was added. After 2 hours on ice with occasional mixing, the sample was poured into a column (4 x 1.3 cm) and washed with five times the column volume in buffer B. The membrane proteins were eluted with buffer B plus 0.3 M N-

acetyl glucosamine and fractions equal to the column volume were collected. Most of the protein came off in the second column volume, which also accounted for most of the EGF receptor activity. Aliquots of this fraction were stored at -80°C until use.

Phosphorylation of synthetic polymers and a synthetic peptide using receptors isolated from placenta. The receptor tyrosine kinase preparations from placenta were assayed using $(\text{Glu}_4\text{Tyr}_1)_n$ (Sigma) as a substrate, essentially as described by Zick *et al.* (50). Assays with other polypeptides (Sigma) and a synthetic peptide substrate, the *src* peptide (Peninsula Laboratories) were carried out in a similar manner. The *src* peptide is related to the tyrosine phosphorylation site in pp60^{src}, and has the sequence: Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, as described by Casnellie *et al.* (54). The receptor preparations (about 10 μg in 30-50 μl of the buffer used for column elution) were incubated for 15 min at 22°C with 10 mM MgCl_2 , 2 mM MnCl_2 , and 100 ng of mouse EGF (Bethesda Research Laboratories). The polypeptide or peptide substrate was then added together with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN, 4500 Ci/mmol) (8.8×10^3 cpm/pmole, final concentration = 10 μM) to a final volume of 100 μl , and the incubation was continued for 10 min at 22°C . In the case of the synthetic polymers, the reactions were stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate, and the acid-insoluble fraction was collected by filtration onto Millipore filters and washed with the same solvent. In the assays using the *src* peptide as the substrate, 160 μl of 5% TCA were added and after 10 min on ice, the acid-insoluble material was removed by centrifugation. An aliquot (50 μl) of the supernatant solution was spotted on a disc of phosphocellulose paper (Whatman), and the disc was washed on a filtration apparatus with six aliquots of 5 ml of 75 mM phosphoric acid (H_3PO_4). The

src peptide is acid soluble but able to bind to phosphocellulose paper. The filters were dried and isotope quantified by liquid scintillation counting.

Phosphorylation of synthetic polymers using receptors isolated from A431 cells. The receptor tyrosine kinase preparation from A431 cells was assayed in the same manner as the placenta preparations with the following modifications: the reaction mixtures contained 10 mM MgCl_2 , 2 mM MnCl_2 , 1 mM DTT, 0.1 mM sodium orthovanadate (Na_3VO_4), 250 mM ammonium sulfate, 0.5 μM hEGF, and about 0.4 μg of solubilized receptor preparation in 50 μl of buffer B. After incubation for 15 min at 22°C, the synthetic polymer substrates were added with ATP to a final volume of 0.1 ml. One microcurie of [γ - ^{32}P]ATP was added along with cold ATP to a final concentration of 10 μM . Incubation with ATP and substrate was continued for 10 min at which time the reaction was stopped with 1 ml of 5% TCA containing 10 mM sodium pyrophosphate. The reactions were processed as described earlier.

Autophosphorylation of purified receptors. The incubation mixtures were as described above for the respective receptor preparations except that the specific activity of the [γ - ^{32}P]ATP was 5 times higher. Instead of adding 5% TCA at the end of the reaction, the mixtures were made 1% in sodium dodecyl sulfate (SDS), 0.35 M in β -mercaptoethanol, and 10% in glycerol. The mixtures were heated 5 min in a boiling water bath and electrophoresed using an 8% polyacrylamide gel prepared as described by Laemmli (55). The gels were stained and destained as described (55), dried, and exposed to Kodak X-OMAT AR-5 film with intensifying screens at -80°C.

Results

Features of inhibitory activity of tyrosine-rich polymers with placental EGF receptors.

The rate of phosphorylation of five tyrosine-containing polypeptides by the solubilized membrane receptor kinase preparation of human placenta was examined as a function of substrate concentration (Fig. 2-1). $(\text{Glu}_4\text{Tyr}_1)_n$ and $(\text{Glu}_6\text{Ala}_3\text{Tyr}_1)_n$ are both good substrates, yielding high levels of phosphorylation in the presence of EGF. Eighty percent of the kinase activity of the receptor preparation can be attributed to the EGF receptor kinase as shown by the gel analysis described below. $(\text{Glu}_1\text{Tyr}_1)_n$ and $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ were found to be phosphate acceptors at concentrations of 1-5 $\mu\text{g}/0.1$ ml, but they became inhibitory at higher concentrations. $(\text{Lys}_1\text{Tyr}_1)_n$ was a poor substrate. Because of their possible inhibition at higher concentrations, the three tyrosine-rich polymers were then tested as inhibitors of the protein kinase activity with 20 μg of $(\text{Glu}_4\text{Tyr}_1)_n$ as substrate (Fig. 2-2). All three tyrosine-rich polymers significantly inhibited the enzyme activity. One μg of $(\text{Glu}_1\text{Tyr}_1)_n$ or $(\text{Lys}_1\text{Tyr}_1)_n$ inhibited the phosphorylation of $(\text{Glu}_4\text{Tyr}_1)_n$ by 50%, and higher concentrations led to 80-90% inhibition. $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ was about one fifth as effective as an inhibitor, needing 5 μg to cause 50% inhibition of $(\text{Glu}_4\text{Tyr}_1)_n$. Figure 2-3 Panel A is the concentration curve of the reaction showing the phosphorylation the *src* peptide. As Fig. 2-3 Panel B shows, the phosphorylation of 100 μg of the *src* peptide was also strongly inhibited by $(\text{Glu}_1\text{Tyr}_1)_n$ at 1 $\mu\text{g}/0.1$ ml. Inhibition plots have a negative control blank subtracted, representing counts contributed by the autophosphorylation and phosphorylation of the tyrosine-rich polymers. Lineweaver-Burk plots of data for the inhibition of $(\text{Glu}_4\text{Tyr}_1)_n$ and the *src* peptide by $(\text{Glu}_1\text{Tyr}_1)_n$ are shown in Fig. 2-4 panels A and B. The type of inhibition appears to be mixed.

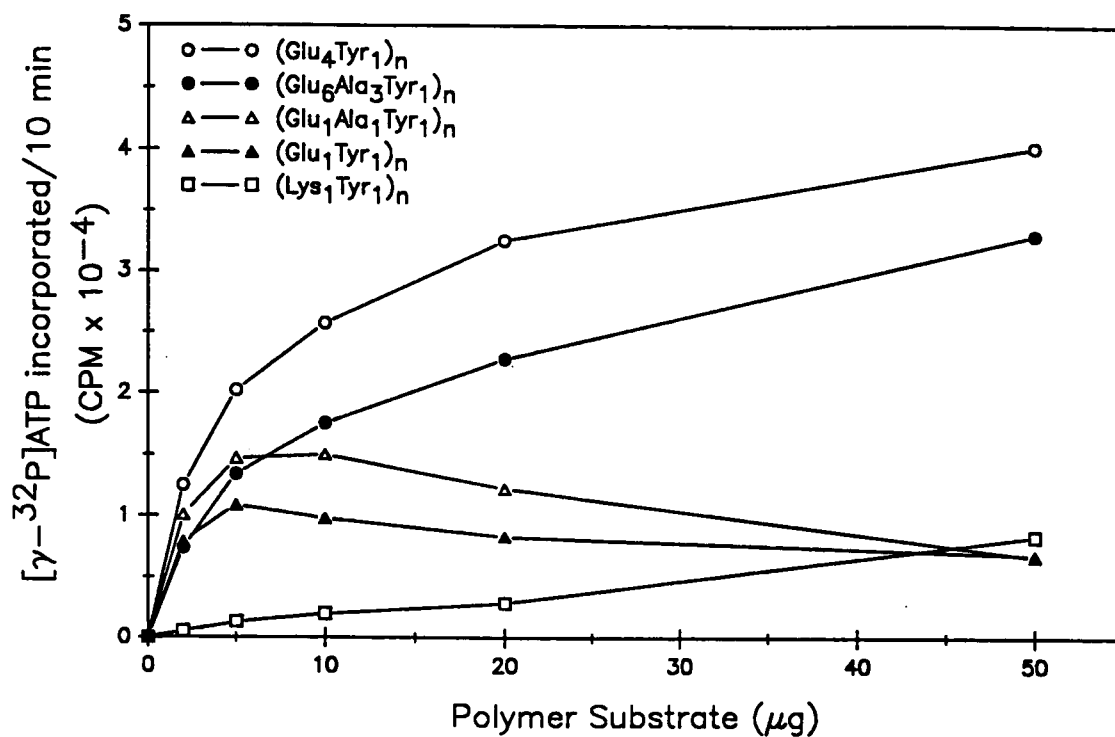


Figure 2-1. Dose response curves of the tyrosine kinase activity of the EGF receptor purified from placenta using tyrosine-containing polymers as substrates.

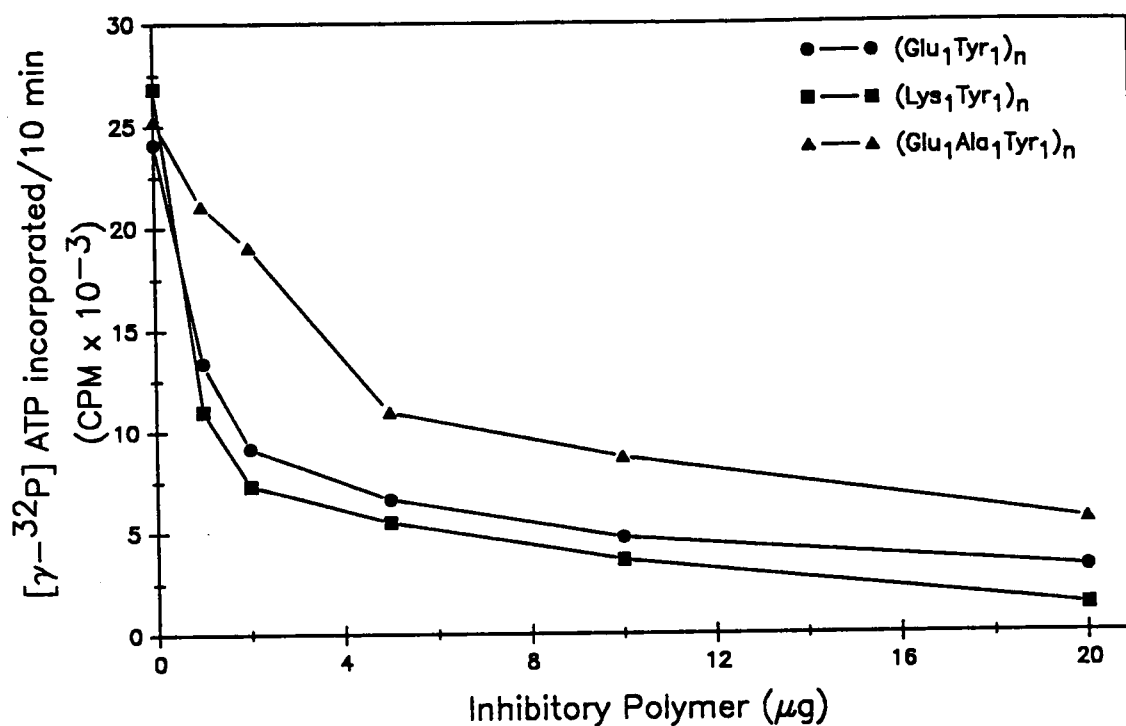


Figure 2-2. Inhibition of $(\text{Glu}_1\text{Tyr}_1)_n$ phosphorylation by tyrosine-rich polymers using EGF receptors purified from placenta. The amount of $(\text{Glu}_1\text{Tyr}_1)_n$ was kept at 20 μg in each reaction, and the amount of the other polymers were increased as shown.

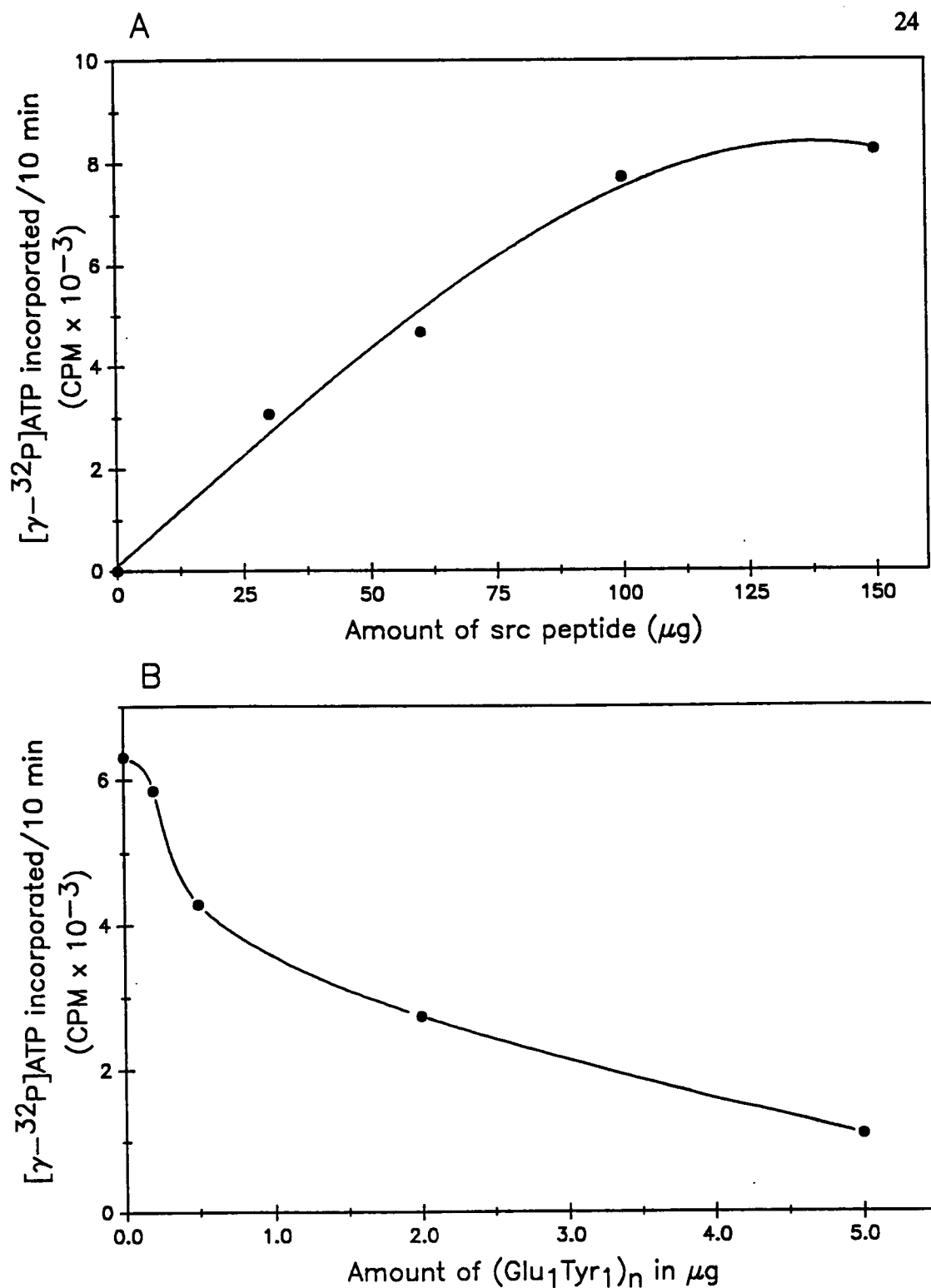


Figure 2-3. Protein tyrosine kinase activity of the EGF receptor purified from placenta using the *src* peptide as a substrate. Panel A; dose response curve using increasing amounts of the *src* peptide. Panel B; inhibition of 100 $\mu\text{g}/0.1$ ml of the *src* peptide by $(\text{Glu}_1\text{Tyr}_1)_n$.

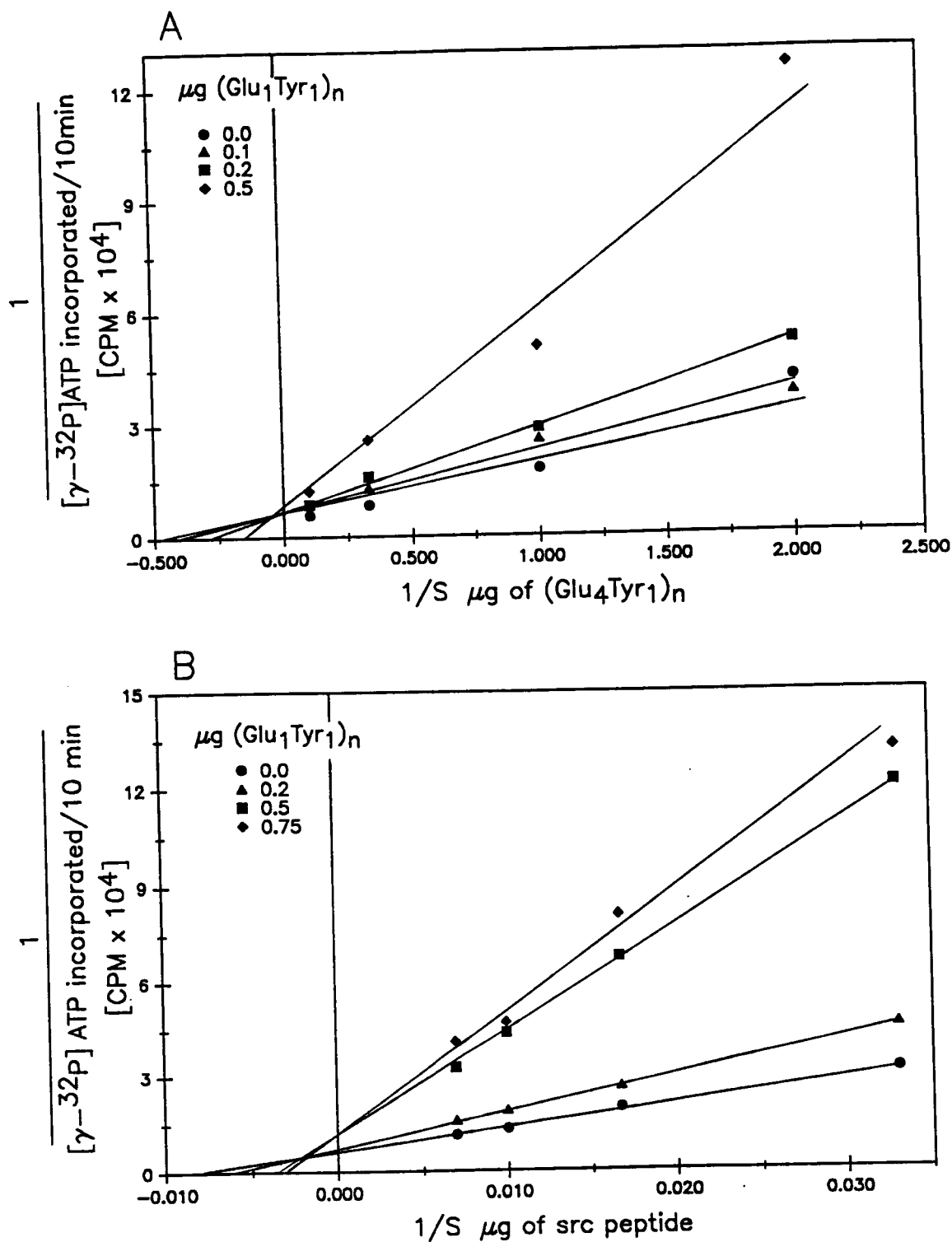


Figure 2-4. Lineweaver-Burk plots of the inhibition of $(\text{Glu}_4\text{Tyr}_1)_n$ and the *src* peptide by $(\text{Glu}_1\text{Tyr}_1)_n$ using EGF receptors purified from placenta. *Panel A*, inhibition using $(\text{Glu}_4\text{Tyr}_1)_n$ as a substrate; *panel B*, inhibition using the *src* peptide as a substrate.

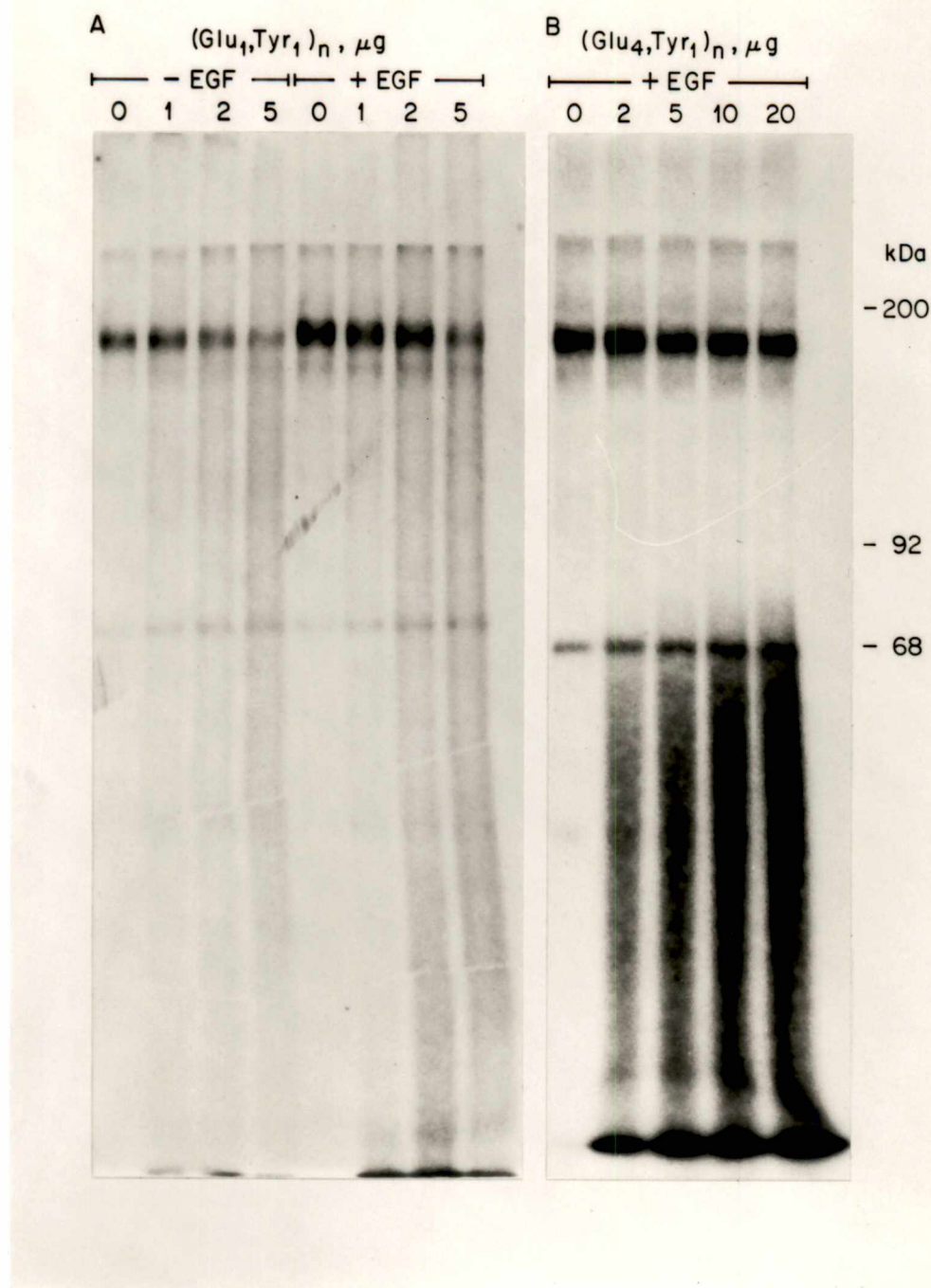


Figure 2-5. Inhibition of the autophosphorylation of the EGF receptor kinase purified from placenta. *Panel A*; the EGF receptor kinase preparation was incubated in reaction mixtures containing $(\text{Glu}_1, \text{Tyr}_1)_n$ in the amounts shown in both the absence and presence of EGF. *Panel B*; $(\text{Glu}_4, \text{Tyr}_1)_n$ in the amounts shown was used as a substrate in the kinase reaction.

Acrylamide gel analyses of reaction mixtures containing increasing amounts of $(\text{Glu}_1\text{Tyr}_1)_n$ (0-5 μg) and $(\text{Glu}_4\text{Tyr}_1)_n$ (0-20 μg) are shown in Fig. 2-5. Fig. 2-5 panel A shows that $(\text{Glu}_1\text{Tyr}_1)_n$ significantly inhibited the autophosphorylation of the EGF receptor band (170 kDa) both in the absence and the presence of EGF. Determination of label in the EGF receptor band obtained from a gel such as that shown in Fig. 2-5 showed that 5 $\mu\text{g}/0.1$ ml of $(\text{Glu}_1\text{Tyr}_1)_n$ led to 60% inhibition and 15 $\mu\text{g}/0.1$ ml to 90% inhibition of the autophosphorylation. Fig. 2-5 panel B shows that $(\text{Glu}_4\text{Tyr}_1)_n$ did not inhibit the autophosphorylation of the EGF receptor kinase.

Features of inhibitory activity of tyrosine-rich polymers with EGF receptors isolated from A431 cells. The same five tyrosine containing polymers tested in the assays using placenta preparations were tested with A431 EGF receptor kinase preparations. As with the placental EGF receptor fraction, but using high-salt assay conditions of Koland and Cerione (56) which give higher activity levels than the conditions used with placenta, the $(\text{Glu}_4\text{Tyr}_1)_n$ polymer was the best substrate for the A431 EGF receptor fraction as shown in Figure 2-6. $(\text{Glu}_1\text{Tyr}_1)_n$, $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ and $(\text{Lys}_1\text{Tyr}_1)_n$ proved to be very poor substrates, yielding less than 10% of the activity of $(\text{Glu}_4\text{Tyr}_1)_n$. $(\text{Glu}_6\text{Ala}_3\text{Tyr}_1)_n$ was a fair substrate, but was still only 30% as good as $(\text{Glu}_4\text{Tyr}_1)_n$. As described above, $(\text{Glu}_1\text{Tyr}_1)_n$, $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ and $(\text{Lys}_1\text{Tyr}_1)_n$ were tested to see if they could inhibit the receptor's ability to phosphorylate the $(\text{Glu}_4\text{Tyr}_1)_n$ substrate. Figure 2-7 shows the inhibition concentration curves of these polymers using 10 μg of $(\text{Glu}_4\text{Tyr}_1)_n$ as substrate. $(\text{Glu}_1\text{Tyr}_1)_n$ proved to be the strongest inhibitor, being able to reduce the $(\text{Glu}_4\text{Tyr}_1)_n$ activity 50% at 0.1 $\mu\text{g}/0.1$ ml. It took ten times more of the $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ polymer, 1 $\mu\text{g}/0.1$ ml to inhibit 50%. Both of these polymers were able to decrease the activity of

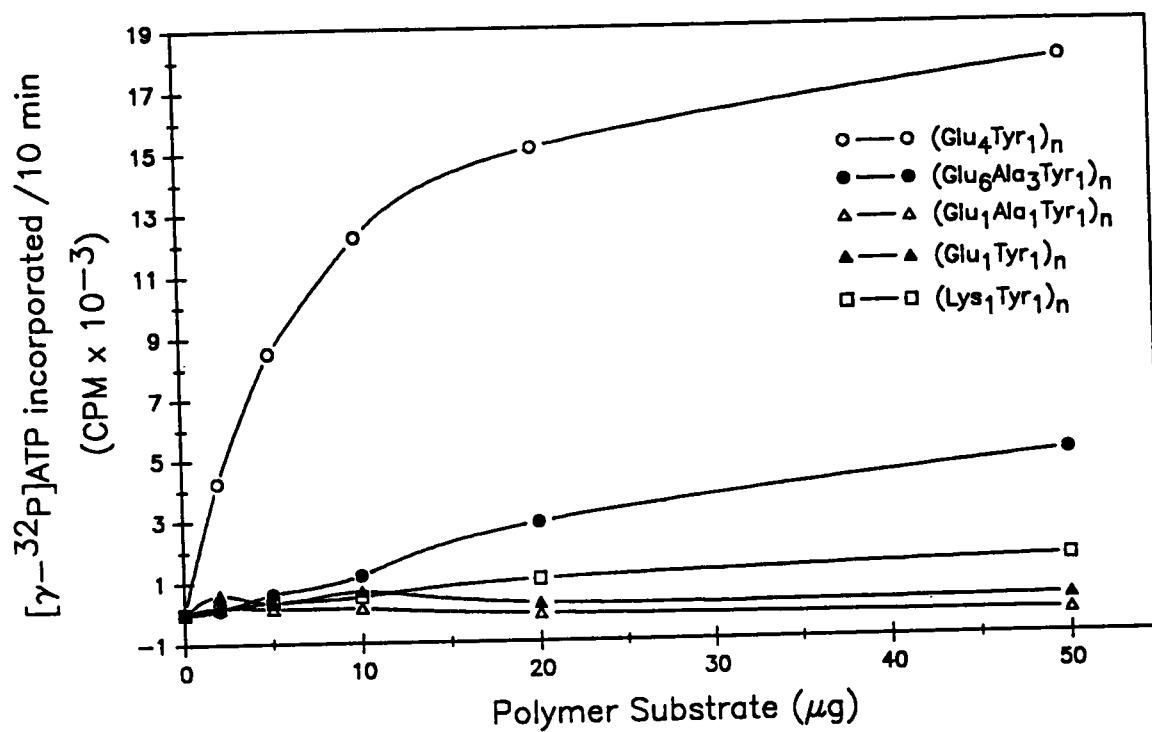


Figure 2-6. Dose response curves of the tyrosine kinase activity of the EGF receptor purified from A431 cells using tyrosine-containing polymers as substrates.

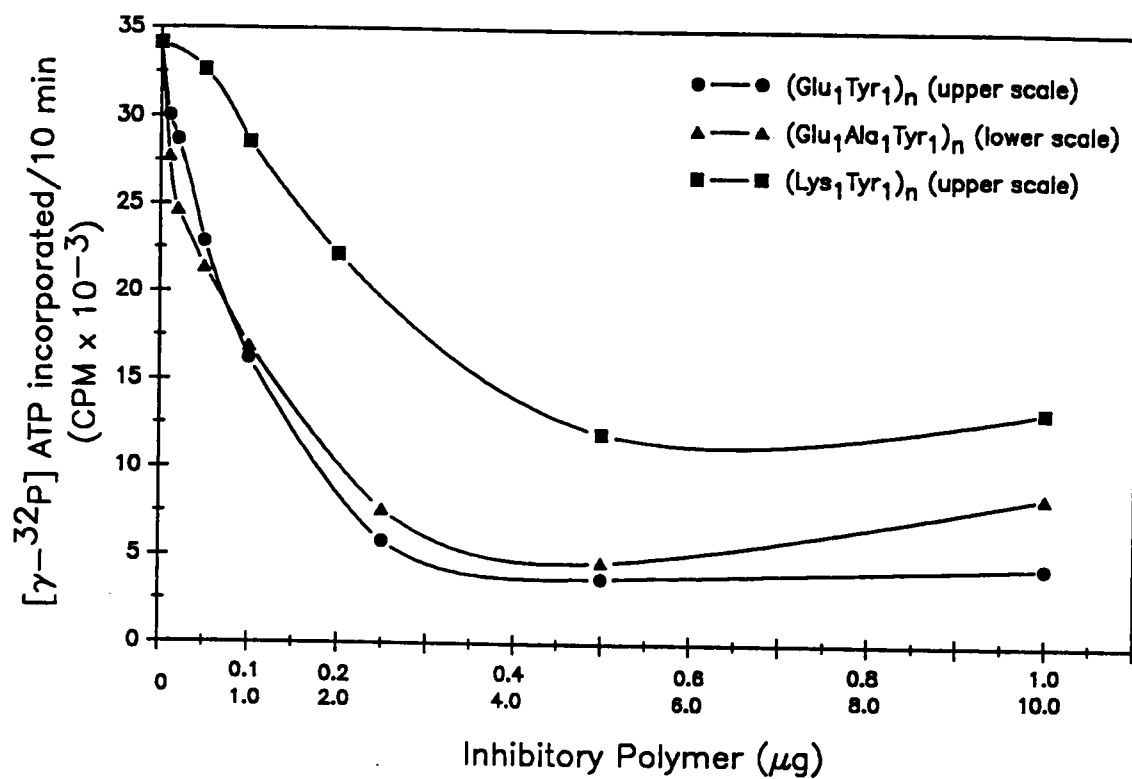


Figure 2-7. Inhibition of $(\text{Glu}_1\text{Tyr}_1)_n$ phosphorylation by tyrosine-rich polymers using EGF receptors purified from A431 cells. The amount of $(\text{Glu}_1\text{Tyr}_1)_n$ was kept at $10 \mu\text{g}/0.1 \text{ ml}$, and the amount of the other polymers were increased as shown.

the $(\text{Glu}_4\text{Tyr}_1)_n$ to 13% of the maximum. The $(\text{Lys}_1\text{Tyr}_1)_n$ polymer caused 50% inhibition at $0.3 \mu\text{g}/0.1 \text{ ml}$, but was unable to inhibit as fully yielding 40% maximum inhibition instead of the 13% shown by the other two polymers. Lineweaver-Burk plots of $(\text{Gly}_1\text{Tyr}_1)_n$ and $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ inhibition of the $(\text{Glu}_4\text{Tyr}_1)_n$ substrate (Fig. 2-8, panels A and B) show the inhibition to be a mixed type.

Discussion

Braun *et al.* (49) found that several synthetic polymers with ordered sequences were inhibitors of different tyrosine-specific protein kinases. These polymers contained Ala, Glu, Gly, and Tyr in equal amounts in different repeated sequences. We have found that $(\text{Glu-Tyr-Ala-Gly})_n$, which these authors found to be a good inhibitor of a crude EGF receptor kinase preparation (membrane preparation), is only 10% as potent as $(\text{Glu}_1\text{Tyr}_1)_n$ in inhibiting the partially purified EGF receptor kinase of placenta.

EGF receptor kinase fractions from human placenta and from A431 cells showed a preference for the $(\text{Glu}_4\text{Tyr}_1)_n$ polymer as a substrate, and were inhibited by the $(\text{Glu}_1\text{Tyr}_1)_n$, $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$ and $(\text{Lys}_1\text{Tyr}_1)_n$ polymers. The differences found in the inhibitory properties of the polymers is most likely due to differences in the preparation and purity of the enzyme and to the differences in the reaction mixtures, as discussed below.

The placental enzyme preparations used in this study also have insulin receptor kinase activity which can be detected by gel electrophoresis of the $[^{32}\text{P}]$ products formed when insulin is added to the reaction mixtures. (The autophosphorylation of the β subunit of the insulin kinase in the presence of insulin is about 5-10% that of the EGF receptor in the presence of EGF). The phosphorylation of the insulin receptor band was also

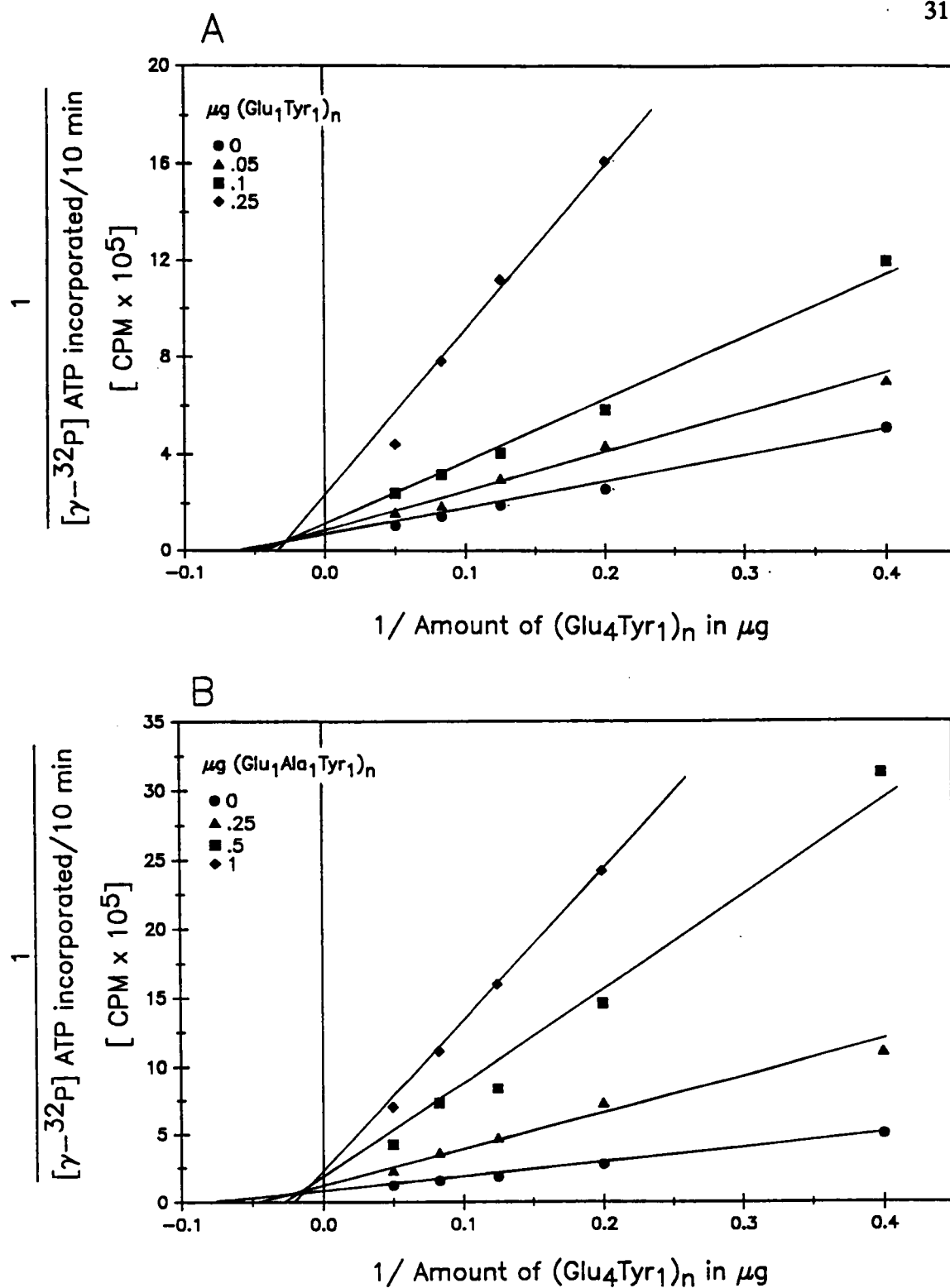


Figure 2-8. Lineweaver-Burk plots of the inhibition of $(\text{Glu}_4\text{Tyr}_1)_n$ by tyrosine-rich polymers using EGF receptors purified from A431 cells. Panel A, inhibition caused by $(\text{Glu}_1\text{Tyr}_1)_n$; panel B, inhibition caused by $(\text{Glu}_1\text{Ala}_1\text{Tyr}_1)_n$.

inhibited by $(\text{Glu}_1\text{Tyr}_1)_n$ (data not shown). Autoradiography also showed the presence of an undefined band at 68 kDa. Studies involving the A431 purified receptors were carried out several years after the studies involving placenta purified receptors. The human carcinoma cell line, A431, (because of its high concentration of EGF receptors (57)) was subsequently chosen for these studies in an effort to obtain an EGF receptor preparation free of other contaminating tyrosine kinases found in the placental preparations.

Kinase reaction mixtures using purified receptors from A431 cells were run on a polyacrylamide gel followed by autoradiography. The only visible bands are those associated with the EGF receptor at 170,000 and its slight breakdown product at 150,000 (58). Thus one can assume that greater than 95% of the kinase activity seen in the assays is due to the EGF receptor kinase. No bands were visible in Commassie blue-stained gels. A much lower levels of A431 protein, 0.4 μg of the A431 fraction compared to 10 μg of placental fraction, was used in the assays.

A major difference between the assays described above with the two receptor preparations is the composition of the reaction mixtures. A431 receptors were assayed in the presence of 250 mM ammonium sulfate, 0.1 mM Na_3VO_4 , and 1 mM DTT. The decision to add ammonium sulfate was based on the work of Koland and Cerione (56) who showed that its addition increased the EGF-dependent phosphorylation of a $(\text{Glu}_6\text{Ala}_3\text{Tyr}_1)_n$ substrate four-fold. In our hands it increased the $(\text{Glu}_4\text{Tyr}_1)_n$ phosphorylation by two and a half-fold. Sodium orthovanadate has been shown to inhibit some phosphatases (59), so its presence in the reaction mixtures may prevent phosphatase activity.

As described earlier, the type of inhibition exhibited by the tyrosine-rich polymers appears to be mixed, in that both the $V_{\max}$ of the phosphorylation reaction and the K_m of the peptide substrates are affected (60). This is not surprising considering the complexity of the assay system. The system used here to measure the inhibition of the tyrosine-poor polymers involves three potential substrates: the autophosphorylation sites on the receptor itself, the tyrosine-poor polymer, and the tyrosine-rich polymer; and two potential inhibitors: the autophosphorylation sites and the tyrosine-rich polymer. All three of the phosphorylation substrate molecules must compete for the same active site. Bertics and Gill (61) have demonstrated that the autophosphorylation sites of the receptor can act as competitive inhibitors of peptide substrates such as angiotensin II, gastrin and the *src* peptide. The inhibition by the tyrosine-rich polymers may be caused by a stretch of tyrosines since high concentrations of Tyr-Tyr and Tyr-Tyr-Tyr are inhibitory to the $(\text{Glu}_4\text{Tyr}_1)_n$ phosphorylation (data not shown because the insolubility of the peptides prevented reproducible quantitative studies). Assuming that the $(\text{Glu}_4\text{Tyr}_1)_n$ is helical in structure and that helicity contributes to its being a good substrate, the addition of tyrosine, which has been reported to disrupt the helicity of peptides (62), could explain why tyrosine-rich polymers are not good substrates.

The peptide sequence of a glutamic acid followed by a tyrosine is found in all three of the autophosphorylation sites of the EGF receptor, as well as the *src* peptide and can be presumed to be in those polymers containing both glutamic acid and tyrosine. This specific sequence on a substrate may be necessary for the binding of a substrate to the active site of the kinase domain. Therefore, the higher content of glutamic acid in a polymer may be the reason why it is a good substrate, and why a polymer poor in glycine is not.

The inhibitory synthetic polymers or similar peptide analogs may be useful as specific tyrosine kinase inhibitors, and for examining and modifying the response of cell extracts and permeabilized cells to tyrosine phosphorylation.

CHAPTER 3

CLONING OF HUMAN EPIDERMAL GROWTH FACTOR AS A SECRETORY PROTEIN AND INITIAL SITE-DIRECTED MUTAGENESIS STUDIES

Introduction

EGF-like growth factors elicit a variety of responses within the cell, some responses identical to EGF and some differing in their specificity and intensity (6). Since all EGF-like proteins exert their effects upon the cell via the EGF receptor, elucidation of how EGF differs in its mechanism of action compared to other EGF-like molecules necessitated the understanding of how EGF binds to its receptor. There are two general ways in which this can be accomplished. One is to define what region/regions of the receptor is necessary for binding and to ascertain whether the same region is used in the binding of the other EGF-like molecules. The second is to determine what region of the ligand is necessary for binding. Since the EGF molecule is the smaller and less complicated of the two proteins, it was chosen to study this problem.

In an effort to determine what domain of the EGF ligand is important for binding to the receptor, several groups used synthetic peptide fragments to see if any specific peptide could bind to the receptor. Komoriya *et al.* (63) found that a peptide corresponding to loop B of EGF (containing amino acid residues 20-31, see Fig. 1-1, page 3), whether in the linear or cyclic form, could bind to the receptor and stimulate cell growth, though poorly (only 0.003% as active as the full length EGF). Fragments corresponding to the other loop regions of EGF were inactive. Likewise, Heath and Merrifield (64) showed that a fragment containing residues 15-33 was 0.0001 as active as EGF in receptor binding and stimulation of DNA synthesis, and other fragments were even less active (residues 32-53) or inactive (residues 45-53).

The importance of the amino and carboxyl termini in receptor binding has also been investigated. Deletion of up to three amino acids of the NH₂-terminus of EGF appears to have no effect on biological activity (65,66), and naturally occurring EGF molecules are frequently isolated as such. Extensions of the NH₂-terminus of EGF do not appear to have much of an effect on ligand binding (67,68,69) even though the additions can be quite large, 450,000 daltons. Those EGF-like molecules capable of binding to the EGF receptor are quite variable in the length of their carboxyl tail (see Fig. 1-3, page 6), and purification of EGF from some species results in the presence of one or more variants with shortened carboxyl ends (4,70,71). Amino acids 48-53 can be removed with little effect on binding (72,73,74).

Since peptide fragments of EGF were unable to bind efficiently to the EGF receptor, it was assumed that some higher order structure is necessary for receptor recognition. A rigorous determination of which residues are necessary for binding involves examination of the whole intact molecule with specific amino acid alterations at various positions. This can be accomplished by site-directed mutagenesis techniques. However, before site-directed mutagenesis could be undertaken, the gene coding for EGF first had to be cloned.

Several laboratories had cloned EGF and obtained pure protein by various methods. Some (75,76,77) had produced EGF as a fusion protein which was enzymatically cleaved after purification to generate the correct length EGF molecule. Human EGF has been produced as a secretory protein in yeast where it is secreted into the medium (78), and *E. coli*, where it is secreted into the periplasm (79). It was felt that secretion of EGF into the periplasm of *E. coli* might yield better results since it could produce the authentic molecule with the fewest purification steps. This chapter reports the cloning, purification,

and biochemical characterization of hEGF and two site-directed mutants of hEGF, at position 47 and 7. Details of this work including additional mutations within the molecule have been previously published (80).

Materials and Methods

Synthesis and purification of oligonucleotides. Twelve single-stranded deoxyoligonucleotides, ranging in length from 34 to 40 bases, were synthesized utilizing phosphoramidite chemistry (81) (see Fig 3-1). Oligonucleotides were purified by electrophoresis through 16% polyacrylamide gels containing 8 M urea. To check the purity of the final product each oligonucleotide was phosphorylated at the 5' terminus (80), enzymatically hydrolysed with nuclease P₁ (Pharmacia LKB Biotechnology Inc.) and subjected to thin layer chromatography. Autoradiography showed the labeled 5'-mononucleotide of the oligonucleotide hydrolysate, and it was compared to cold 5'-deoxynucleoside monophosphate standards.

*Construction of *phoA* signal/hEGF gene.* Each single-stranded oligonucleotide, 3,5,7 or 9, was annealed with its respective complementary single-stranded oligonucleotide, 4,6,8 or 10, to form double-stranded oligonucleotides with 5'-overhangs of 6-9 bases. Annealing was performed in a stepwise fashion by heating the annealing pairs to 10-15°C above the empirically calculated temperature of dissociation (T_d) (82) followed by incubation at 20°C below the T_d and finally slow cooling at room temperature. The annealed pairs were phosphorylated at their 5' termini as described (80). Oligonucleotides 2 and 11 were 5' phosphorylated and annealed to their respective complementary strands, 1 and 12, that were left as the 5'-OH species. The six double-stranded oligonucleotides were ligated together to form the *phoA* signal/hEGF gene construct.

Transfer of phoA signal/hEGF genetic construct into expression vector. The *phoA* signal/*hEGF* gene construct was ligated to a 2731-base pair *NcoI-EagI* restriction fragment from the expression vector pFL142 (A gift of Dr. F.W. Larimer, Biology Division, Oak Ridge National Laboratory), which contains the *tac* promoter under the regulatory control of the lactose repressor-operator and two Shine-Dalgarno sequences. Various dilutions of the ligation mixture were transformed into *E. coli* BNN45 (*metB*, *thi*-, *hsdR*, *lacY*, *supE44*, *supF*) by the procedure of Hanahan (83) and plated onto LB medium containing 25 μ g/ml ampicillin. Plasmid DNA was prepared from ampicillin-resistant colonies by the alkaline lysis method (84) and screened by digestion with *HindIII*. Plasmid DNA containing the *hEGF* insert, called pEGF-1, was digested with *Bam*HI to liberate a 300-base pair fragment which was subcloned into the *Bam*HI site of M13mp19RF DNA (purified from CsCl-banded phage as described (85)). *E. coli* JM107 ($\Delta(lac-pro)$, *endA1*, *gyrA96*, *thi1*, *hsdR17*, *supE44*, *relA1*, (*F'**traD36*, *proAB*⁺, *lacI*^q Δ M15)) was transfected (83) with this DNA preparation and plated on LB medium using top agar containing 0.3 mM IPTG and 0.03% X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside) to select for plaques containing the *Bam*HI fragment. Single stranded phage DNA was isolated and sequenced by the dideoxynucleotide chain termination protocol (86) to verify the correct sequence of the *phoA* signal/*hEGF* gene construct.

Expression of hEGF. Overnight cultures of *E. coli* JM107 harboring the pEGF-1 plasmid were grown in LB medium containing 25 μ g/ml ampicillin and diluted 100-fold into the same medium. In general, cultures were grown at 37°C and 250 rpm in a rotary shaker for three hours after which time chloramphenicol was added to a final concentration of 5 μ g/ml. They were allowed to grow for one to two more hours when

the expression of hEGF was induced by the addition of IPTG to a 1 mM final concentration. The cultures were allowed to grow for an additional 10-12 hours or until hEGF production was maximal, at which time the cells were harvested by centrifugation. Details and rationale of this expression regime are discussed elsewhere (87).

Purification of hEGF. The hEGF segregated in the periplasm was isolated from the cell pellet by resuspending the pellet in cold 1 M Tris-Cl (pH 9.0), 2 mM EDTA, followed by incubation on ice for 20 min. The hEGF in the Tris/EDTA fraction was precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ (80% saturation) and stirred at 4°C for 1 hour. After centrifugation at 39,000 x g for 50 min, the pellet was resuspended in and dialyzed against a 25 mM sodium phosphate (pH 7.2) buffer. The dialyzed fraction was loaded onto a column (2.3 x 40 cm) of Sephadex G-75 equilibrated with 25 mM sodium phosphate (pH 7.2) and eluted with the same buffer. The fractions containing hEGF activity were pooled and further purified by reverse-phase chromatography on a Waters Model 600E HPLC system utilizing an 18-34% CH_3CN gradient in 10 mM sodium phosphate (pH 7.2) buffer.

Amino acid composition analysis and protein sequencing. Analysis of amino acid composition was carried out according to standard methods (88). Cysteine was determined as cysteic acid after dimethyl sulfoxide oxidation (89), and tryptophan determination was accomplished after hydrolysis in mercaptoethanesulfonic acid (90). Sequencing was performed on carboxymethylated hEGF using an Applied Biosystems 470A gas-phase protein sequenator.

EGF radioreceptor competition binding assay. Specific binding of hEGF to the EGF receptor of purified membrane fractions from the human epidermoid carcinoma cell line

A431 (ATTC) were as described (91). Briefly, A431 membrane fractions (approximately 0.2 μ g protein/ml) were incubated with ^{125} I-EGF in a reaction mixture containing 20 mM Hepes, pH 7.4, 0.1% (w/v) BSA, and various dilutions of the unlabeled EGF protein. Mixtures were allowed to reach equilibrium (30 min at 30°C) after which the receptor bound ^{125} I-EGF was collected on cellulose acetate filters (Millipore GVWP) and washed with a buffer containing 20 mM Hepes, pH 7.4 and 0.1% BSA (w/v) to remove unbound ligand. Filters were dried and the radioactivity quantified by liquid scintillation spectrometry. A431 cells were grown and cell membranes isolated as described in Chapter 2. ^{125} I-hEGF was prepared by the chloramine-T method (92) to an average specific activity of 120,000 cpm/pmol hEGF.

Assay of EGF receptor tyrosine kinase activity. The ability of hEGF to stimulate the tyrosine kinase activity of the EGF receptor was assayed by measuring the incorporation of ^{32} P_i from [γ - ^{32} P]ATP (ICN, 4500 Ci/mmol) into the synthetic polypeptide substrate (Glu₄Tyr₁)_n (Sigma) as described in Chapter 2. Solubilization of the receptor with Triton X-100, receptor preincubation, and assay conditions were similar to those described in Chapter 2 for the tyrosine kinase assay using receptors purified from A431 cells with these changes: the final ATP concentration was 100 μ M and all reactions contained (Glu₄Tyr₁)_n at a final concentration of 0.5 mg/ml.

Oligonucleotide-directed mutagenesis of hEGF gene. Deoxyoligonucleotides, degenerate at 1 or 2 nucleotides corresponding to the targeted codon and/or restriction site (see Results), were synthesized and purified, and phosphorylated at the 5' termini. RF I DNA was synthesized utilizing the mutant oligonucleotides as primers and M13 ssDNA containing the hEGF insert as the template. Reaction were carried out according

to the modifications of Niyogi *et al.* (93,94) of the "boost protocol" (95). Alkaline sucrose gradient purified RF I DNA (96) was used to transfect *E. coli* JM107.

Screening and characterization of mutant hEGF clones. Plaques were transferred to nitrocellulose membranes, and the filters hybridized to ^{32}P -labeled mutant oligonucleotide probes as previously described (93). Briefly, hybridization was carried out for 1 h at 20°C below the empirically calculated T_d after which time filters were washed under stringent temperature conditions specific for each mutation and subjected to autoradiography. Candidate mutant phage from individual plaques were grown and RF DNA was isolated and digested with the appropriate restriction endonuclease to screen for plaques harboring the mutation. Phage whose RF DNA lacked the restriction site in question were propagated for the isolation of ssDNA and sequenced to confirm the mutations and the absence of secondary mutations in other areas of the gene.

Transfer of mutant hEGF clones into expression vector. *Bam*HI fragments of each mutant clone were removed from the phage RF DNA, ligated into pEGF-1 lacking the 300-base pair *Bam*HI fragment carrying the wild-type *hEGF* gene, and screened for proper orientation by *Eco*RI digestion and agarose gel electrophoresis.

Expression of mutant hEGF genes. Expression vectors containing the mutations in the *hEGF* gene were transformed into *E. coli* JM107 (83). Induction, expression, and isolation of mutant proteins were as described for the wild-type hEGF.

Results

Special features of phoA signal/hEGF genetic construct. Twelve deoxyoligonucleotides were constructed and ligated as shown in Fig. 3-1, to form a

Alkaline Phosphatase Signal Peptide → 42



Figure 3-1. Design of synthetic genetic construct. Numbered lines, delineate the single-stranded oligonucleotides synthesized. Arrows, above the nucleotide sequence, delineate amino acid sequences for alkaline phosphatase signal peptide and hEGF. Restriction sites are italicized.

synthetic gene that codes for the *E. coli phoA* signal peptide fused to the amino terminal end of hEGF. Within these deoxyoligonucleotides are unique restriction enzyme recognition sites. The initiator codon for N-formylmethionine of the *phoA* signal peptide is, in this case, ATG, to allow for the insertion of the *phoA* signal/hEGF construct into the *NcoI* site of the expression vector, pFL142, to form pEGF-1 (Fig. 3-2). The correct sequence and position of the genetic construct in the recombinant plasmid, pEGF-1, were verified by DNA sequencing.

Expression, purification, and characterization of hEGF. After testing several strains of *E. coli* under different media and growth conditions (data not shown), it was decided to use JM107 as the host for the expression of hEGF. Time course studies to determine the optimal times for the addition of chloramphenicol, IPTG, and harvesting (described under "Methods") were carried out by David Engler and are described elsewhere (87). Typically 600-800 μ g of purified EGF were liberated per liter of cultured cells (approximately 2-3 g of cells, wet weight).

When the samples are purified, as described under "Methods", the HPLC fraction containing receptor binding activity yields a single protein migrating with the apparent M_r of hEGF on SDS-PAGE, see Fig 3-3. A typical HPLC elution profile is shown in Fig. 3-4. Amino acid composition analysis (see Table 3-1) and protein sequencing by Edman degradation were performed on the isolated protein and the results of these analyses show that the purified recombinant hEGF is identical to authentic human EGF (97). The recombinant hEGF competed with mEGF on an equimolar basis for binding to the EGF receptor and in stimulating the EGF receptor tyrosine kinase activity, (data not shown). Previous studies have shown that mEGF and hEGF are equipotent in their receptor binding ability (70).

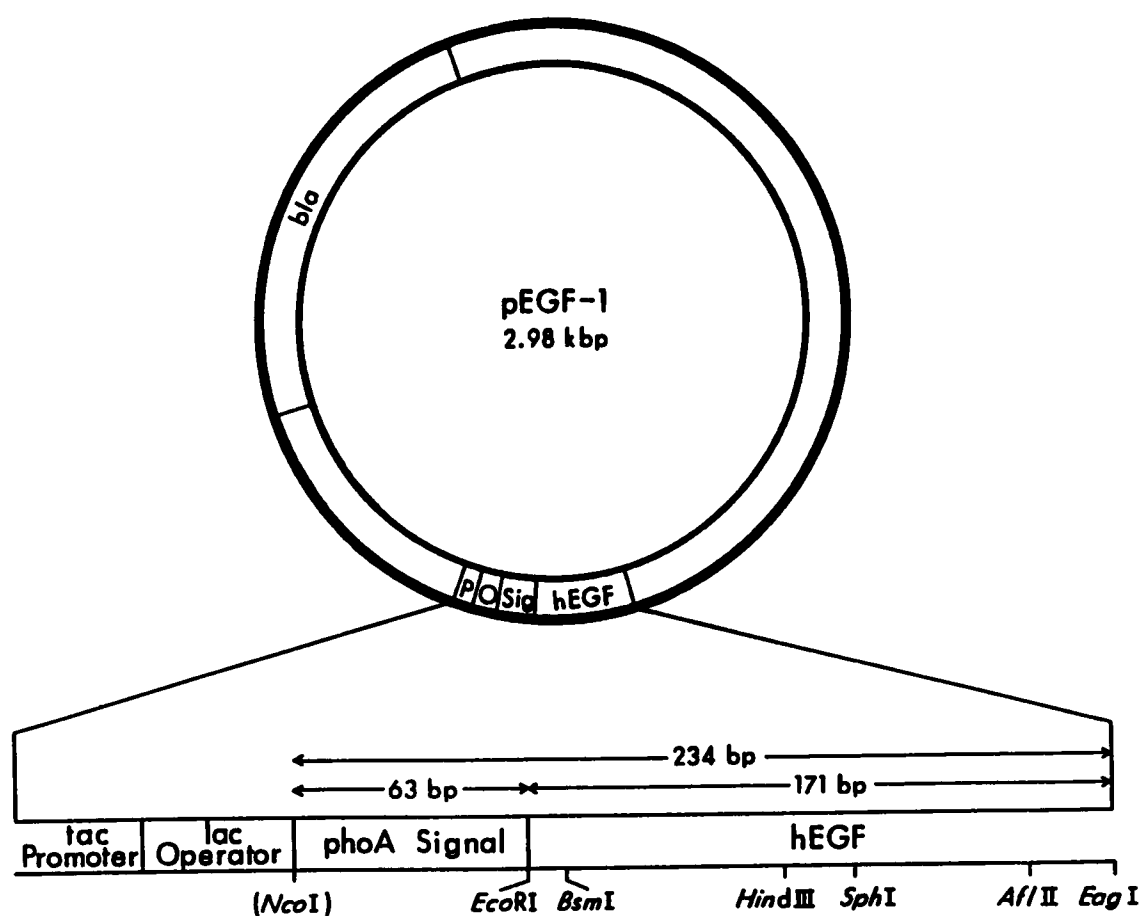


Figure 3-2. Expression plasmid pEGF-1 showing relative size of insert and positions of restriction sites. *Parenthesis* indicate the loss of the restriction site *NcoI* upon insertion into the vector.

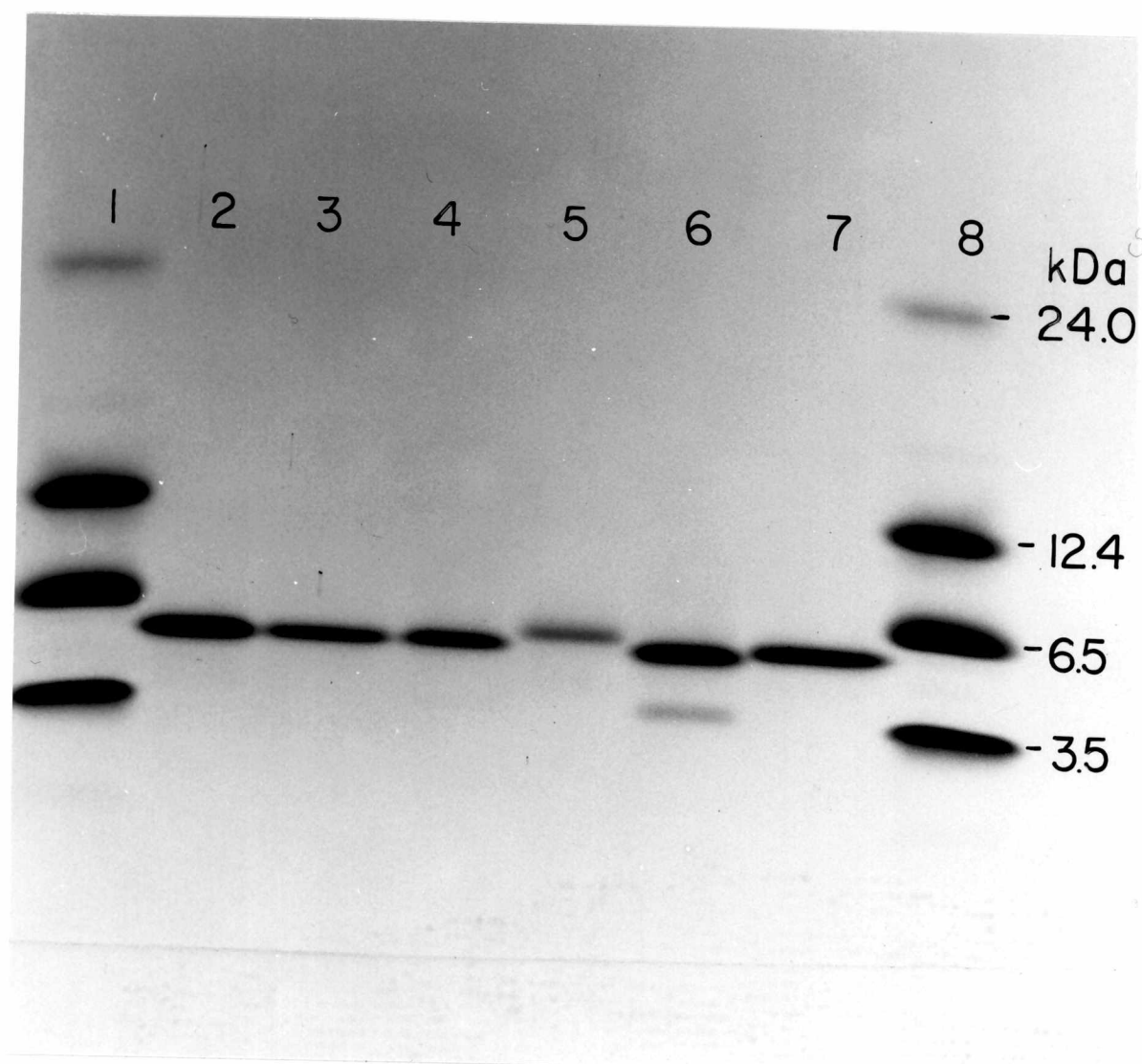


Figure 3-3. Coomassie Blue-stained SDS-PAGE of recombinant wild-type hEGF and mutant EGFs. Lanes 1 and 8, molecular mass markers; lane 2, 5 μ g of wild-type hEGF; lane 3, 3 μ g of Pro7Thr mutant; lane 7, 3.7 μ g of Leu47His mutant. Other lanes are mutants not mentioned in this thesis. Polyacrylamide concentration in the stacking gel was 5%, and the separating gel consisted of a linear gradient from 10 to 22% polyacrylamide. Electrophoretic conditions were essentially those of Laemmli (55).

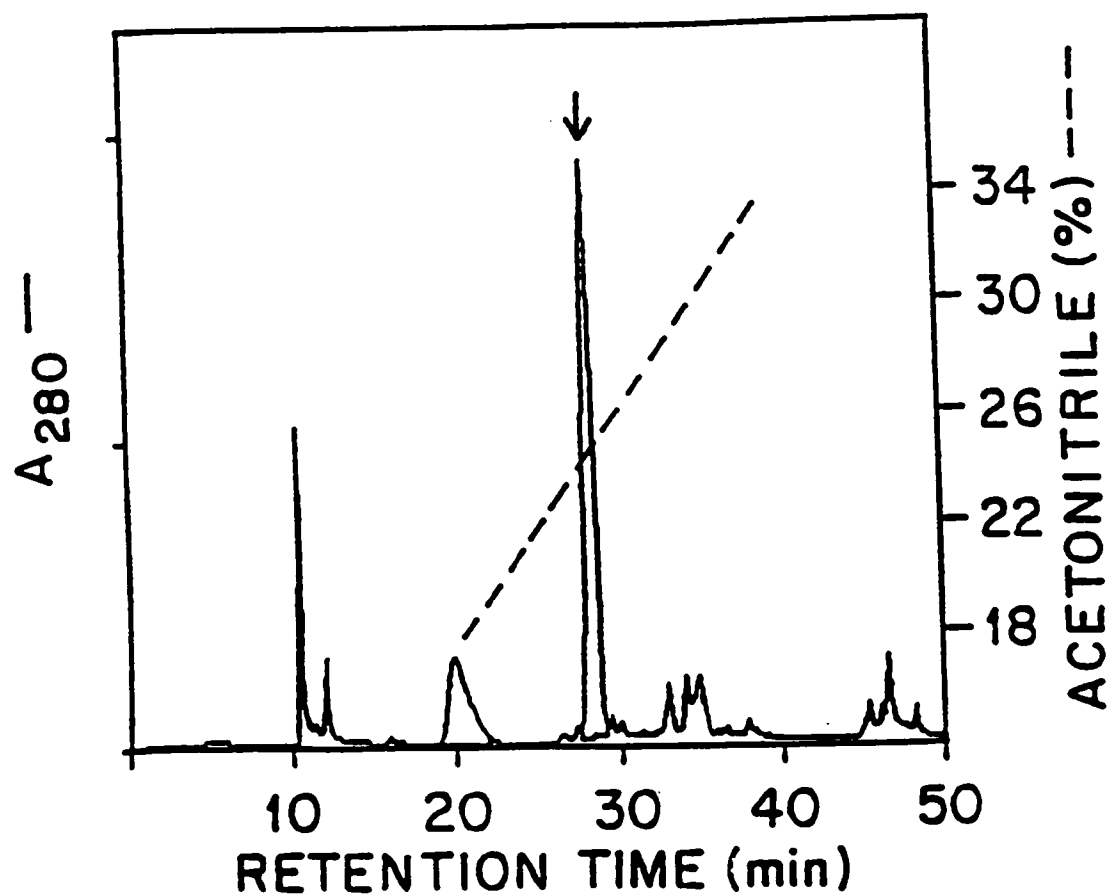


Figure 3-4. Typical elution profile of recombinant hEGF off Vydac 218TPS HPLC column. Arrow indicates peak containing EGF receptor binding activity.

Table 3-1

Amino Acid Composition of Recombinant Wild Type hEGF

Amino acid residue	Observed ^a	Calculated ^b
residues/mole		
Asx ^c	7.3	7
Thr	0.3	0
Ser	2.9	3
Glx ^d	4.7	5
Pro ^e	1.3	1
Gly	4.1	4
Ala	2.5	2
Cys ^f	4.7	6
Val	2.8	3
Met	0.6	1
Ile	2.1	2
Leu	5.3	5
Tyr	5.0	5
Phe	0.2	0
His	2.1	2
Lys	2.1	2
Trp ^g	1.8	2
Arg	3.0	3
Total		53

^aBased on a calculated M_r of 6217 after 6N HCl hydrolysis under reducing conditions, except where noted.

^bCalculated from nucleotide sequence.

^cFive aspartic acid residues and 2 asparagine residues.

^dFour glutamic acid residues and 1 glutamine residue.

^eDetermined after oxidation in dimethyl sulfoxide to remove coeluting cysteine.

^fDetermined as cysteic acid after dimethyl sulfoxide oxidation.

Generation of EGF mutants by oligonucleotide-directed mutagenesis. Single amino acid substitutions within hEGF were produced, as described under "Methods", utilizing the oligonucleotide primers shown in Table 3-2. The detection of mutant clones was done using differential hybridization of a mutant oligonucleotide probe to *hEGF* ssDNA, and by monitoring the altered restriction site. In the Leu47His mutant the *Afl*III site was destroyed in the creation of the new codon, and in the Pro7Thr mutant a *Bsm*I site was destroyed by creating a silent mutation in another codon in the oligonucleotide.

Purity and yield of isolated mutant proteins. Mutant EGF proteins were purified as described for the wild-type protein. All proteins eluted at the same position during gel filtration on Sephadex G-75 and showed similar retention times during HPLC (data not shown). These data suggest the absence of polymerization, of mispairing of sulfhydryls, and of gross conformational differences from wild-type EGF. Fig. 3-3 shows the SDS-PAGE of the mutant proteins, and scanning laser densitometry showed that the purified proteins were typically greater than 98% homogeneous.

Biochemical properties of mutant EGF proteins. Specific binding of the mutant species to the EGF receptor was calculated based on the ability of various concentrations of the mutants to compete with ^{125}I -labeled hEGF in the radioreceptor binding assay (as described under "Methods"). The concentration of the competitor giving 50% inhibition of binding (IC_{50}) was estimated graphically for each protein (see Fig. 3-5). The relative receptor affinity was calculated from this concentration compared to that obtained with wild-type EGF. Similarly, the effective concentration of each mutant hEGF resulting in 50% maximal stimulation of the EGF receptor tyrosine kinase activity (EC_{50}) was estimated from its curve representing EGF-dependent kinase activity (see Fig. 3-6).

Table 3-2
Features of Mutagenic Oligonucleotides

Mutation	Length	Sequence ^a	Monitored restriction site
Pro7Thr	19	5'-ACTCTGAATG <i>TACG</i> CTGTC-3'	<i>Bsm</i> I (-)
Leu47His	18	5'-TACCGTGACC <i>ATA</i> AGTGG-3'	<i>Afl</i> III (-)

^aMismatches are italic and boldface, and amino acid codon is underlined

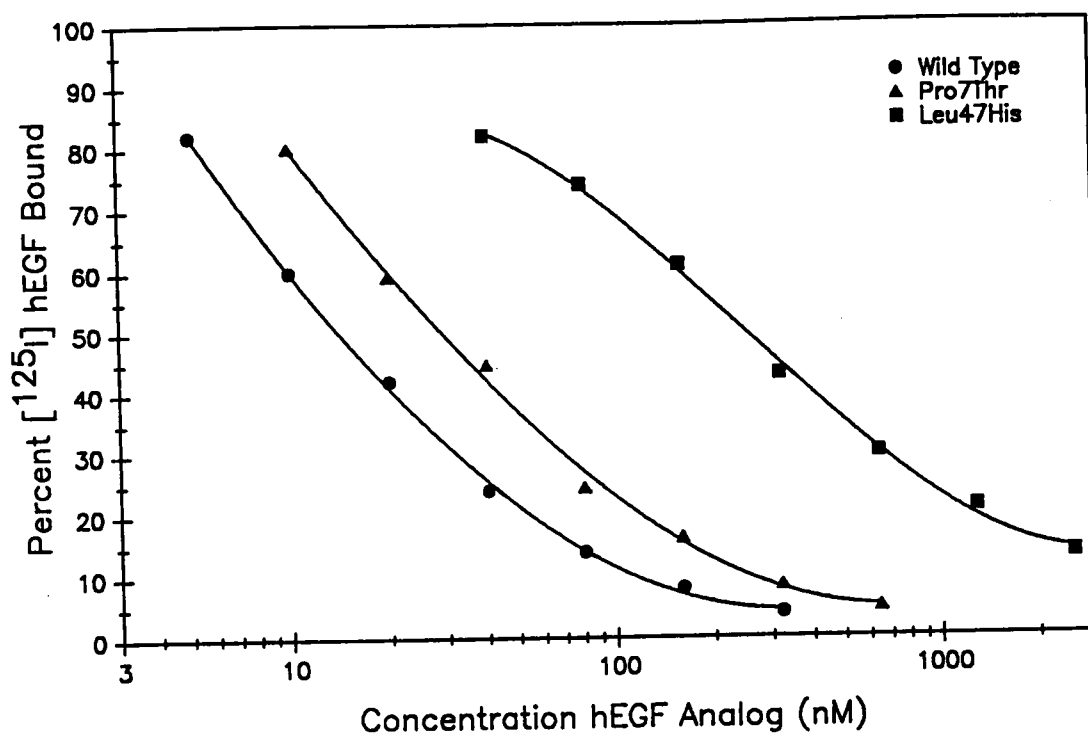


Figure 3-5. Radioreceptor competition binding curves of ^{125}I -hEGF *versus* wild type and mutant EGFs. All curves represent the best fit through the average data points of two or more experiments.

Relative receptor affinity was determined by comparison of this value with that of wild-type EGF. Table 3-3 shows a comparison of the relative receptor affinities of the two mutants compared to wild-type, by both assays. The relative values obtained by the two assay methods were in agreement.

Discussion

Previous work by Oka *et al.* (29), who had constructed a secretion system placing hEGF behind a *phoA* signal and under a *phoA* promoter, produced EGF as a secretory protein under phosphate limiting conditions. We chose to regulate the secretion of hEGF to the periplasmic space of *E. coli* by placing the EGF gene under the transcriptional control of the *tac* promoter and *lac* repressor/operator. The signal peptide for alkaline phosphatase, a natural constituent of the periplasm, allowed for the secretion of EGF across the bacterial inner membrane. This system was advantageous for several reasons. These are 1) The *tac* promoter and *lac* repressor/operator allow transcriptional control of the gene by induction with IPTG. This allowed an easier means of inducing EGF expression than altering phosphate concentration. 2) Enzymatic cleavage *in vivo* of the signal peptide left the proper amino terminal residue on hEGF. 3) Isolation of periplasmic protein was a simple one step procedure. 4) An initial purification of the EGF was obtained at this step since only 4% of *E. coli* proteins are found in the periplasm (98). 5) Localization in the periplasm protected hEGF from cytoplasmic proteases (99,100,101) and provided a lower reducing potential to facilitate proper folding. Insulin, another small protein, was found to be 10 times more resistant to proteolysis in the periplasm than in the cytoplasm (102).

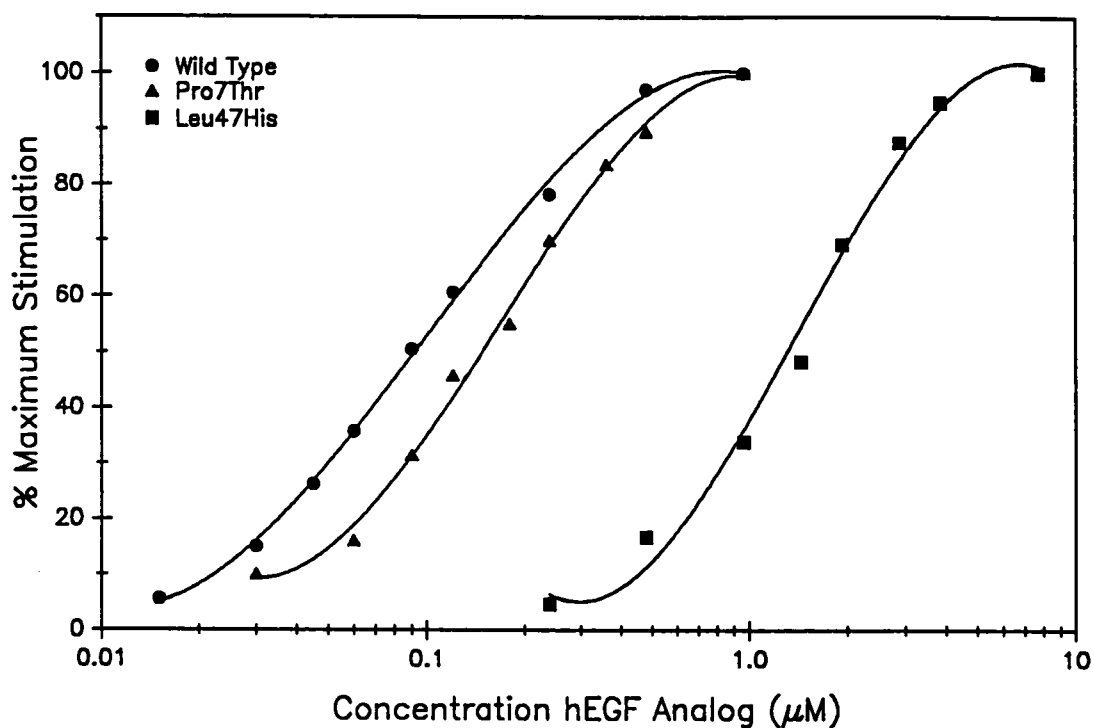


Figure 3-6. Dose-response curves for the stimulation of the EGF receptor tyrosine kinase activity by wild type and mutant EGFs. Curves are plotted as percent of maximum stimulation, and represent the best fit through the average data points of two or more experiments.

Table 3-3
Comparison of Receptor Affinity

EGF species	Relative receptor affinity	
	Radioreceptor competition assay ^a	Tyrosine kinase stimulation assay ^b
	<u>% of wild type</u>	
Wild type	100	100
Pro7Thr	55	53
Leu47His	7	11

^aRelative receptor affinity = $IC_{50}(\text{wild type})/IC_{50}(\text{mutant}) \times 100\%$. Values were derived from Fig. 3-5.

^bRelative receptor affinity = $EC_{50}(\text{wild type})/EC_{50}(\text{mutant}) \times 100\%$. Values were derived from Fig. 3-6.

Chemical synthesis of the gene allowed for the placement of unique restriction sites which facilitated the screening process during mutagenesis. These restriction sites also render the gene suitable for cassette mutagenesis (103) in which mutant sections of the gene can be introduced through synthetic double stranded oligonucleotides placed between two successive restriction sites. Also, an *EcoRI* restriction site was engineered at the junction of the hEGF and *phoA* signal genes which would allow the insertion of other cloned genes in the place of EGF, thus creating a standard vector for the secretion of any given protein to the periplasm.

Two residues, proline at position 7 and leucine at position 47, were initially chosen as target sites for site-directed mutagenesis studies. The reasons for choosing these sites were based on: 1) species conservation studies showed that both of these positions were highly conserved throughout all known EGFs and EGF-like species (1); 2) data obtained with peptide fragments (see "Introduction"); and 3) solution structures of mEGF (16) and hEGF (19), from two-dimensional nuclear magnetic resonance (NMR) spectroscopy and computer modeling.

Proline 7 in the A-loop (containing amino acid residues 6-14) was replaced with a threonine, an amino acid lacking the structural characteristics of the imino acid proline. Structural NMR data have placed Proline 7 in close proximity to the aromatic residues of both the A- and B-loops (16). Proline 7 resides next to cysteine 6, which is involved in an intramolecular disulfide bond thought to stabilize interactions between the A- and B-loops, therefore, a Pro7Thr mutation might be expected to have considerably impaired function. However, from both the competition binding and kinase stimulation assays, the Pro7Thr mutant was greater than 50% as effective as wild type (Table 3-3). The substantial activity displayed by the Pro7Thr mutant suggests an apparent lack of

requirement for this highly conserved and structurally distinctive residue for receptor binding.

The leucine 47 residue was not expected to be necessary for receptor binding based on NMR data and computer generated structural models (16,19). The loss of activity (Table 3-3) through the introduction of a histidine indicates that there is a requirement for a "leucine type" residue at this position. The histidine residue may introduce structural constraints in binding due to its aromatic side chain. The data obtained from other mutants having more conservative changes at this site will be discussed in the next chapter.

The lowered binding affinities of these mutants may come about from either loss of an amino acid needed for ligand-receptor interaction, or loss of a residue needed for maintaining some feature of the three-dimensional structure. Physical analysis of the mutant hEGFs was done utilizing NMR spectroscopy to determine if the mutants were structurally intact. Results from these studies will be discussed in the next chapter.

CHAPTER 4

THE IMPORTANCE OF THE LEUCINE RESIDUE AT POSITION 47 OF HUMAN EPIDERMAL GROWTH FACTOR

Introduction

The results found for the Leu47His mutant described in Chapter 3 suggested that it may be critical for receptor binding. The importance of this leucine residue for receptor binding has also been suggested by results of others. Burgess *et al.* (73) and Gregory *et al.* (103) have treated mEGF or hEGF with trypsin to produce a fragment containing amino acids 1-48. This was further digested with carboxypeptidase to produce fragments of residues 1-47 and 1-46. The 1-48 fragment had full potency *in vivo* (73) while the 1-47 fragment had 10% activity and the 1-46 fragment had less than 1% activity of the intact molecule. These results imply that either the leucine residue itself is necessary for activity, or that the active EGF molecule has a required length that includes amino acid 47. Structural models based on nuclear magnetic resonance (16,19) have not predicted a defined structural placement for leucine 47.

To address the importance of the leucine residue, several other groups have mutated this site in mouse EGF (mEGF) and human TGF- α (hTGF- α) (see Fig. 1-3, page 6 for protein sequences). Their work was published as the studies detailed in this chapter were being done. Lazar *et al.* (105) expressed mutants of hTGF- α in a yeast expression system in which the leucine at position 47 was changed into an isoleucine, alanine and methionine. Their results show that the alanine mutant was unable to compete against labeled EGF in competition binding assays, nor was it able to stimulate the formation of colonies in soft agar in the presence of TGF- β , which is a characteristic of transformed

cells and an assay often used to determine TGF- α activity (106). The methionine and isoleucine mutants were reported as having low activity. Site-specific mutagenesis of mEGF was done by Ray *et al.* (107), and leucine 47 was changed to a serine and a valine. The activities of these mutants, measured as receptor affinities using membrane fractions and whole cells, and by receptor autophosphorylation, were 20-33% for the valine and 6-14% for the serine. A more detailed account of these studies is addressed in the "Discussion"

In this chapter, the results of seven amino acid changes at the leucine 47 site in hEGF are described. The studies were done to determine the structural requirements of the amino acid at this site needed to elicit full biological activity. Besides the competition binding assay and the receptor tyrosine kinase assay, wild type EGF and these mutants were assayed for their ability to stimulate the uptake of thymidine in tissue culture cells. In addition, the structural integrity of some of these mutants were examined through the use of NMR spectroscopy.

Materials and Methods

Construction of mutant genes and generation of mutant proteins. The genes coding for the mutant EGF proteins were constructed according to the procedures outlined in Chapter 3. Mutant proteins were isolated and purified in the manner also detailed in Chapter 3.

Radioreceptor competition binding assays and tyrosine kinase stimulation assays. The procedures for these assays are described in Chapter 3.

Tissue culture studies. Stimulation of mitosis was measured by the uptake of [^3H] 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 Carpenter and Cohen (108) that 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 EGF proteins were added at different concentrations and the cells were incubated for 20-22 hours, after which time [^3H] thymidine was added (specific activity 0.4 Ci/mmol) to a final concentration of 5 μM . After incubation at 37°C for four additional hours, the media was siphoned off and the cells were washed two times with one ml (PBS). Cells were rinsed with T+V (0.025% trypsin, 0.02% EDTA) and incubated at 37°C to allow the cells to detach from the dishes. Cells were resuspended in 2 ml of cold PBS. One ml (or less) was diluted with 10 ml of Isoton II (Coulter Diagnostics) and the number of cells determined using a Coulter counter. One ml 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 liquid scintillant, and counted. The amount of thymidine incorporated per cell was calculated, and the values as a percent of maximum stimulation was determined.

Nuclear magnetic resonance 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 D₂O 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 by sitting at 22°C for at least 3 hours before spectral analysis. NMR spectra were acquired at 30°C on a Varian VXR 500 (500 MHz) spectrometer. One dimensional spectra were collected using the pulsed Fourier mode with 512 scans per spectra, and with pre-irradiation of the residual solvent resonance. These experiments were carried out under the direction of and in the laboratory of Dr. Gaetano Montelione, Department of Chemistry, Rutgers University and the New Jersey Center for Advanced Biotechnology and Medicine.

Results

Oligonucleotide primers. Table 4-1 shows the oligonucleotide primers that were synthesized in order to produce the mutant replicative form DNA. All oligonucleotides were 18 bases long with one or two misparings, and all destroyed the *Afl*III restriction site.

Radioreceptor competition binding assay. The mutants were assayed for their ability to compete with ¹²⁵I-labeled wild type EGF in binding to the EGF receptor of A431 membrane fractions. Binding curves are shown in Fig. 4-1 and are plotted as μ M EGF analog vs % maximum binding with the maximum [¹²⁵I]-EGF binding in the absence of cold EGF protein representing 100%. The curves presented are an average of two or more experiments. The IC₅₀ value, or the amount of protein needed to compete with 50% of the labeled EGF, is reported in Table 4-2 for each mutant, as well as its relative receptor affinity compared to wild type. The most conservative change to an isoleucine lowered binding to 16.9% of that of wild type. As the side chain is shortened to an alanine and

Table 4-1
Features of Position 47 Mutagenic Oligonucleotides

Mutation	Length	Sequence ^a
Wild Type (Leu)	--	5'-TAC CGT GAC CTT AAG TGG-3'
Gly	18	5'-TAC CGT GAC <u>GGT</u> AAG TGG-3'
Ala	18	5'-TAC GCT GAC <u>GCT</u> AAG TGG-3'
Ile	18	5'-TAC CGT GAC <u>ATT</u> AAG TGG-3'
Pro	18	5'-TAC CGT GAC <u>CCT</u> AAG TGG-3'
Asp	18	5'-TAC CGT GAC <u>GAT</u> AAG TGG-3'
Arg	18	5'-TAC CGT GAC <u>GGT</u> AAG TGG-3'

^aMismatches are underlined and boldface

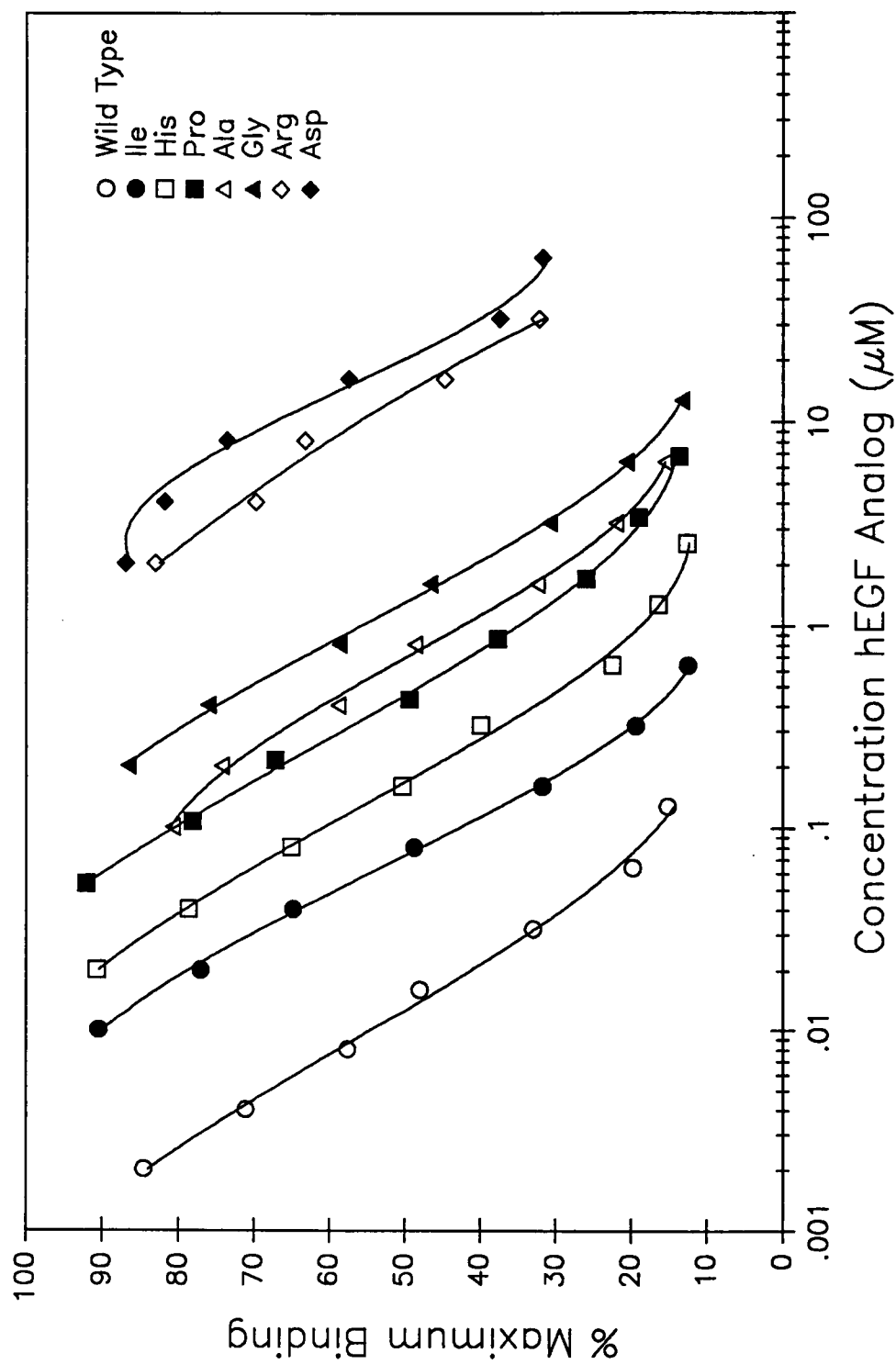


Figure 4-1. Radioreceptor competition binding curves for leucine 47 hEGF mutants plotted as a percent of maximum binding. Curves represent the best fit through the average data points of two or more experiments.

Table 4-2
Biochemical Properties of Leucine 47 hEGF Mutants

Residue at Position 47 of hEGF	Radioreceptor Competition assay		Tyrosine Kinase Stimulation assay				Tissue Culture	
	IC ₅₀ μ M	Relative Receptor affinity ^a	EC ₅₀ μ M	Relative Receptor affinity ^b	V _{max} cpm x 10 ⁻³	Relative Stimulation ^c	[³ H]Thymidine uptake 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

^aRelative receptor affinity = IC₅₀(wild type)/IC₅₀(mutant) x 100%. Values were derived from Fig. 4-1

^bRelative receptor affinity = EC₅₀(wild type)/EC₅₀(mutant) x 100%. Values were derived from Fig. 4-3

^cRelative stimulation = V_{max}(wild type)/V_{max}(mutant) x 100%. Values were derived from Fig. 4-2

^dRelative mitogenic activity = ED₅₀(wild type)/ED₅₀(mutant) x 100%. Values were derived from Fig. 4-6

^end = not determined

glycine the binding was further diminished to 1.8% and 0.94%, respectively. The charged amino acids, of arginine and aspartic acid were the least tolerated mutations leading to binding values of less than 0.1% of wild type. The proline and histidine mutations were poorly tolerated at 2.3% and 7.2% of wild type, respectively.

Stimulation of the EGF receptor tyrosine kinase. Five of the mutants were assayed for their ability to stimulate the EGF receptor's protein tyrosine kinase. Determination of this stimulation was measured by the amount of ^{32}P label incorporated into exogenously added $(\text{Glu,Tyr})_n$ polymer substrate. Fig. 4-2 shows the phosphorylation curves of wild type and five leucine 47 mutants plotted as concentration of analog vs $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ incorporated. A noticeable feature of this graph is that the maximum velocity of the reactions with the mutants is lower than the maximum velocity with wild type. The lower V_{max} is seen with many, of the poorer binding mutants, and a more detailed study is described in Chapter 5. Table 4-2 lists the V_{max} values and how they compare to wild type.

To determine the EC_{50} values, the concentration of mutant EGF that gives 50% maximum stimulation of the kinase reaction, the curves were plotted as percent of maximum stimulation and are shown in Fig. 4-3. The EC_{50} values and the relative receptor affinities are shown in Table 4-2. The aspartic acid and arginine mutants could not be assayed in this manner due to their low receptor affinities; the kinase reaction was unreliable at the high protein concentrations used. This will be discussed later in this chapter. The order in which the mutants stimulate the tyrosine kinase activity follows the same order as in the radioreceptor binding assay, and their relative receptor affinities are also similar, differing by less than 2%.

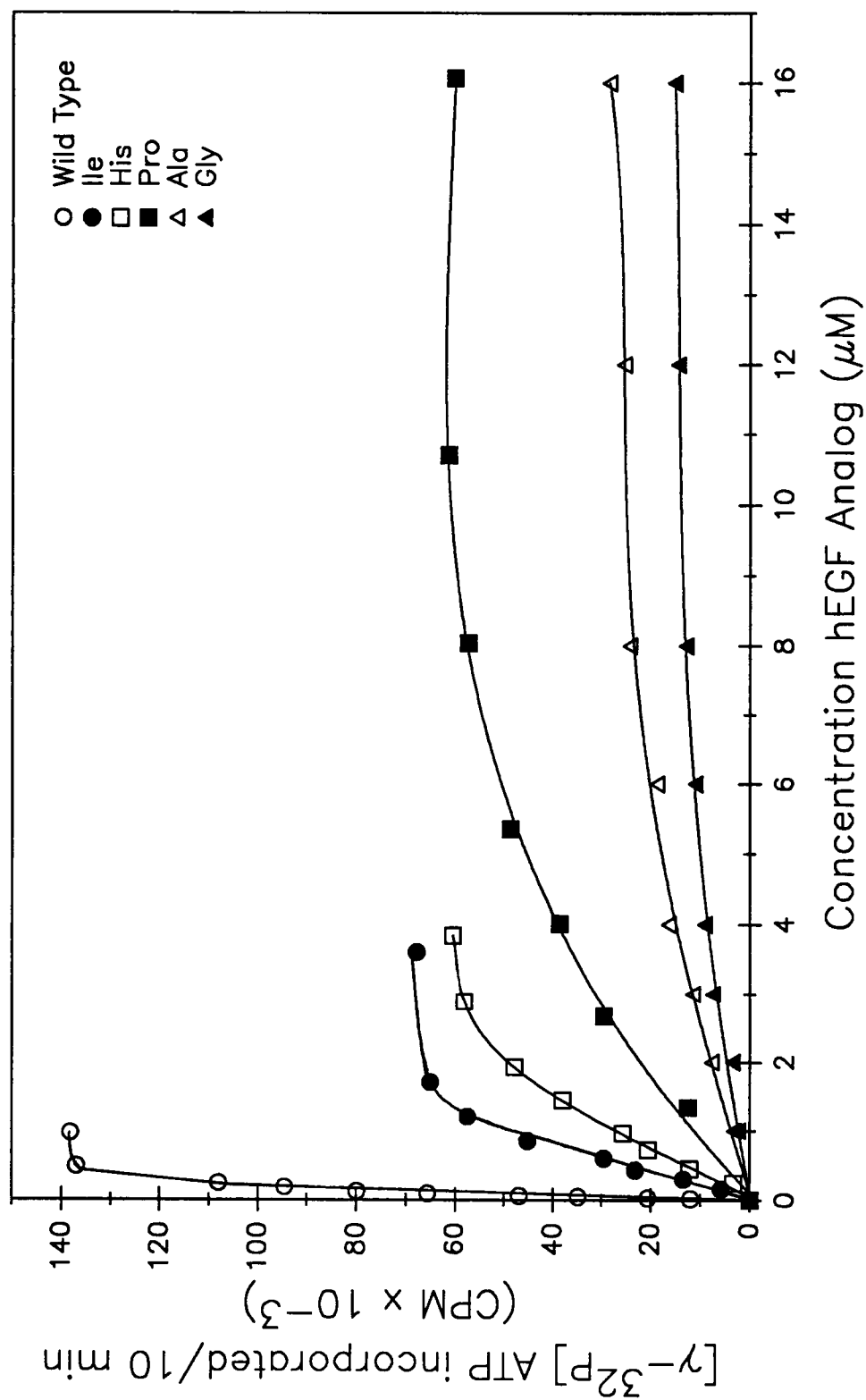


Figure 4-2. Stimulation of the EGF receptor's protein tyrosine kinase activity by the leucine 47 mutant proteins. Curve represent the best fit through the average data points of two or more experiments.

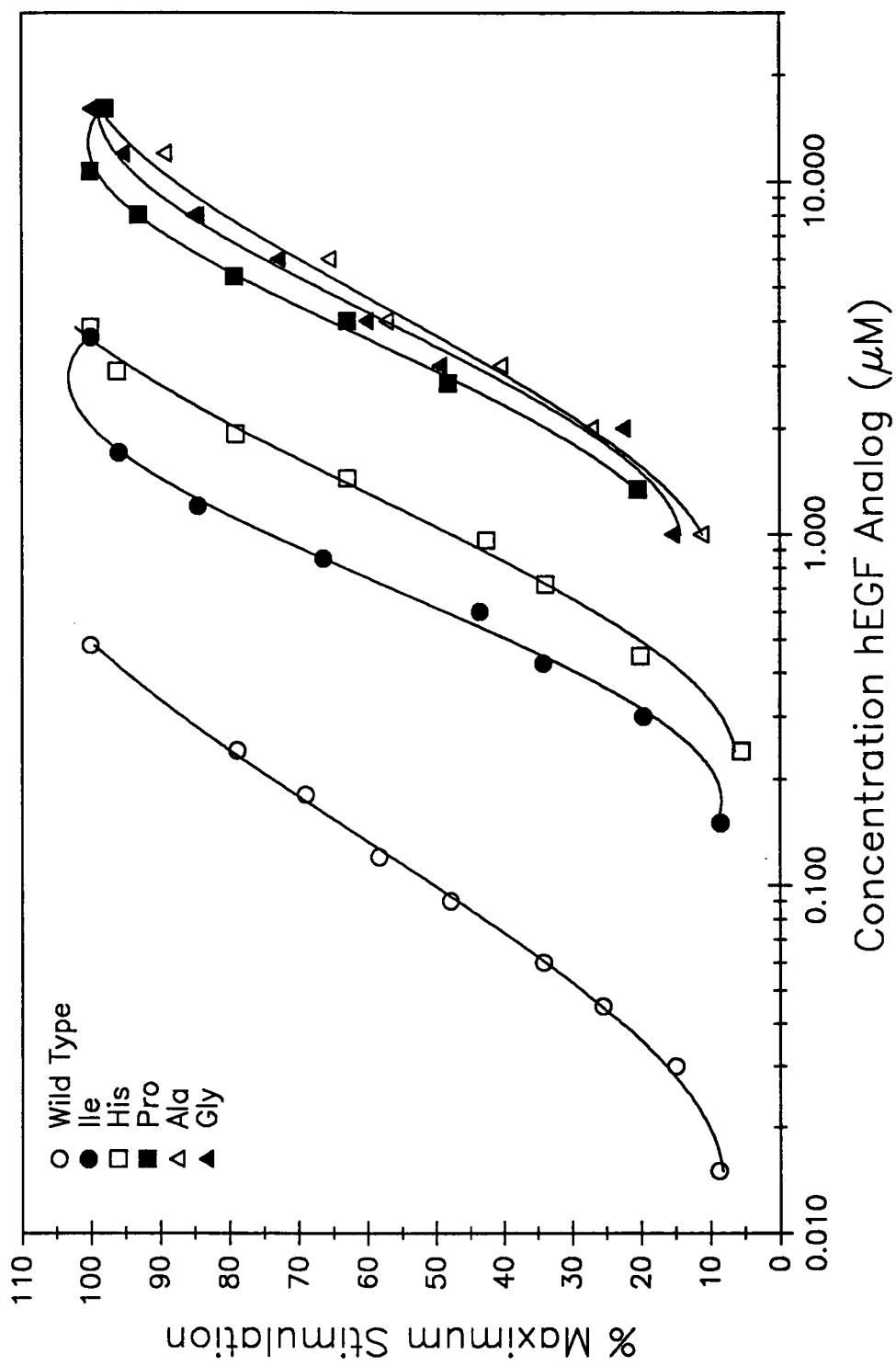


Figure 4-3. EGF receptor kinase activity of the leucine 47 mutants plotted as a percent of maximum stimulation. Curves represent the best fit through the average data points of two or more experiments.

Breakdown of the kinase assay at high EGF concentrations. With some of the mutants that had a lower receptor affinity it was observed that the maximum velocity of the kinase reaction was lower than that of wild type. In addition, the velocity reached a maximum and then decreased as the protein concentration increased. To test whether this was a direct effect of the EGF mutant analog, or some inhibitory factor in the assay due to the use of large amounts of protein, several parameters were tested. The HPLC-purified proteins are in 10 mM phosphate buffer (1-2 ml), which was lyophilized to remove acetonitrile and to concentrate the protein. It seemed possible that sodium phosphate, also concentrated upon lyophilization, might inhibit the phosphorylation reaction; therefore, the effect of phosphate concentration on the reaction was determined (Fig 4-4). Phosphate concentrations above 5 mM inhibited the reaction, so the lyophilized proteins were routinely dialyzed against water prior to use.

The next parameter examined was the effect of high concentrations of protein on the reaction. Both specific (wild type hEGF) and non-specific binding proteins, were tested in the reaction. Bovine serum albumin (BSA) at concentrations as high as 400 μ g (in the presence of 90 μ M hEGF) showed no inhibitory or stimulatory effect (data not shown). A wild type hEGF curve (to 450 μ M) showed that stimulation was fairly constant from 0.5 μ M to 10 μ M, but then gradually decreased until at 450 μ M the counts incorporated were 35% of the $V_{\max}$ (Fig. 4-5). Why high concentrations of EGF protein should have an inhibitory effect on the assay is unknown, but may be due to an aggregation of the EGF molecules or the EGF/receptor complex, or inhibition by contaminating material.

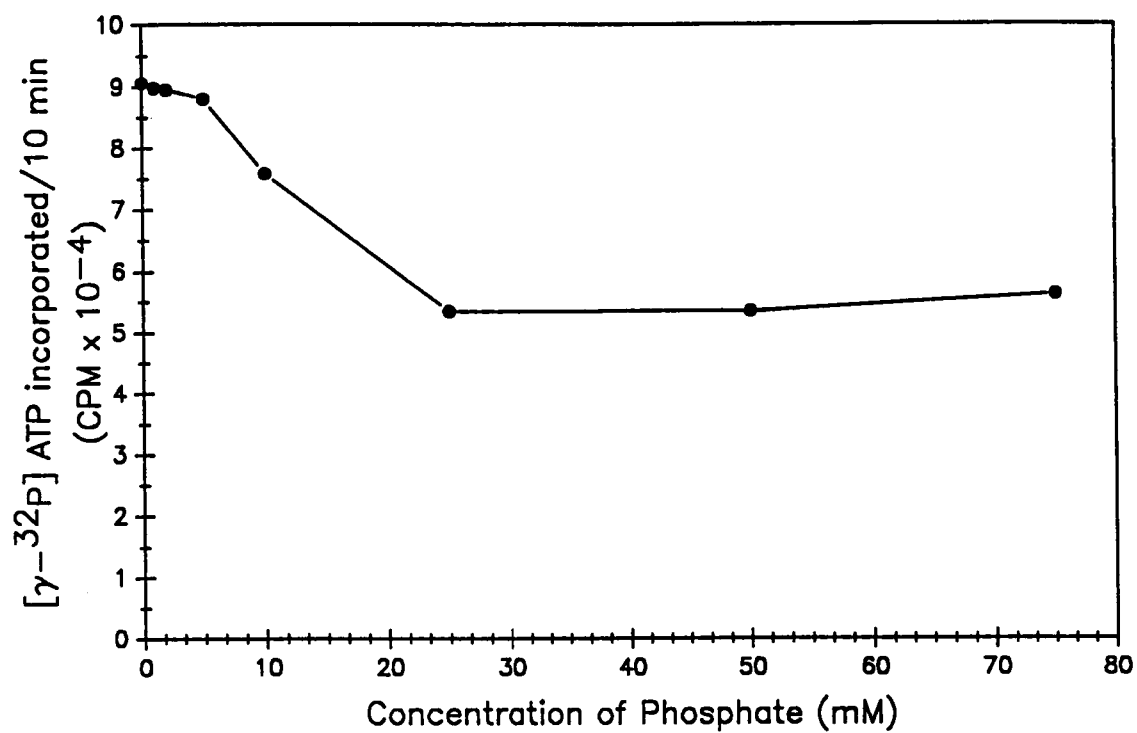


Figure 4-4. EGF receptor kinase activity in the presence of an increasing concentration of sodium phosphate. The reactions were done in the presence of 90 nM hEGF.

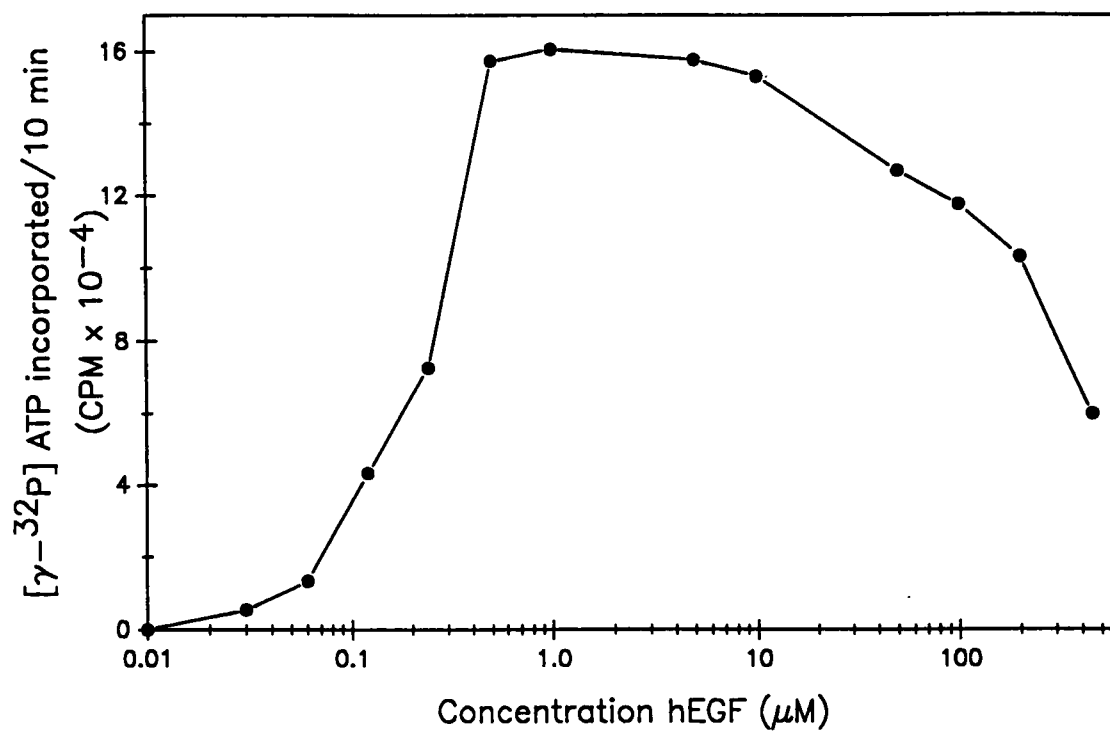


Figure 4-5. Inhibition of the EGF receptor tyrosine kinase activity using high concentrations of wild type hEGF.

Tissue culture results. All seven mutants were assayed for their ability to stimulate mitosis in a fibroblast cell line as measured by [^3H] thymidine incorporation. The cells incorporated [^3H] thymidine in response to EGF in a concentration dependent manner, as shown in Fig. 4-6. 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 4-2 as well as their relative percentage compared to 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. The alanine, at 25%, and proline, at 22.6%, had a noticeably better activity than displayed in the other assays. All the mutants tended to be more active in their response (as a percent of wild type) in this assay as compared to the *in vitro* receptor binding and phosphorylation assays.

NMR results--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 shows the resonances for the protons from aromatic residues, are shown in Fig. 4-7. The spectra are arranged from bottom to top in the order of best binding to worst binding EGF 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 plots tend to look less "wild type-like" as the binding affinities decrease, but the mutant EGFs still contain most of the wild type structure. Assignments for the peaks have not yet been determined.

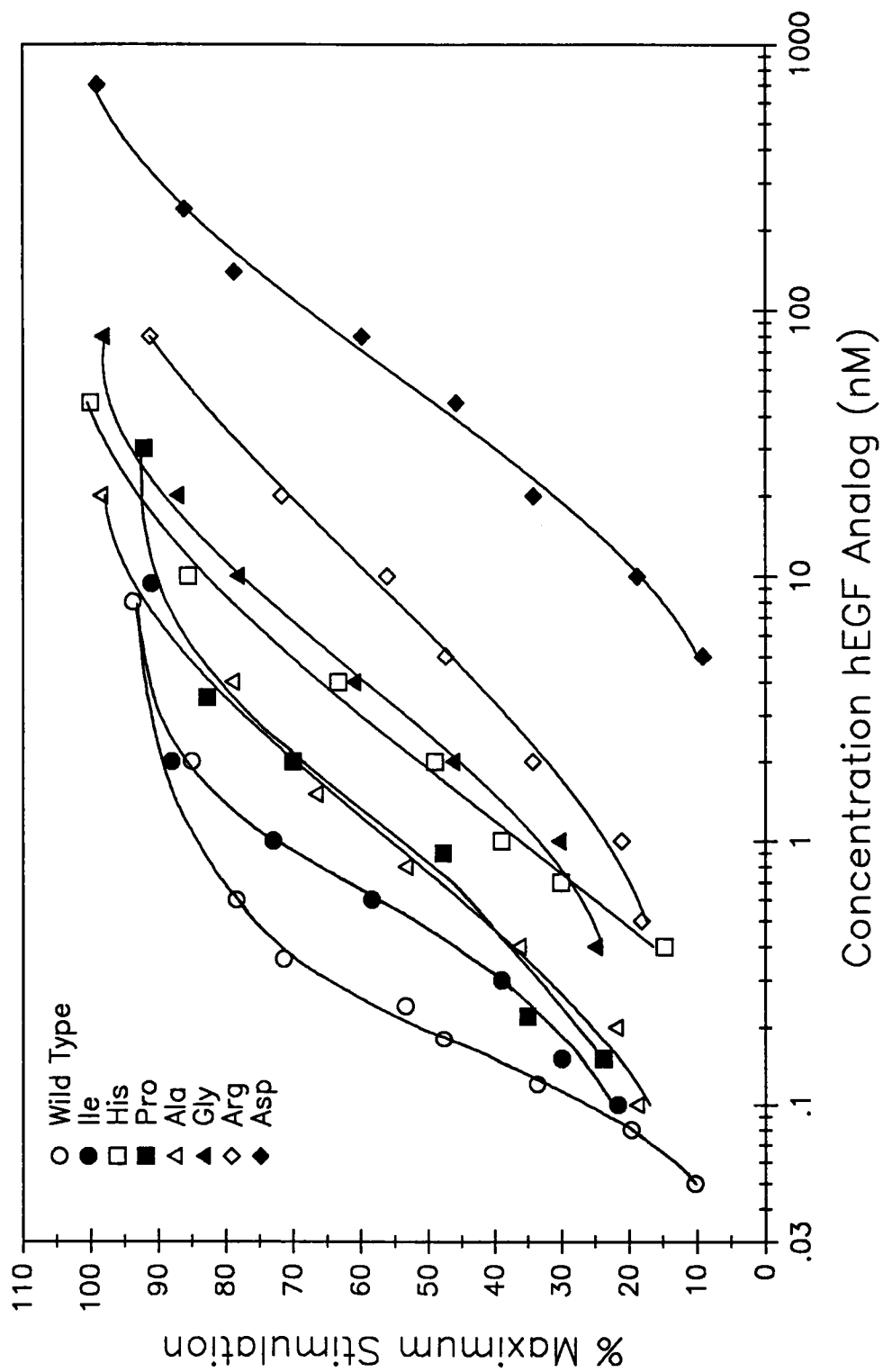


Figure 4-6. Stimulation of ^3H thymidine uptake of A31 cells by leucine 47 mutants of hEGF; percent maximum stimulation. Curves represent the best fit through the average data points of three experiments.

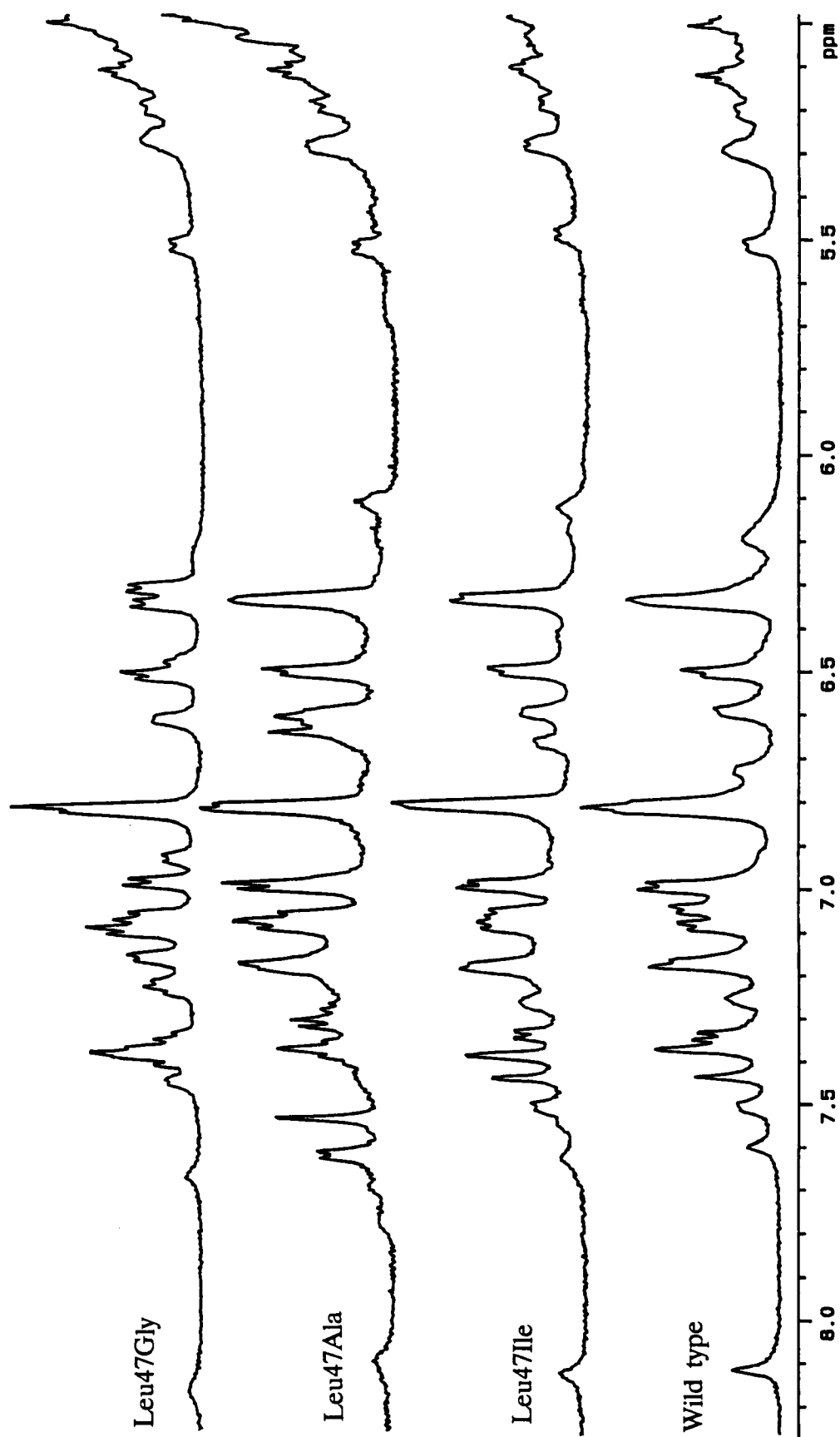


Figure 4-7. Comparison of the downfield resonances of 1D ¹H-NMR spectra of wild type and leucine 47 mutants. All spectra represent the average of 512 scans on a Varian VXR500 spectrometer under the conditions described in the text.

Discussion

The results of the three assays, receptor binding, phosphorylation, and stimulation of mitosis, suggest that the leucine residue itself is necessary for full receptor affinity and activation of the other responses, but the residue is not exclusively needed for activity since other mutations are tolerated. The changes to an aspartic acid or arginine are the least tolerated, implying that there may be some type of hydrophobic interaction between the receptor and the ligand at this position of the ligand.

In an attempt to see if the lower receptor affinities of the isoleucine, alanine, and glycine mutants were due to structural changes induced in the molecule by the mutations, one dimensional proton NMR analysis was performed. 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.* (109) who showed that the 1D spectrum is a reliable indicator of structural changes that are seen in follow-up 2D NMR analysis. Fig 4-7 shows the mutant and wild type spectra in the region where aromatic protons resonate. This region was chosen for comparison because aromatic residues are spread throughout the molecule, thus any structural perturbations would be detected by these residues.

The decision to look at the isoleucine, alanine and glycine mutants for NMR analysis was partly based on the inappropriateness of structural determination with charged amino acid substitutions. If charged residues had been introduced in this area, chemical shift differences seen might have been attributed to local electric field effects on the surrounding resonances and not to structural changes. Another reason for choosing isoleucine, alanine and glycine was to ascertain whether any loss of structural integrity of the molecule could be correlated with chain shortening and loss of activity. This appears

to be the case, but it should be noted that the overall structure is still well preserved, and that structural perturbations are not the cause for lower biological activity.

If the IC_{50} 's of receptor binding and the EC_{50} 's of tyrosine kinase stimulation for each of the EGF analogs are compared, it is seen that it takes from three to eight fold more EGF to cause 50% maximum stimulation in tyrosine kinase activity than it takes to cause 50% competition in the binding assay. There are several reasons which may explain this discrepancy. First, the physical environment of the receptor protein is different in the two assays. The binding assay is done using membrane fractions and the receptor is still in a specified orientation and is free to move within the liquid membrane. The phosphorylation assay is done with solubilized receptors. Another reason for the quantitative differences may be that different types of activities are measured. The binding assay is based on equilibrium binding measurements, while the phosphorylation assay measures the ability of the EGF ligand to stimulate an enzymatic reaction--the receptors ability to phosphorylate itself and other substrate molecules. The binding of one ligand to the receptor does not necessarily correspond to the addition of one phosphate group to the substrate; the binding may trigger one or many such actions of the receptor.

The lower V_{max} of the mutants in the phosphorylation assay did not appear to have any effect on their stimulation of thymidine incorporation under the assay conditions used here. Although time courses were not run, others have reported that cells need to be exposed to EGF for at least 8 hours before they are committed to mitosis (108,110,111). The mutants with the lower V_{max} may require more time than wild type to commit to mitogenesis, and preliminary work on other lower V_{max} mutants indicate that this may be the case (Melinda Hauser, personal communication). Others have reported that only a small percentage of the receptors need to be occupied for full biological effect (112,22); thus the binding of some of the mutant ligand would be enough to trigger mitogenesis.

Since the binding of EGF to its receptor triggers a variety of cellular responses (6) it is reasonable to assume that there is more than one pathway by which EGF acts. The stimulation of mitosis is the end result of one or more of these pathways. The binding of EGF is known to stimulate the receptor's tyrosine kinase, and this is necessary for mitogenesis (43,113). The substrates for the EGF receptor kinase then act upon other molecules in the cell. Other events which happen between the time of EGF binding and the eight hours needed for commitment to mitosis remain a mystery. Because there are an unknown number of steps from the time of EGF binding to the receptor to the time of DNA synthesis, one cannot compare the effective concentrations of EGF in the binding and phosphorylation assays *in vitro* to the effective concentration for stimulating DNA synthesis *in vivo*. In addition, EGF is not the only growth factor responsible for the stimulation of mitogenesis. Other growth factors can act synergistically with as well as antagonistically to EGF in determining cell growth rates. These include transforming growth factor- β , platelet derived growth factor, fibroblast growth factor, insulin, and insulin-like growth factors (for review see 114). Some of these factors exist in the serum added to the cells. The action of these growth factors depends upon cell type and temporal restrictions. These facts may explain why there is such a difference between the effective doses *in vitro*, which are on a micromolar level, to the effective concentration for DNA synthesis, which are on the nanomolar level.

As mentioned in the "Introduction", leucine 48 in hTGF- α was mutated to an isoleucine, alanine and methionine, and these variants were reported as having low to no activity. Although the conclusion of Lazar *et al.* (105) supports the results found in this thesis with regard to the importance of the leucine residue for binding, the data upon which they were based are questionable. The hTGF- α used was not highly purified, and

in quantification of the protein, it was assumed that all the protein detected was properly folded, which may not have been the case. Also in the case of the methionine mutant, it did not have the correct amino and carboxyl terminal ends.

Site-specific mutagenesis of mouse EGF done by Ray *et al.* (107), used an expression system similar to the one described in this study in that they secreted the protein into the periplasm of *E. coli*. The activities reported for their mutants are similar to those reported here. In a follow up paper, Moy *et al.* (109), show one and two dimensional NMR spectra of the serine mutant. Results suggest no significant structural change in this mutant compared to wild type EGF.

While this thesis was in preparation, Dudgeon *et al.* (115) published work on mutagenesis of the leucine 47 site in hEGF. Using a yeast expression system they were able to purify EGF molecules that were 52 amino acids in length, lacking residue 53. Four variants of leucine 47 were created, changing the leucine to an alanine, glutamic acid, aspartic acid and valine. Results from their binding assays showed that the valine mutant had 14% of the activity of wild type and the other 3 mutants only about 2%. 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 looked at the one-dimensional NMR spectra of the four variants (although their study was done at non-physiological pH 3.0) and they 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 importance of the leucine 47 site in hEGF and EGF-like molecules for receptor binding has been confirmed by the present work and the work of others (73,104,105,107,115). The fact that it is highly conserved lends credence to its importance.

It is apparent that the leucine residue itself is necessary for full activity, for even the most conservative changes to a valine or an isoleucine diminish the activity to less than one third that of wild type. However, studies with an EGF-like molecule, amphiregulin, show that other structural features may replace the leucine.

Amphiregulin, an 84 amino acid glycoprotein, is a bifunctional cell growth modulator for it is able to stimulate the growth of some cells while inhibiting the growth of others (116). It was first isolated from a human adenocarcinoma cell line. The carboxyl-terminal half of amphiregulin (residues 46-84) shows a 38% homology to hEGF with the correct alignment of cysteines, but its sequence ends at residue 45 of hEGF, thus lacking the leucine 47 residue. Despite this truncation, amphiregulin is able to bind to the EGF receptor (15). However, it is capable of displacing only 75% and 50% of ^{125}I -EGF in competition binding assays using whole cells and membrane fractions, respectively. The competition binding curves for amphiregulin suggest a lower receptor affinity (10% of wild type EGF) for the EGF receptor than EGF itself. In cell culture, amphiregulin can fully supplant the requirement for EGF or TGF- α for murine keratinocyte growth, but was unable to induce anchorage-independent growth of rat kidney cells, unlike EGF or TGF- α .

These results indicate that the leucine 47 residue is not essential for amphiregulin to bind to the EGF receptor, which contradicts the work done with EGF (73,104) that showed little binding capability of truncated forms of EGF lacking leucine 47. There must be additional residues in amphiregulin that may explain its ability to bind to the EGF receptor, albeit with lower affinity. The first 45 amino acids residues at the NH_2 -terminal end of amphiregulin are not homologous to EGF, and may have an influence on receptor binding. This amino terminal end is very hydrophilic and contains unusually high numbers

of lysine, asparagine and arginine residues. The fact that amphiregulin can replace EGF in tissue culture studies of some cell lines and not others may be due to the presence of a separate receptor for amphiregulin that is found on some cell types and not others, although there is no conclusive evidence for this at present.

CHAPTER 5

ANALOGS OF HUMAN EPIDERMAL GROWTH FACTOR WHICH PARTIALLY INHIBIT THE GROWTH FACTOR-DEPENDENT TYROSINE KINASE ACTIVITY OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

Introduction

As shown in Chapter 4, some mutants of hEGF were unable to stimulate the EGF receptor's protein tyrosine kinase activity to the same $V_{\max}$ as that of wild type, even though saturating amounts of mutant growth factor were used. One of these mutants, Leu47Ala, and two other mutants, Leu26Gly and Ile23Thr (117), were more thoroughly examined in this chapter for their ability to stimulate the receptor's protein tyrosine kinase activity.

The process by which kinase activation is achieved has been explained as resulting either from dimerization of inactive receptor monomers to form activated dimers (118-121) or from dissociation of latent receptor dimers to monomers having high tyrosine kinase activity (122). A third model invokes a ligand-induced intramolecular conformational change in the receptor's extracellular domain leading to the activation of the kinase activity of the receptor (56,123). While this matter is still being resolved, it is clear that the tyrosine kinase activity of the receptor is essential for the mitogenic response of the cells to EGF (43,112). The mutants mentioned here are interesting in that the lower $V_{\max}$ may indicate an altered, novel pathway through which the receptor is activated, making it less active. These mutants may then be potentially important as growth inhibitors that could block the action of wild type hEGF. Details of this work have been published (124).

Materials and Methods

Mutagenesis, protein purification, and competition binding assays. These techniques have been described in Chapter 3. The Leu26Gly and Ile23Thr mutant proteins were kindly provided by Dr. Stephen R. Campion (Biology Division, Oak Ridge National Laboratory).

Assay of EGF receptor kinase activity. The phosphorylation assays were carried out in the same manner as described in Chapter 3. In competition kinase reactions the concentration of the wild type protein was kept constant at two different concentrations, while that of the mutants were varied from 0 to 16 μ M. Each point shown on the curves is an average value determined from two or more experiments. Analysis of the time courses of the reactions with wild type and the mutants showed that they were linear.

SDS gel electrophoresis of the phosphorylation reaction mixtures. To measure the extent of autophosphorylation of the EGF receptor, phosphorylation reactions were performed as described above, with the following modifications: $(\text{Glu}_4\text{Tyr}_1)_n$ substrate was not added, the specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was 1 Ci/mmol, and the reactions were stopped by the addition of 3x Laemmli loading buffer (55), and heating in a boiling water bath for 5 min. The samples were then electrophoresed according to the procedure of Laemmli (55). The amount of each EGF protein used in the autophosphorylation assay was the same amount that was determined saturating in the phosphorylation assays using $(\text{Glu}_4\text{Tyr}_1)_n$ as a substrate. The gel was stained, dried, and processed for autoradiography. The autoradiograph was then scanned using a laser densitometer to determine the amount of radioactivity incorporated into the EGF receptor band. Greater than 95% of the

radioactivity on the gel was associated with a 170 kDa band, which was assumed to be the EGF receptor. Each reaction was done in duplicate and the average of the two values obtained by densitometry scans was used to calculate the values. The negative control of no EGF added was subtracted from each of the values.

Results

Relative receptor affinities. The relative receptor affinities of the three mutants of hEGF described in this paper are shown in Table 5-1. The values were obtained by using the radioreceptor competition assay and by measuring their stimulation of the receptor protein tyrosine kinase activity. Both assays show that the Ile23Thr and Leu26Gly mutants have about 5% of the receptor affinity of wild type, as determined from the values of the dose response curves, while the Leu47Ala mutant has only about 2% of wild type. Despite their lower receptor affinities, each mutant was able to fully displace wild type hEGF in the competition binding assay, indicating that the mutants bound to the EGF in a specific manner.

Mutants have a lower V_{max} of substrate phosphorylation and autophosphorylation. As shown in Fig. 5-1, the V_{max} of the receptor tyrosine kinase activity was markedly less with the three mutants than that obtained with wild type hEGF. Values, as % of wild type (Table 5-2) were 22 for the Leu26Gly mutant, 24 for the Leu47Ala mutant, and 31 for the Ile23Thr mutant, in the phosphorylation reaction in which $(\text{Glu}_4\text{Tyr}_1)_n$ was used as a substrate.

Using saturating concentrations of each mutant, the velocity of the EGF receptor autophosphorylation reaction was also measured. The results show that auto-

Table 5-1
**Receptor Affinities of hEGF Species as Measured by
 Radioreceptor Competition and Receptor Tyrosine Kinase Stimulation**

hEGF Species	<u>Relative Receptor Affinity</u>	
	Radioreceptor Competition Assay ^a	Tyrosine Kinase Stimulation Assay ^b
	<u>% of Wild Type</u>	
Wild Type	100	100
Leu26Gly	5	4
Leu47Ala	2	2
Ile23Thr	3	5

^aRelative receptor affinity = $IC_{50}(\text{wild type})/IC_{50}(\text{mutant}) \times 100\%$

^bRelative receptor affinity = $EC_{50}(\text{wild type})/EC_{50}(\text{mutant}) \times 100\%$

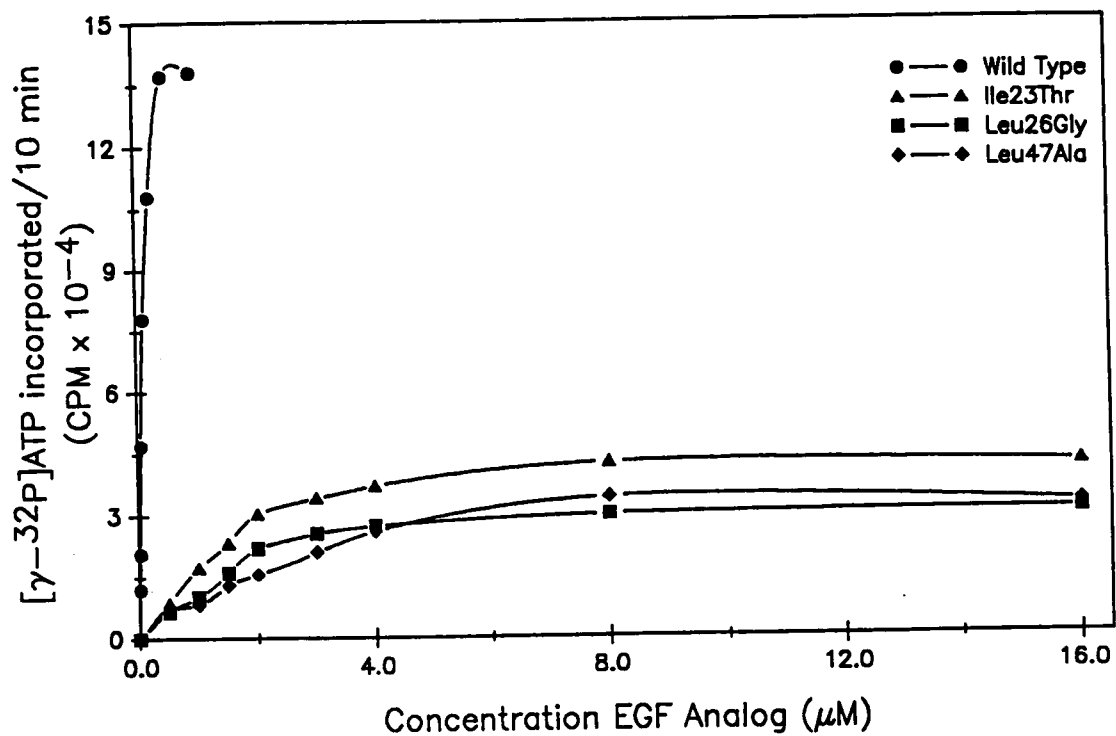


Figure 5-1. Dose response curves of wild type and low V_{max} mutants in the receptor tyrosine kinase assay. Plots are the average of two or more experiments.

Table 5-2

**$V_{\max}$ Values Obtained for Stimulation of the
Receptor Tyrosine Kinase by hEGF Analogs**

hEGF Species	<u>$V_{\max}$ of Receptor Tyrosine-Kinase</u>	
	(Glu ₄ Tyr ₁) _n as Substrate	Receptor Autophosphorylation
	<u>% of Wild Type</u>	
Wild Type	100	100
Leu26Gly	22	23
Leu47Ala	24	17
Ile23Thr	31	38

phosphorylation of the EGF receptor was significantly lower when stimulated by the mutant hEGFs as compared with wild type hEGF. Fig 5-2 is a typical autoradiograph showing the decreased autophosphorylation. Densitometry analysis shows that the Leu26Gly mutant stimulated autophosphorylation of the EGF receptor band (170 kDa) 23% that of wild type, the Table 5-1 Leu47Ala mutant 17% of wild type, and Ile23Thr, 38% of wild type. The autophosphorylation values, as percent of wild type, are similar to the values obtained using the $(\text{Glu}_4\text{Tyr}_1)_n$ substrate (see Table 5-2).

Low $V_{\max}$ mutants can competitively inhibit wild type hEGF. It was decided to examine whether the hEGF mutant proteins, having lower stimulatory capacity as demonstrated in the receptor protein tyrosine kinase assay, would act as competitive inhibitors of wild type hEGF under the phosphorylation assay conditions. A modification of the equation from Segel (60) for the velocity of a reaction with one enzyme and mixed alternative substrates was used to evaluate the competitive nature of the mutant hEGF analogs:

$$v_i = \frac{V_{\max A}([A]/Kd_A) + V_{\max B}([B]/Kd_B)}{1 + [A]/Kd_A + [B]/Kd_B}$$

where A and B are two different activators, and $V_{\max A}$ is the maximum velocity of the reaction with A as the activator, and $V_{\max B}$ is the maximum velocity of the reaction with B as the activator, Kd_A and Kd_B are the apparent dissociation constants and v_i is the observed velocity under the given conditions.

Fig. 5-3, panels A,B, and C, show the dose-response curves for stimulation by mutant hEGFs of the receptor tyrosine kinase with $(\text{Gly}_4\text{Tyr}_1)_n$ as a substrate and the

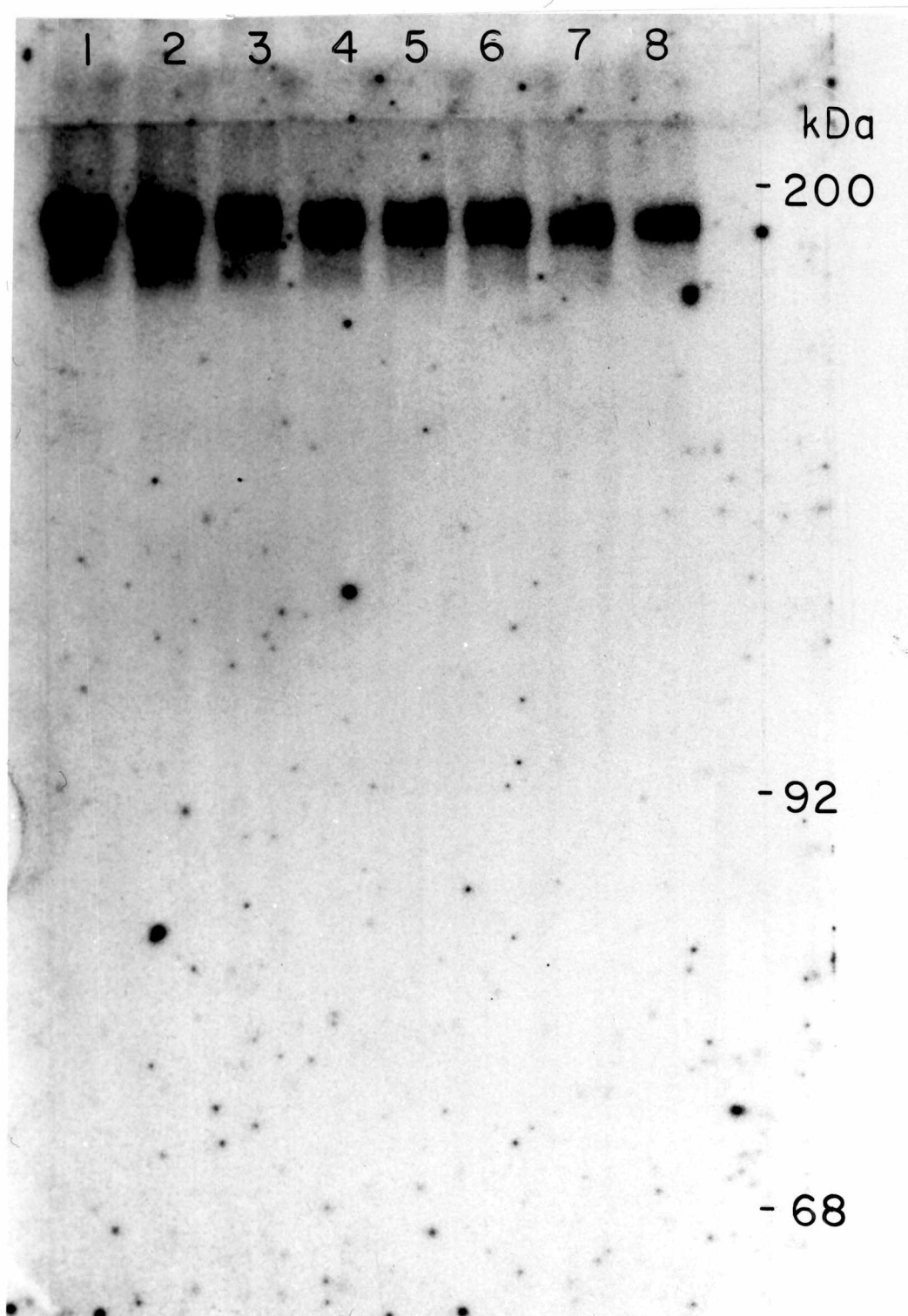


Figure 5-2. Autoradiograph of wild type and low $V_{\max}$ mutants. Lane 1 and 2, 0.48 μM wild type; lanes 3 and 4, 8 μM Ile23Thr; lanes 5 and 6, 8 μM Leu26Gly; lanes 7 and 8, minus EGF.

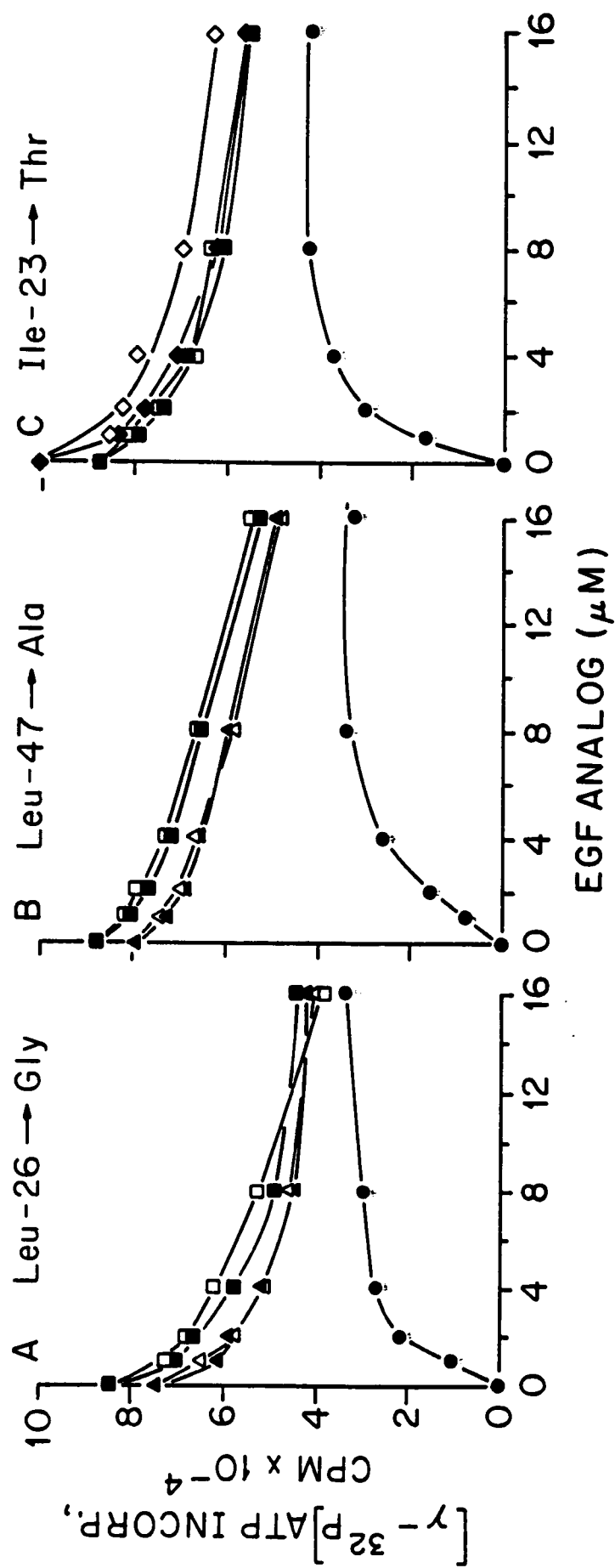


Figure 5-3. Dose-response curves and competition plots for the low V_{max} mutants. ● Dose-response curves; ▲ theoretical competition curves against 45 nM wild type; ■ actual and theoretical competition curves against 60 nM wild type; ◊ actual and theoretical competition curves against 75 nM wild type.

competition plots for each of the mutants at two different concentrations of wild type hEGF. Equilibrium constants and velocity values were obtained from the concentration curves. Also plotted are the theoretical curves predicted by the above equation using the values obtained for the individual mutants. These theoretical curves closely resemble the actual curves and demonstrate that the inhibitory nature of the mutant analogs is competitive. A mutant hEGF, Tyr22Asp, that has a lower relative receptor affinity (6% that of wild type) but not a lower $V_{\max}$ was tested as a competitive inhibitor of wild type hEGF at the same concentrations as the other mutants. The competition by this mutant is also predicted by the equation, but since the $V_{\max}$ is not lower, no inhibition should be observed. This proved to be true (data not shown), indicating that the competition seen with the other mutants is not due to an artifact of the assay.

Discussion

The results reported in this chapter demonstrate that three site-specific mutants of hEGF stimulate the receptor's protein-tyrosine kinase activity to a lower $V_{\max}$ than the wild type growth factor, and that these mutants can competitively inhibit the wild type hEGF's phosphorylation of a tyrosine-containing synthetic substrate. For each mutant the same value for relative $V_{\max}$ of the kinase reaction as compared to wild type was found for both the autophosphorylation reaction and the phosphorylation of the $(\text{Glu}_4\text{Tyr}_1)_n$ substrate.

The competitive inhibition of wild type hEGF-stimulated tyrosine kinase activity by the mutant hEGF species confirms their lower stimulatory capacity. These results clearly demonstrate a partial "uncoupling" of growth factor binding and kinase stimulation. The observed decrease in the maximal rate of phosphorylation catalyzed by the EGF/receptor complex activated by mutant hEGF species suggests that the activated receptor-growth

factor complexes formed with these mutants have a conformation slightly altered from the complex formed with the wild type growth factor. Obviously, structural studies are necessary to determine the nature of the conformational change(s). Such studies, as done recently with complexes formed between wild-type EGF and the external domain of the EGF receptor (125), are promising avenues to pursue. Our findings also suggest that the design of growth-inhibitory EGF analogs may be feasible.

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