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I am submitting herewith a dissertation written by Krishnadas Nandagopal entitled "The roles of leucine 15 and histidine 16 of human epidermal growth factor in the reception and relay of mitogenic signals by the epidermal growth factor receptor." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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**THE ROLES OF LEUCINE 15 AND HISTIDINE 16 OF HUMAN EPIDERMAL
GROWTH FACTOR IN THE RECEPTION AND RELAY OF MITOGENIC
SIGNALS BY THE EPIDERMAL GROWTH FACTOR RECEPTOR**

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DEDICATION

To my parents *Sundari* and *Krishnadas* for all their love and encouragement

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ABSTRACT

The solution structure of human epidermal growth factor (hEGF), as determined by nuclear magnetic resonance (NMR) spectroscopy, indicates that L15 and H16 of hEGF are solvent-exposed and are in spatial proximity to other residues implicated in receptor recognition and activation. Therefore, they may be involved in receptor-ligand interactions.

Although site-specific mutagenesis does not indicate an essential role for the imidazole functionality of H16 in mediating biochemical and biological activity, optimal receptor recognition may rely on hydrogen-bond donor/acceptor interactions involving a polar, isosteric side-chain at this site. Furthermore, the increased relative affinity (272%) and agonist activity (263%) of the H16Q mutant suggest that H16 could be a target for the engineering of superagonists with therapeutic potential.

The geometry and hydrophobicity of the γ -branched L15 side-chain of hEGF promote formation of a catalytically active receptor-ligand complex. The L15A mutant partially impairs allosteric activation of the EGFR in a (Glu₄,Tyr₁)_n substrate phosphorylation assay and is also defective in stimulating receptor dimerization / oligomerization *in vitro*. Gross structural perturbations are not evident in the ¹H-NMR spectrum of L15A compared to wild-type, thereby indicating a functional role for L15 of hEGF in receptor-ligand interactions.

Stimulation of murine keratinocytes with either the partial agonist (L15A) or wild-type hEGF reveals a lack of linear correlation between the activation of p42 MAP kinase and levels of receptor autophosphorylation. The percentages of cells which proliferate in response to 1 nM L15A and their rate of entry into S phase are both decreased relative to the response elicited by 1 nM wild-type. Higher concentrations of L15A reverse this effect, showing that L15A and wild-type differ in the number of receptors that they must activate to induce the threshold response. Biological thresholds may be attained by cooperative activation of pre-existing receptor dimers / oligomers by van der Waal's weak forces of attraction. The receptor reserve in intact cells thus points to an elegant strategy in cell survival, which ensures the mitogenic response to receptor activation by both L15A and wild-type hEGF. This study shows that L15 of hEGF is important for the reception and relay of mitogenic signals by the epidermal growth factor receptor (EGFR).

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ABBREVIATIONS

Amino acids : Ala or A, alanine	Arg or R, arginine
Asn or N, asparagine	Asp or D, aspartate
Cys or C, cysteine	Gln or Q, glutamine
Glu or E, glutamate	Gly or G, glycine
His or H, histidine	Ile or I, isoleucine
Leu or L, leucine	Lys or K, lysine
Phe or F, phenylalanine	Pro or P, proline
Thr or T, threonine	Trp or W, tryptophan
Tyr or Y, tyrosine	Val or V, valine
BCA : bicinchonic acid	
BSA : bovine serum albumin	
cAMP : adenosine-3',5'-cyclic monophosphate	
cpm : counts per minute	
dNTP : deoxynucleoside triphosphate	
DMEM : Dulbecco's modified Eagles's medium	
DTT : dithiothreitol	
ECL : enhanced chemiluminescence	
EDC : 1-ethyl-3-[3-(dimethylamino) propyl]carbodiimide	
EDTA : ethylenediaminetetraacetic acid	
EGF : epidermal growth factor; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; HB-EGF, heparin-binding epidermal growth factor	
EGFR : epidermal growth factor receptor	
EGTA : ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid	
EMEM : Eagle's minimal essential medium	
erbB : erythroblastosis virus B homologue; v-erbB, erythroblastosis virus	
FBS : fetal bovine serum	
Grb2 : growth factor receptor bound 2	
HEPES : N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid	
HPLC : high performance liquid chromatography	
HRP : horse radish peroxidase	

Hz : Hertz

kDa : kilo Dalton

k_D : equilibrium dissociation constant

LB : Luria broth

MAP 2 : microtubule-associated protein 2 / mitogen-activated protein 2

MDCK : Madin Darby Canine Kidney

MES : [2(N-morpholino)ethanesulfonic acid]

NMR : nuclear magnetic resonance spectroscopy

PBS : phosphate buffered saline

PCR : polymerase chain reaction

PI-3 kinase : phosphatidylinositol-3 kinase

PKC : protein kinase C

PLC $_{\gamma 1}$: phospholipase C $_{\gamma 1}$

PMSF : phenyl methyl sulfonyl fluoride

RNaseA : ribonuclease A

SDS : sodium dodecyl sulfate

SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHC : src homology and collagen

SH2 : src homology 2

TCA : trichloroacetic acid

TGF α : transforming growth factor α ; hTGF α , human transforming growth factor α

Tris : Tris(hydroxymethyl)aminomethane

V_{max} : maximal velocity

Chapter I

EPIDERMAL GROWTH FACTOR AND ITS RECEPTOR

Literature survey

EGF is a 6-kDa polypeptide mitogen which interacts with the EGFR, a 170-kDa transmembrane receptor glycoprotein expressed in epithelial and mesenchymal cell types (1). Discovered initially as a contaminant of nerve growth factor preparations obtained from murine submaxillary glands, mEGF was shown to induce early incisor eruption, precocious eyelid opening (2) and keratinization of the epidermis (3). Human EGF or β -urogastrone, which was isolated and purified from urine (4, 5), inhibits gastric acid secretion and promotes proliferation and epithelialization of cells lining the gastrointestinal tract (5). These studies led to the isolation and characterization of EGF from other sources including rat, guinea pig and dog (6, 7).

Determination of the primary sequence of EGF shows that it is composed of 53 amino acids and 3 intramolecular disulfide bonds (8). The spacing of cysteine residues ($CX_{4-14}CX_{3-8}CX_{4-14}CXCX_{8-14}C$, where X represents any non-cysteine residue) defines an 'EGF-like' structural unit characteristic of polypeptides such as TGF α (9-11), amphiregulin (12, 13), HB-EGF (14), betacellulin (15, 16), epiregulin (17, 18) and the pox virus family of growth factors (19-23). These proteins are encoded by distinct genes and resemble EGF in their sequence, disulfide bond arrangement and interaction with the EGFR. 'EGF-like' units are also found in proteins that do not bind the EGFR. These include proteases (e.g., thrombin), protease cofactors (e.g., thrombomodulin), developmental proteins (e.g., Notch) and proteins that mediate interactions between cells and the extracellular matrix (e.g., laminins) (24).

The gene for hEGF is present on chromosome 4 and codes for a glycosylated, membrane-anchored precursor molecule (proEGF) consisting of 1207 amino acids (25). Other EGF-related polypeptide precursors range from 160 to 252 amino acid residues (26-28). There are 9 'EGF-like' repeats in the extracellular domain of proEGF. Mature EGF which corresponds to amino acids 971-1023, is derived by proteolytic processing of the 'EGF-like' unit proximal to the membrane (26). As proEGF is capable of interacting with the EGFR (29), intercellular communication may proceed via juxtacrine and/or paracrine mechanisms.

The biosynthesis and release of EGF has been studied in some detail. In the salivary gland, mature EGF (6-kDa) is secreted after intracellular cleavage by an arginine esterase-like activity (30, 31). The predominant species released from most epithelial cells *in vivo* is the 160-kDa form which is found in high concentrations in urine and milk (32-34). This species is biologically less active than the mature 6-kDa EGF (33, 34). proEGF is expressed exclusively on the luminal surface of epithelial cells in the distal convoluted tubule of the kidney (35-37) and on the luminal surface of alveolar cells in the mammary gland (38, 39).

The biological consequences of differential sorting and processing of proEGF have been investigated in polarized epithelial cells where EGFRs are spatially restricted to basolateral surfaces. There is an apical enrichment of proEGF in MDCK cells due to the activity of a metalloprotease which preferentially cleaves the basolateral ectodomain (40). This suggests that loss of epithelial cell polarity, as in cell transformation, may deregulate EGFR activity as a result of direct access to ligand. Kallikreins and endoproteases release mature EGF from high-molecular weight precursors (41) which are reservoirs of latent growth factor in the luminal environment. Such mechanisms may permit EGF to interact with EGFR populations distant from its site of synthesis. Alternatively, proEGF itself may function as a receptor (27) in the apical environment or may promote cell adhesion (34).

The mitogenic activity of EGF and TGF α may be only one of numerous physiological roles ascribed to these molecules. The mRNA for EGF is maternally inherited in the ovulated, unfertilized egg and pre-implantation mouse embryo (42). EGF and TGF α are both involved in cavitation (blastocyst formation) and promote polarization of the trophectoderm, which is both the first epithelium of development and the outer cell layer surrounding the inner cell mass of the blastocyst (43). Functional EGFR activity is detectable in the skin, developing skeletal muscle, amnion and placenta of 13-day mouse and 16-day rat fetuses (44), and correlates with the onset of organogenesis. EGF and TGF α stimulate proliferation of hepatocytes *in vivo* and in culture (45). They also inhibit differentiation of adipocyte precursors in primary culture (46). TGF α is a chemoattractant for perioptic mesenchymal cells in developing mouse eyes (47). Some studies indicate that intravenous infusion of EGF in conscious rats and monkeys induces hypotension (decrease in blood pressure) with modest

tachycardia (rapid heart rate) (48).

Cloning and sequencing of the human EGFR revealed that it is a cellular homologue of the avian v-erbB oncoprotein (49, 50). The EGFR / erbB1 is a member of the superfamily of proto-oncogenes which constitute the Class I receptor tyrosine kinases; these include erbB2/neu, erbB3 and erbB4, all of which are distinct vertebrate gene products - none are orthologues (51). While the ligand for erbB2 has not yet been identified, erbB3 and erbB4 are activated by numerous splice variants of heregulin / neuregulin which possess 'EGF-like' receptor recognition domains (52-54). Genetic analyses of mice lacking individual erbB receptor genes have revealed the spectrum of targets affected *in vivo*. Depending on penetrance and the genetic background, EGFR null mutants exhibit defects of the skin, gut, lung, liver, kidney and cerebral cortex (55-57). Mice harbouring the *waved-1* (*wa-1*) and *waved-2* (*wa-2*) mutations reveal eye and skin abnormalities. While TGF α and *wa-1* are allelic (58), the *wa-2* phenotype results from a point mutation which leads to a V-G substitution in the kinase domain of the EGFR (59). The neuregulins and their receptors are involved in cardiac and neural development (60-62).

Significant progress has been made with regard to the biochemistry of EGF action. Specific binding of EGF to the EGFR stimulates the latter's intrinsic protein-tyrosine kinase activity (63). EGF-induced receptor dimerization (64, 65) coincides with the activation process (66) and results in receptor autophosphorylation (63). This renders the receptor competent to interact with and phosphorylate intracellular substrates like PLC γ_1 (67-69), and the GRB2/sem-5 (70) and SHC (71, 72) adaptor proteins, which couple to the GTPase activating protein of *ras* (ras-GAP) [73, 74]. These interactions are mediated by phosphotyrosine-binding SH2 domains in these proteins (75). Signal transduction by the EGFR may be subject to feed-back control by protein-tyrosine phosphatases (76, 77). Downstream effector molecules like PKC and MAP-2 kinase effect rapid receptor desensitization by phosphorylating the EGFR on T654 and T659, respectively (78, 79). Ligand-induced receptor internalization (80-82) is also involved in the attenuation of EGF signaling. The gamut of biochemical responses elicited in EGF-treated cells, includes a rise in intracellular pH and Ca²⁺ levels, increased rates of glycolysis, DNA, RNA and protein syntheses (24).

Deregulation of normal expression of TGF α contributes to neoplastic transformation *via* autocrine mechanisms (83, 84). The constitutively active v-erbB oncoprotein, which lacks a ligand-binding region as well as C-terminal autophosphorylation sites, signals aberrantly in transformed cells (85). The EGFR is also frequently over-expressed in epidermoid carcinomas, glioblastomas, squamous lung cancers and immature melanomas (24). Thus, it is of considerable interest to elucidate mechanisms underlying growth factor control over the formation of catalytically active ligand-receptor complexes. Investigation of structure-activity relationships would aid the rational design of EGFR antagonists.

Although high-resolution crystal structures for EGF or the ligand-receptor complex are not available, the solution structure of EGF has been determined by two-dimensional NMR (86-92). The overall conformation of EGF is defined by its two-domain structure, i.e., two anti-parallel β -sheets (residues 19-31 and 37-45) which are linked by a tight turn of type II (residues 34-36) [86]. Both β -sheets have a right-handed twist (93). Conformational dynamics suggest the existence of a motion between the two domains corresponding to the opening and closing of the palm and thumb; the importance of this conformational flexibility is explained in terms of the 'mitten model' (94). Structure-function analyses have identified several residues in hEGF that participate in the recognition and activation of the EGFR. These include Y13, I23, L26, R41 and L47, all of which are solvent-exposed and lie on the same face of the molecule (reviewed in 95-97). Double mutants involving these sites display cumulative effects on receptor binding, indicating that each of these residues interacts independently with the EGFR (98).

Characterization of chimeric receptor constructs of chicken/human EGFRs show that domain III (residues 310-474) is a major ligand-binding region of the EGFR (99). A 40-kDa hEGF/TGF α binding fragment (C302-R503) generated by limited proteolysis of the extracellular domain of the EGFR, binds hTGF α with 100-fold lower affinity as compared to the intact EGFR (100). Chemical cross-linking studies have identified Y101 (101), K336 (102) and K465 (103) as potential contact sites. Continuous epitopes (as opposed to juxtaposed assembly or carbohydrate determinants on the EGFR) recognized by monoclonal antibodies that are competitive with EGF have been localized to a 14 amino-acid region

(A351-D364) in the EGFR (104). The molecular determinants involved in EGFR recognition and activation continue to be the focus of active research in many laboratories.

The clinical importance of studying EGF-EGFR interactions stems from the involvement of EGF in pathophysiological states. In autosomal recessive polycystic kidney disease, which is associated with overexpression and mislocalization of the EGFR, EGF in the cyst fluid stimulates cystogenesis (105). The candidate gene for Rieger syndrome, an autosomal dominant disorder, maps to the human chromosome 4q region and has been identified with tight linkage to the gene for EGF (106). Interestingly, the syndrome is associated with the absence of maxillary incisor teeth, malformations of the anterior chamber of the eye and umbilical abnormalities (106). EGF reverses the syndrome of X-linked ectodermal dysplasia in Tabby (Ta) mice by promoting the development of dermal ridges and functional sweat glands (107). This finding offers promise for the cure of congenital X-linked hypohidrotic ectodermal dysplasia; the absence of sweat glands causes hyperpyrexia (high fever), a clinical emergency in infants. EGF also promotes wound healing and has been used in the treatment of burns (108, 109), corneal traumas (110, 111) and gastric ulcers (112).

The biology of receptor tyrosine kinases and their activating ligands is unique to metazoans. Although tyrosine kinase activities have been reported in *S. cerevisiae* (113), none has been identified as a receptor tyrosine kinase. The low activity in crude lysates has precluded direct biochemical analyses (114). Expression of the pp60^{v-src} oncoprotein in *S. cerevisiae* is lethal and is associated with a loss of cell cycle control at the G1/S and G2/M checkpoints (115). Signal transducing cascades are conserved in *C. elegans*, *D. melanogaster* and mammals. In *C. elegans*, vulval induction is initiated by the lin-3 gene product (116), a molecule similar to EGF / TGF α and it acts through the *let-23* gene which is homologous to the EGFR (117). At least three ligands, similar to EGF / TGF α , can activate the *Drosophila* EGFR in a tissue specific manner. These include Spitz (118), Gurken (119) and Vein (120). Recent studies show that regulation of expression and activity of these ligands increases the repertoire of functions associated with the *Drosophila* EGFR (121, 122). Not surprisingly then, mammals utilize 4 distinct 'EGFR-like' tyrosine kinases (erbB1-4) which can interact with one another (123, 124), 6 ligands (EGF, TGF α ,

amphiregulin, HB-EGF, betacellulin and epiregulin) that activate the EGFR and several splice variants of neuregulin (125, 126) which stimulate erbB3 and erbB4. How does the mammalian cell integrate physiological processes in terms of specificity and redundancy, as well as regulation in time and space?

Purpose of study

Several factors merit consideration when an attempt is made to understand how a cell elicits an EGF-dependent response. It is essential to first define the nature of ligand-receptor interactions. This has been addressed by the structure-function analyses of L15 and H16 of hEGF in 'cell-free' systems. The next step is to explain the link between recognition by the ligand and subsequent receptor activation in terms of a model which is plausible both energetically and mechanistically. The L15A mutant of hEGF has been used to glean information in this regard. The validity of the proposed model must be examined in a cellular context. To this end, the mitogenic response to L15A and wild-type hEGF has been examined in murine keratinocytes (Balb/MK) which are EGF-dependent for survival. Most importantly, this study seeks to identify variables which limit our understanding and thereby pave the way for future experiments.

References

1. Carpenter, G. and Cohen, S. (1990) *J. Biol. Chem.*, **265**, 7709-7712
2. Cohen, S. (1962) *J. Biol. Chem.*, **237**, 1555-1562
3. Cohen, S. and Elliot, G. (1963) *J. Invest. Dermatol.*, **40**, 1-5
4. Starkey, R. H., Cohen, S. and Orth, D. N. (1975) *Science* **189**, 800-802
5. Gregory, H. (1975) *Nature* **257**, 325-327
6. Simpson, R. J., Smith, J. A., Moritz, R. L., O'Hara, M. R., Rudland, P. S., Morrison, J.R., Lloyd, C. J., Grego, B., Burgess, A. W., and Nice, E. D. (1985) *Eur. J. Biochem.*, **153**, 629-637
7. Kobayashi, R., Reeves, J. R. Jr., and Walsh, J. H. (1985) *Biochem. J.*, **229**, 611-619
8. Savage, C. R. Jr., Inagami, T., and Cohen, S. (1972) *J. Biol. Chem.*, **247**, 7612-7621

9. Anzano, M. A., Roberts, A. B., Smith, M. J., Sporn, M. B., and DeLarco, J. E. (1983) *Proc. Natl. Acad. Sci., USA*, **80**, 6264-6288
10. Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., DeLarco, J. E., Stephenson, J. R., and Todaro, G. J. (1983) *Proc. Natl. Acad. Sci., USA*, **80**, 4684-4688
11. Marquardt, H., Hunkapiller, M. W., Hood, L. E., and Todaro, G. E. (1984) *Science* **223**, 1079-1082
12. Shoyab, M., McDonald, V. L., Bradley, J. G., and Todaro, G. J. (1988) *Proc. Natl. Acad. Sci., USA*, **85**, 6528-6532
13. Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G., and Todaro, G. J. (1989) *Science* **243**, 1074-1076
14. Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C., and Klagsbrun, M. (1991) *Science* **251**, 936-939
15. Sasada, R., Ono, Y., Taniyama, Y., Shing, Y., Folkman, J., and Igarashi, K. (1993) *Biochem. Biophys. Res. Commun.*, **190**, 1173-1179
16. Shing, Y., Christofori, G., Hanahan, D., Sasada, R., Igarashi, K., and Folkman, J. (1993) *Science*, **259**, 1604-1607
17. Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K. (1995) *J. Biol. Chem.*, **270**, 7495-7500
18. Toyoda, H., Komurasaki, T., Uchida, D., and Morimoto, S. (1997) *Biochem. J.*, **326**, 69-75
19. Todaro, G. J., and DeLarco, J. E. (1976) *Nature* **264**, 26-31
20. DeLarco, J. E., and Todaro, G. J. (1978) *Proc. Natl. Acad. Sci., USA*, **75**, 4001-4005
21. Blomquist, M. C., Hunt, L. T., and Barker, W. C. (1984) *Proc. Natl. Acad. Sci., USA*, **81**, 7363-7367
22. Brown, J. P., Twardzik, D. R., Marquardt, H., and Todaro, G. J. (1985) *Nature* **313**, 491-492
23. Reisner, A. H. (1985) *Nature* **313**, 801-803

24. Carpenter, G., and Wahl, M. I. (1990) *Handb. Exp. Pharmacol.*, **95/1**, 69-171
25. Bell, G. I., Fong, N. M., Stempie, N. M., Wormsted, M. A., Caput, D., Ku, L., Urdea, M.S., Rall, L. B., and Sanchez-Pescador, R. (1986) *Nucl. Acids Res.*, **14**, 8427-8446
26. Barnard, J., Beauchamp, R., Russell, W., DuBois, R., and Coffey, R. (1995) *Gastroenterology* **108**, 564-580
27. Derynck, R. (1992) *Adv. Cancer Res.*, **58**, 27-52
28. Lee, D., Fenton, S., Berkowitz, E., and Hissong, M. (1995) *Pharmacol. Rev.*, **47**, 51-85
29. Mroczkowski, B., Reich, M., Whittaker, J., Bell, G., and Cohen, S. (1988) *Proc. Natl. Acad. Sci., USA*, **85**, 126-130
30. Carpenter, G., and Cohen, S. (1979) *Annu. Rev. Biochem.*, **48**, 193-216
31. Jorgensen, P., Hilchey, S., Nexø, E., Poulsen, S., and Quilley, C. (1993) *J. Endocrinol.*, **130**, 227-234
32. Laksmanan, J., Salido, E., Lam, R., and Fischer, D. (1992) *Am. J. Physiol.*, **26**, E142-E150
33. Mroczkowski, B., and Reich, M. (1993) *Endocrinology* **132**, 417-425
34. Parries, G., Chen, K., Misono, K., and Cohen, S. (1995) *J. Biol. Chem.*, **270**, 27954-27960
35. Rall, L., Scott, J., Bell, G., Crawford, R., Penschow, J., Niall, H., Coghlan, J. (1985) *Nature* **313**, 228-231
36. Salido, E., Barajas, L., Lechago, J., Laborde, N., and Fischer, D. (1985) *J. Histochem. Cytochem.*, **34**, 1155-1160
37. Salido, E., Yen, P., Shapiro, L., Fischer, D., and Barajas, L. (1989) *Am. J. Physiol.*, **256**, F632-F638
38. Brown, C., Teng, C., Pentecost, B., and DiAugustine, R. (1989) *Mol. Endocrinol.*, **89**, 1077-1083
39. Jahanke, G., Chao, J., Walker, M., and DiAugustine, R. (1994) *Growth Fact.*, **11**, 113-123

40. Dempsey, P. J., Meise, K. S., Yoshitake, Y., Nishikawa, K., and Coffey, R. J. (1997) *J. Cell Biol.*, **138**, 747-758
41. Margolius, H. (1984) *Annu. Rev. Physiol.*, **46**, 309-326
42. Johnson, S. E., Rothstein, J. L., and Knowles, B. B. (1994) *Dev. Dyn.*, **201**, 216-226
43. Watson, A. J. (1992) *Mol. Reprod. Dev.*, **33**, 492-504
44. Hortsch, M., Schlessinger, J., Gootwine, E., and Webb, C. G. (1983) *EMBO J.*, **2**, 1937-1941
45. Fausto, N., Laird, A. D., and Webber, E. M. (1995) *FASEB J.*, **9**, 1527-1536
46. Serrero, G., and Lepak, N. (1996) *Int. J. Obes. Relat. Metab. Disord.*, **20**, S58-S64
47. Reneker, L. W., Silversides, D. W., Patel, K., and Overbeek, P. A. (1995) *Development* **121**, 1669-1680
48. Keiser, J. A., and Ryan, M. J. (1996) *Proc. Natl. Acad. Sci., USA*, **93**, 4957-4961
49. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M. D. (1984) *Nature* **307**, 521-527
50. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) *Nature* **309**, 418-424
51. van der Geer, P., Hunter, T., and Lindberg, R. (1994) *Annu. Rev. Cell Biol.*, **10**, 251-337
52. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, M., Ben-Levy, R., Koski, R. A., Lu, H. S., and Yarden, Y. (1992) *Cell* **69**, 559-572
53. Wen, D., Suggs, S. V., Karunakaran, D., Liu, N., Cupples, R. L., Luo, Y., Janssen, A. M., Ben-Baruch, N., Trollinger, D. B., Jacobsen, V. L., Meng, S. Y., Lu, H. S., Chang, D., Yang, W., Yanagihara, D., Koski, R. A., and Yarden, Y. (1994) *Mol. Cell. Biol.*, **14**, 1909-1919
54. Tzahar, E., Levkowitz, G., Karunakaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994) *J. Biol. Chem.*, **269**, 25226-25233
55. Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D.,

- LaMantia, C., Mourton, T., Herrup, K., Harris, R. C., Barnard, J. A., Yuspa, S. H., Coffey, R. J., and Magnuson, T. (1995) *Science* **269**, 230-234
56. Sibilila, M., and Wagner, E. F. (1995) *Science* **269**, 234-238
57. Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pederson, R. A., Werb, Z., and Derynck, R. (1995) *Nature* **376**, 337
58. Luetkeke, N. C., Qiu, T. H., Pfeiffer, R. L., Oliver, P., Smithies, O., and Lee, D. C. (1993) *Cell* **73**, 263-278
59. Luetkeke, N. C., Phillips, H. K., Qiu, T. H., Copeland, N. G., Earp, H. S., Jenkins, N. A., and Lee, D. C. (1994) *Genes and Dev.*, **8**, 399-413
60. Carraway, K. L. III, and Burden, S. J., (1995) *Curr. Opin. Neurobiol.*, **5**, 606-612
61. Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995) *Nature* **378**, 394-398
62. Gassmann, M., Casagrande, F., Orioli, D., Simon, H., Lai, C., Klein, R., Lemke, G. (1995) *Nature* **378**, 390-394
63. Ushiro, H. and Cohen, S. (1980) *J. Biol. Chem.*, **255**, 8363-8365
64. Yarden, Y. and Schlessinger, J. (1987a) *Biochemistry* **26**, 1434-1442
65. Yarden, Y. and Schlessinger, J. (1987b) *Biochemistry* **26**, 1443-1451
66. Canals, F. (1992) *Biochemistry* **31**, 4493-4501
67. Wahl, M. I., Nishibe, S., Suh, P. G., Rhee, S. G., and Carpenter, G. (1989) *Proc. Natl. Acad. Sci., USA*, **86**, 1568-1572
68. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1989) *Cell* **57**, 1101-1107
69. Meisenhelder, J., Suh, P. G., Rhee, S. G., and Hunter, T. (1989) *Cell* **57**, 1109-1122
70. Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnick, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992) *Cell* **70**, 431-442
71. Ruff-Jamison, S., McGlade, J., Pawson, T., Chen, K., and Cohen, S. (1993) *J. Biol. Chem.*, **268**, 7610-7612

72. Donaldson, R. W., and Cohen, S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8477-8481
73. McCormick, F. (1989) *Cell* **56**, 5-8
74. Soler, C., Beguinot, L., Sorkin, A., and Carpenter, G. (1993) *J. Biol. Chem.*, **268**, 22010-22019
75. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. J., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. (1993) *Cell* **72**, 767-778
76. Feng, G. S., Hui, C. C., and Pawson, T. (1993) *Science* **259**, 1607-1611
77. Vogel, W., Lammers, R., Huang, J., and Ullrich, A. (1993) *Science* **259**, 1611-1614
78. Hunter, T. N., Ling, N., and Cooper, J. A. (1984) *Nature* **311**, 480
79. Rossomando, A. J., Payne, D. M., Weber, M. J., and Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci., USA*, **86**, 6940-6943
80. Wiley, H. S., Herbst, J. J., Walsh, B. J., Lauffenburger, D. A., Rosenfeld, M. G., and Gill, G. N. (1991) *J. Biol. Chem.*, **266**, 11083-11094
81. Baulida, J., Kraus, M. H., Alimandi, M., DiFiore, P. P., and Carpenter, G. (1996) *J. Biol. Chem.*, **271**, 5251-5257
82. Worthylake, R., and Wiley, H. S. (1997) *J. Biol. Chem.*, **272**, 8594-8601
83. Sporn, M. B. and Roberts, A. B. (1985) *Nature*, **313**, 745-747
84. Matsui, Y., Halter, S., Holt, J., Hogan, B., and Coffey, R. (1990) *Cell*, **61**, 1147-1155
85. McManus, M. J., Connolly, D. C. and Maihle, N. J. (1995) *J. Virol.*, **69**, 3631-3638
86. Cooke, R. M., Wilkinson, A. J., Baron, M., Pastore, A., Tappin, M. J., Campbell, I. D., Gregory, H. and Sheard, B. (1987) *Nature* **327**, 339-341
87. Cooke, R. M., Tappin, M. J., Campbell, I. D., Kohda, D., Miyake, T., Fuwa, T., Miyazawa, T. and Inagaki, F. (1990) *Eur. J. Biochem.* **193**, 807-815
88. Hommel, U., Harvey, T. S., Driscoll, P. C. and Campbell, I. D. (1992) *J. Mol. Biol.* **227**, 271-282

89. Kohda, D., Go, N. and Inagaki, F. (1988) *J. Biochem.* **103**, 741-743
90. Kohda, D. and Inagaki, F. (1992) *Biochemistry* **31**, 11928-11938
91. Montelione, G. T., Wüthrich, K., Nice, E. C., Burgess, A. W. and Scheraga, H. A. (1987) *Proc. Natl. Acad. Sci., USA*, **84**, 5226-5230
92. Montelione, G. T., Wüthrich, K., Burgess, A. W., Nice, E. C., Wagner, G., Gibson, K. D. and Scheraga, H. A. (1992) *Biochemistry* **31**, 236-249
93. Chothia, C. (1984) *Annu. Rev. Biochem.*, **53**, 537-572
94. Ikura, T., and Go, N. (1993) *Proteins: Structure, Function and Genetics* **16**, 423-436
95. Champion, S. R. and Niyogi, S. K. (1994) In Cohn, W. E. and Moldave, K. (eds), *Prog. Nucl. Acid Res. Mol. Biol.* Academic Press, New York, Vol. **49**, pp. 653-683
96. Groenen, L. C., Nice, E. C. and Burgess, A. W. (1994) *Growth Factors*, **11**, 235-257
97. Tadaki, D. K. and Niyogi, S. K. (1996) In Leroith, D. and Bondi, C. (eds), *Growth Factors and Cytokines in Health and Disease*, JAI Press Inc., Vol **1A**, pp. 85-121
98. Champion, S. R., Geck, M. K., and Niyogi, S. K. (1993) *J. Biol. Chem.*, **268**, 1742-1748
99. Lax, I., Bellot, F., Howk, R., Ullrich, A., Givol, D., and Schlessinger, J. (1989) *EMBO J.*, **8**, 421-427
100. Kohda, D., Odaka, M., Lax, I., Kawasaki, H., Suzuki, K., Ullrich, A., and Schlessinger, J. (1993) *J. Biol. Chem.*, **268**, 1976-1981
101. Woltjer, R. L., Lukas, T. J., and Staros, J. V. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7801-7805
102. Wu, D., Wang, L., Sato, G. H., and Sato, J. D. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3151-3155
103. Summerfield, A. E., Hudnall, A. K., Lukas, T. J., Guyer, C. A., and Staros, J. V. (1996) *J. Biol. Chem.*, **271**, 19656-19659
104. Wu, D., Wang, L., Sato, G. H., West, K. A., Harris W. R., Crabb, J. W., and Sato, J. D. (1989) *J. Biol. Chem.*, **264**, 17469-17475
105. Orellana, S. A., Sweeney, W. E., Neff, C. D., and Avner, E. D. (1995) *Kidney Int.*, **47**,

490-499

106. Slavkin, H. C. (1993) *Am. J. Med. Genet.*, **47**, 689-697
107. Blecher, S. R., Kapalanga, J., and Lalonde, D. (1990) *Nature* **345**, 542-544
108. Brown, G. L., Curtsinger, L., Brightwell, J. R., Ackerman, D. M., Tobin, G. R., Polk, H. C., George-Nascimento, C., Valenzuela, P., and Schultz, G. S. (1986) *J. Expt. Med.*, **163**, 1319-1324
109. King, L. E. Jr., Ellis, D. L., Gates, R. E., Inman, W. H., Stoscheck, C. M., Fava, R. A., and Nanney, L. B. (1988) *Am. J. Med. Sci.*, **31**, 154-158
110. Carter, M. M., Balin, A. K., Gottlieb, A. B., Eisinger, M., Lin, A., Pratt, L., Sherbany, A., and Caldwell, D. (1988) *Prog. in Clin. Res.*, **266**, 303-319
111. Gospodarowicz, D., Bialecki, H., and Thakral, T. K. (1979) *Exp. Eye Res.*, **28**, 501-514
112. Schultz, G. S., Rotatori, D. S., and Clark, W. (1991) *J. Cell. Biochem.*, **45**, 346-352
113. Schieven, G., Thorner, J., and Martin, G. S. (1986) *Science* **231**, 390-393
114. Adamikova, L., Resnick, R. J., and Tomaska, L. (1996) *Yeast* **12**, 833-838
115. Boschelli, F., Uptain, S. M., and Lightbody, J. J. (1993) *J. Cell. Sci.*, **105**, 519-528
116. Hill, R. J., and Sternberg, P. W. (1992) *Nature* **358**, 470-476
117. Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y., and Sternberg, P. W. (1990) *Nature* **348**, 693-699
118. Rutledge, B., Zhang, K., Bier, E., Jan, Y. N., and Perrimon, N. (1992) *Genes and Dev.*, **6**, 1503-1517
119. Neuman-Silberberg, F. S., and Schupbach, T. (1993) *Cell* **75**, 165-174
120. Schnepf, B., Grumblin, G., Donaldson, T., and Simcox, A. (1996) *Genes and Dev.*, **10**, 2303-2313
121. Perrimon, N., and Perkins, L. A., (1997) *Cell* **89**, 13-16
122. Schweitzer, R., and Shilo, B-Z. (1997) *Trends in Genetics* **13**, 191-196

123. Carraway, K. L. III., and Cantley, L. C. (1994) *Cell* **78**, 5-8
124. Alroy, I., and Yarden, Y. (1997) *FEBS Lett.*, **410**, 83-86
125. Chang, H., Riese, D. J. 2nd., Gilbert, W., Stern, D. F., and McMahon, U. J. (1997) *Nature* **387**, 509-512
126. Carraway, K. L. III., Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M., and Lai, C. (1997) *Nature* **387**, 512-516

Chapter II

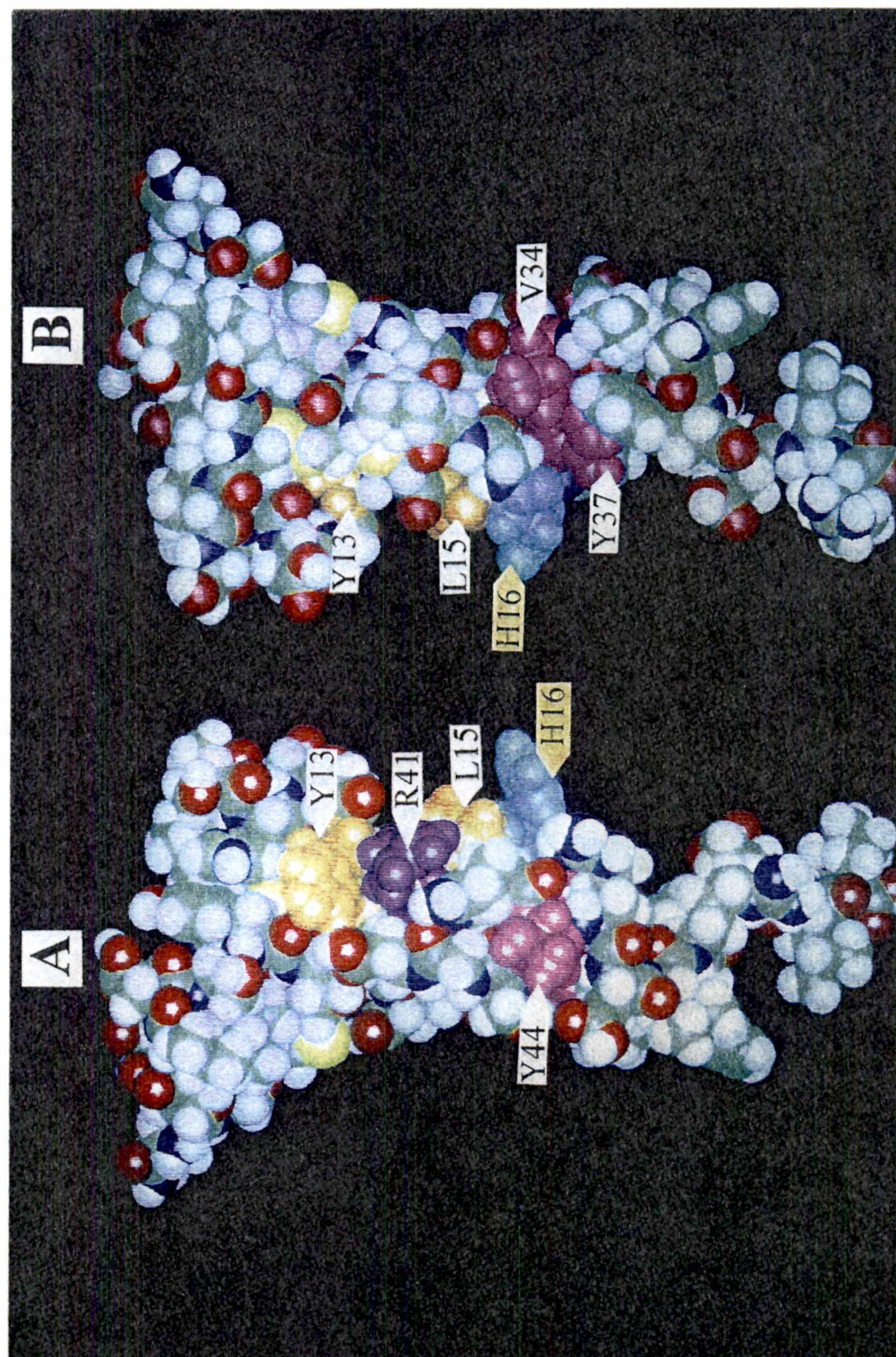
HISTIDINE 16 OF HUMAN EPIDERMAL GROWTH FACTOR MEDIATES RECEPTOR RECOGNITION BY HYDROGEN BOND DONOR / ACCEPTOR INTERACTIONS

Introduction

The interface (comprising residues 13-16, 37 and 41-43; see Figure II-1) between the N- and C-terminal domains of hEGF is stabilized by numerous hydrogen bonding interactions (1). There is also evidence for significant conformational flexibility of the corresponding region in hTGF α and it has been postulated that this region may contribute substantially to the free energy of receptor binding (2). H16 of hEGF is thus implicated in receptor recognition. Sequence alignments and pH titration studies show that the corresponding residue in hTGF α , H18, has a pK_a value of 7.6 (3). The pK_a value of H16 of hEGF is 7.2 (4). This indicates that the H16 side-chain is solvent-exposed, ~50% is protonated at physiological pH and the residue may interact directly with the receptor protein.

Previous studies have suggested that there may be differential contributions of H16 in hEGF and H18 in hTGF α to EGFR binding. Partially purified *Pseudomonas* exotoxin A-hEGF chimeras in which H16 was replaced with Q display reduced binding affinity (19% of wild-type) and cytotoxic activity (5). The H18K hTGF α mutant retains 22% relative binding affinity and 7% relative mitogenic potency (6). These studies suggest a stringent requirement for H since semi-conservative changes to either Q or K result in some loss of biological activity. In contrast, partially purified H18A and H18E mutants of hTGF α exhibit relative binding affinities of 30% and 84% and the H18E mutant is also more potent than wild-type hTGF α in a soft-agar transformation assay (7). In view of these equivocal results, the role of H16 of hEGF was therefore carefully re-examined by site-directed mutagenesis. The L, N, Q, R and Y mutants were expressed as recombinant proteins in *E. coli* and characterized with regard to their native conformation, binding affinity, agonist activity and ability to promote clonal growth of cells in culture. The results indicate that H16 of hEGF, although not essential for biological activity, mediates receptor recognition by hydrogen-bond donor/acceptor interactions and may share this feature with H18 of hTGF α .

Figure II-1. Computer model illustrating the location of H16 in the domain-domain interface of hEGF. NMR coordinates were kindly provided by Dr. G. T. Montelione (Rutgers University). MOLSCRIPT software [Kraulis, P. J. (1991) *J. Appl. Cryst.* **24**, 946-950] was used by Dr. Douglas K. Tadaki to generate the model. (B) is derived from a 180° out-of-plane rotation of (A).



Materials and Methods

Oligonucleotide-directed site-specific mutagenesis

Site-specific mutants were generated by a PCR strategy (8). Deoxyoligonucleotides containing the desired sequences were synthesized in a Milligen/Bioscience Cyclone Plus DNA synthesizer utilizing phosphoramidite chemistry. The sequences of the oligonucleotides, with the mutated codons underlined and the mismatches in boldface, are as follows: H16L, 5'-CCTGCTCGACGGTGTTCATG-3'; H16N, 5'-CCTGAACGACGGTGTTCATG; H16Q, 5'-CCTGCAAGACGGTGTTCATG-3'; H16R, 5'-CCTGCGAGACGGTGTTCATG-3'; H16Y, 5'-CCTGTACGACGGTGTTCATG-3'. Double stranded DNA of the expression vector, pEGF1 (2.9 kb), containing the hEGF insert was used as template. Each deoxyoligonucleotide primer bearing the mutation was utilized in a "back to back" configuration with the wild-type reverse primer 5'-CAGTACCCGTCGTGAGACAG-3'. A thermostable DNA polymerase isolated from *Pyrococcus furiosus* (*pfu* polymerase, Stratagene) was used to incorporate the mutant primer and amplify the *hEGF* gene-containing plasmid. Reactions were carried out in standard buffer (20 mM Tris-Cl, pH 8.2, 10 mM KCl, 6 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1% Triton-X-100, and 10 µg/ml BSA) supplied by the manufacturer. Final concentrations of reagents present in a 100-µl reaction mixture were: 1 pmol each of mutant and reverse primers, 0.25 mM dNTPs, 0.01 pmol template DNA, and 5 units of *pfu* polymerase. Conditions for the first PCR cycle were as follows: denaturation at 94 °C for 3 min, annealing at 40 °C for 1 min, and primer extension at 72 °C for 12 min. Twenty-five successive cycles were performed under the same conditions except that the denaturation time was reduced to 1 min. The linear amplification products were resolved on a 1% agarose gel and isolated by electroelution. The DNA samples were treated with Klenow polymerase (New England BioLabs) to form blunted ends and then circularized by incubation overnight at 14 °C with T4 DNA ligase (New England BioLabs). Transformation of *E. coli* JM107 was according to the procedure of Hanahan (9). All engineered mutations and the absence of any undesired genetic alterations were confirmed by sequencing (10) the *hEGF* gene.

Expression and purification of recombinant hEGF proteins

Overnight cultures of *E. coli* JM107 cells harboring the pEGF1 plasmids were grown at 37 °C to mid-log phase in LB medium containing 25 µg/ml ampicillin and then diluted 100-fold into the same prewarmed medium. After 3 h ($OD_{600}=0.5-0.8$) chloramphenicol was added to a final concentration of 5 µg/ml. When the OD_{600} of the cultures reached 1.2-1.4, expression of protein was induced by the addition of isopropylthiogalactoside to a final concentration of 1 mM. The cultures were allowed to grow until EGF production was maximal, typically 12 h later. The hEGF protein sequestered in the periplasm was isolated from the cell pellet by resuspension in ice-cold buffer (1 M Tris-Cl, pH 9.0, 2 mM EDTA) and incubation on ice for 20 min. Proteins in the Tris-EDTA fractions were precipitated by the gradual addition of $(NH_4)_2SO_4$ to 80% saturation with stirring at 4 °C for 1 h. After centrifugation at 39,000xg for 30 min, the pellets were resuspended in and dialyzed against 25 mM sodium phosphate, pH 7.2. The wild-type or mutant hEGF analogue was first separated by gel filtration chromatography on a Sephadex G-75 column (1x90 cm) using 25 mM sodium phosphate, pH 7.2. Fractions containing the hEGF protein were pooled and loaded onto a reversed-phase HPLC column (Vydac 218TPS, 4.6x250 mm). Elution conditions, using a Waters Model 840 HPLC system, consisted of an isocratic wash of 15% CH_3CN for 15 min followed by a 15-34% CH_3CN gradient in 10 mM sodium phosphate, pH 7.2, increasing linearly at a rate of 1% per min. The fractions containing each hEGF protein were pooled, then lyophilized and stored at -80 °C.

Native gel electrophoresis and immunoblotting of hEGF proteins

Each hEGF mutant protein was examined by non-discontinuous gel electrophoresis in the absence of any detergent / denaturing agent. Samples were resolved in 10% polyacrylamide gels buffered with 75 mM Tris-phosphate, pH 7.0, the same buffer being used for electrophoresis at a constant 200 V for 30 min at 4 °C. Protein samples were subsequently transferred to Immobilon-P membranes (Millipore, Inc.) in the same buffer at a constant 20 V for 16 h at 4 °C. Blots were probed with an anti-hEGF monoclonal antibody [IgG1_k] (Upstate Biotechnology, Inc.) at 1 : 2000 dilution. This high-affinity, neutralizing antibody is specific for hEGF and does not cross react with mEGF or hTGF α . Secondary goat anti-

mouse polyclonal antibody conjugated to HRP (Transduction Labs.) was used at 1 : 2000 dilution as per manufacturer's protocol. ECL (Amersham, Inc.) was used for detection purposes.

Radioreceptor Competition Assay

Membrane-bound hEGF receptors were isolated from A431 (human epidermoid carcinoma) cells according to Akiyama *et al.* (11) with modifications described by Campion *et al.* (12). Specific binding of hEGF proteins to the EGF receptor was determined by a radioreceptor competition assay (13). Purified membrane fractions from A431 cells were resuspended in buffer (20 mM HEPES, pH 7.4, and 10 mM PMSF) and the total membrane protein content was determined by the Bio-Rad Protein Assay. ^{125}I -hEGF was prepared by the chloramine-T method (14) to an average specific activity of $\sim 150,000$ cpm/pmol hEGF. Two μg of the receptor preparation was incubated with 30 nM wild-type ^{125}I -hEGF in a mixture containing 20 mM HEPES, pH 7.4, and 0.1% BSA, and varying concentrations of unlabeled competing hEGF species in a total reaction volume of 100 μl . Incubation was conducted at 37 °C for 30 min. Receptor-bound ^{125}I -hEGF was collected on cellulose-acetate filters (Millipore GVWP). Unbound ligand was removed by washing filters with 20 mM HEPES, pH 7.4, containing 0.1% BSA. Filters were dried and the radioactivity was quantitated by liquid scintillation spectrometry.

Receptor Tyrosine Kinase Stimulation Assay

The ability of wild-type and mutant hEGF analogues to stimulate the protein-tyrosine kinase activity of the EGF receptor was determined by measuring the incorporation of ^{32}P from [γ - ^{32}P]ATP into the synthetic polypeptide substrate $(\text{Glu}_4, \text{Tyr}_1)_n$ (Sigma Chemical Co.) as a function of growth factor concentration. Solubilized and partially purified EGF receptors from A431 cell membranes (11, 12) were preincubated with increasing concentrations of wild-type or mutant hEGF protein for 15 min at room temperature under conditions similar to those described by Akiyama *et al.* (11), with modifications by Koland and Cerione (15). Preincubation in the absence of hEGF protein served as a control. The reaction mixture contained 20 mM HEPES pH 7.2, 250 mM NaCl, 2 mM MnCl_2 , 10 mM MgCl_2 , 1 mM DTT, 250 mM $(\text{NH}_4)_2\text{SO}_4$, 100 μM Na_3VO_4 , 5% glycerol, 0.05% Triton X-100 and ~ 1 μg of total

membrane protein. The kinase reaction was initiated by the addition of [γ - ^{32}P]ATP (1.35 Ci/mmol) and (Glu₄,Tyr₁)_n substrates to final concentrations of 75 μM and 0.5 mg/ml, respectively, in a final reaction volume of 100 μl . After incubation at room temperature for 10 min, the reaction was stopped by the addition of 200 μl 5% TCA containing 10 mM sodium pyrophosphate. The acid-insoluble material was collected on 25-mm Millipore HAWP filters which were then washed extensively with 5% TCA containing 10 mM Na-pyrophosphate. Filters were then dried and the incorporated radioactivity was determined by liquid scintillation spectrometry. The radioactivity incorporated in the absence of hEGF was subtracted from hEGF-stimulated values. The kinase activities reported include the incorporation of ^{32}P into both the polypeptide substrate and the receptor. The contribution of the latter, as determined by assaying the receptor in the absence of the polypeptide substrate, was found to be less than 2% of the total activity.

Cell culture and Clonogenicity assays

C18, an EGF-dependent rat-tracheal epithelial cell line, was a kind gift from Dr. Margaret Terzaghi-Howe, ORNL. Cell stocks were maintained at 37 °C in a humidified atmosphere of 5% CO₂, as proliferating cultures in Ham's F12 medium (GIBCO, NY) supplemented with 2% FBS (Hyclone), 0.1 $\mu\text{g/ml}$ hydrocortisone (Sigma Chemical Co.), 1 $\mu\text{g/ml}$ insulin (Sigma Chemical Co.), 50 $\mu\text{g/ml}$ gentamycin (GIBCO, NY) and 1 ng/ml EGF (complete medium). For clonogenicity assays, single-cell suspensions were plated in 6-well culture dishes (Costar) at a density of 5×10^2 cells/well. The growth medium of experimental cultures contained hEGF variants at the desired concentrations while control samples lacked EGF. After a period of 5-8 days, the dishes were rinsed thoroughly with Ca²⁺/Mg²⁺-free PBS, fixed in methanol, stained with Giemsa and visualized under the light microscope. The % cloning efficiency was calculated from the ratio of discrete colonies scored (criterion : 32-50 cells / colony) to the number of cells seeded per well. The assay permits a distinction between alterations in growth rate (colony size) and survival (colony number) in a given population. Cultures used for these experiments were between passage numbers 26-47 and the cloning efficiency was always compared with sample populations obtained from the same passage.

Results

Purification, electrophoresis and immunoblotting of hEGF mutants

The yields of hEGF mutant proteins obtained after purification by reversed-phase HPLC ranged from 30-75 $\mu\text{g} / \text{l}$. Appropriate fractions were examined by native gel electrophoresis and immunoblotting with a neutralizing anti-hEGF monoclonal antibody. The results, shown in Figure II-2, indicate that the H16L, H16N, H16Q and H16Y proteins co-migrate since they possess the same net charge of -4. The altered electrophoretic mobility of the H16R mutant derives from a net charge of -3. Antibody specificity is demonstrated by the absence of signal in the control lane which is loaded with the N32P hEGF mutant; structural alterations in the native N32P mutant (16), preclude antibody recognition. The immunoassay also confirms the identity of the mutants as hEGF molecules with no major structural perturbations compared to wild-type hEGF.

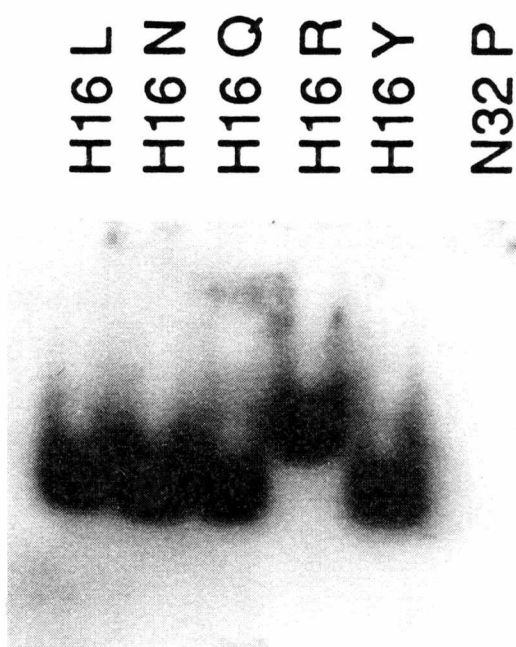
Determination of relative affinities of hEGF mutants

Specific binding of each position 16 mutant was assessed with respect to its ability to compete with ^{125}I -labeled wild-type hEGF bound to EGF receptors in membrane fractions of A431 cells. The competition curves, presented in Figure II-3, permit the estimation of IC_{50} values which indicate the concentration of competing species required for inhibition of binding by 50%. The ratio of wild-type and mutant IC_{50} values was used as an index of the relative affinity of the receptor for each mutant.

Binding data (see Table II-1) for the H16 mutants of hEGF show that their relative receptor affinities range from 7%-272% of wild-type hEGF. Substitution of the polar H16 side-chain with a bulky, hydrophobic residue (H16L) decreases the affinity to 13% of wild type. Interestingly, replacement with an amphipathic tyrosyl moiety also decreases the relative binding affinity of the H16Y mutant to a modest 32%. This suggests a role for the aromaticity and/or hydrogen-bonding potential of H16 in receptor binding. The introduction of a positively charged guanidinium moiety reduces the binding affinity of the H16R variant to 7% of wild-type hEGF. Interactions necessary for optimal receptor recognition may be compromised by the delocalized charge and decreased flexibility of the bulky arginine side-chain at this site. Among this series of mutant proteins, the isosteric N and Q substitutions

Figure II-2. Native gel electrophoresis and immunoblotting of H16 hEGF mutants.

blot: anti-
hEGF



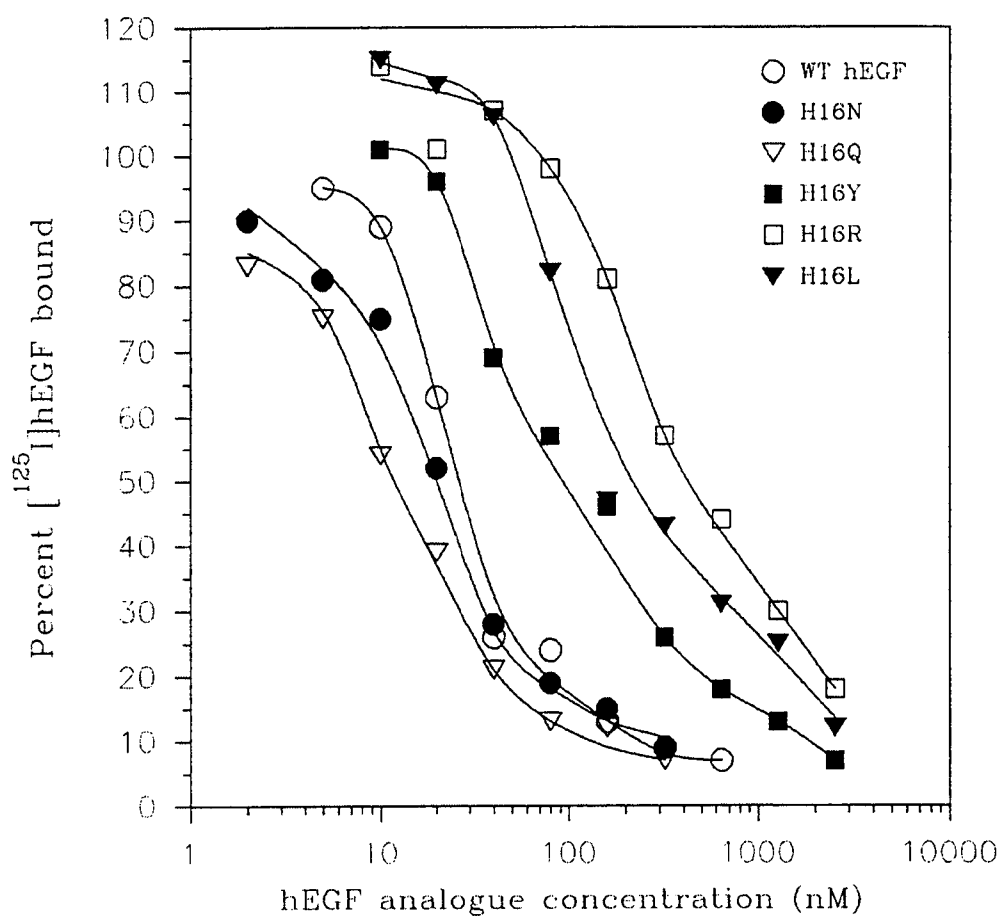


Figure II-3. Radioreceptor competition binding curves for H16 hEGF mutants. Varying concentrations of each mutant were incubated at 37 °C for 30 min with ^{125}I -labeled wild-type hEGF (~150,000 cpm/pmol) and EGF receptor-containing membrane fraction (20 $\mu\text{g}/\text{ml}$ total protein) in 20 mM HEPES (pH 7.4) and 0.1% BSA. All curves shown represent the best fit through the average data points of duplicate experiments.

Table II-1. Biochemical properties of H16 hEGF mutants

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

(a) Relative binding affinity = $IC_{50}(\text{wild-type})/IC_{50}(\text{mutant}) \times 100\%$

(b) Relative agonist activity = $EC_{50}(\text{wild-type})/EC_{50}(\text{mutant}) \times 100\%$

Data are the representative of average values from at least two independent Determinations. See text under **Results** for determination of IC₅₀ and EC₅₀ values.

exhibit elevated receptor affinities (150% and 272%) compared to wild type. This indicates that neither aromaticity nor charge but hydrogen-bonding interactions via the imidazole nitrogen(s) of H16 are important for receptor binding.

Determination of relative agonist activities of hEGF mutants

Using the synthetic polymer $(\text{Glu}_4, \text{Tyr}_1)_n$ as the exogenous substrate, ligand-dependent activation of the EGFR was measured as a function of the concentration of hEGF species. Protein concentrations required for half-maximal stimulation (EC_{50}) were determined from the dose-response curves presented in Figure II-4. The ratio of the EC_{50} value of wild-type compared with that obtained for each mutant was used to assess the relative agonist activity of each mutant under the kinase assay conditions.

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

Clonogenic efficiencies of hEGF mutants

The mitogenic activity of H16 mutants of hEGF was evaluated in a clonogenic assay with C18 cells which are EGF-dependent for growth. Table II-2 shows that H16N, H16Q, H16Y and wild-type hEGF stimulate colony formation with similar efficiencies at doses of 0.9 and 2.7 ng/ml (0.15-0.44 nM). A small number (<0.01%) of colonies appear more elongated and less adherent than cells of normal epithelial morphology, which may reflect the clonal variation in the population. No appreciable differences in growth rates and colony survival were discernable. Cells stimulated with 0.9 ng/ml H16L or H16R form ~10-15 % fewer colonies compared to wild-type. When the dose is increased to 2.7 ng/ml, to compensate for their reduced potency, both H16L and H16R are similar to wild-type hEGF in their activity.

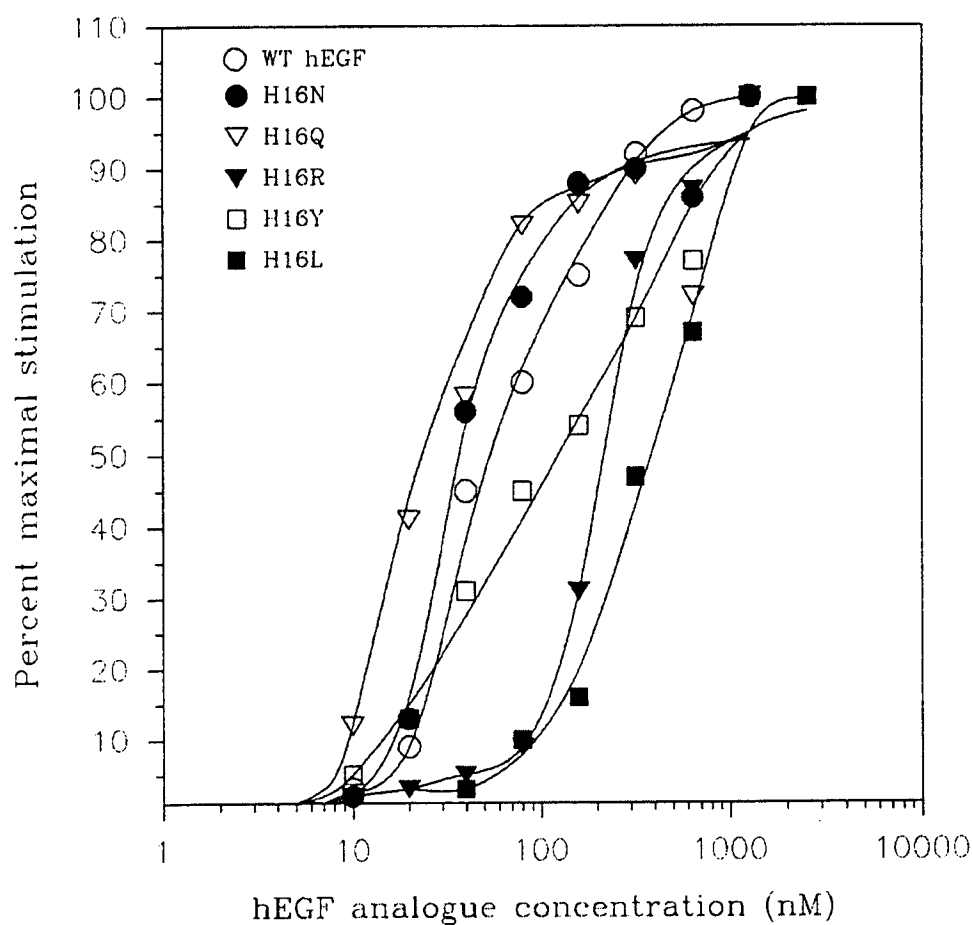


Figure II-4. Receptor tyrosine kinase stimulation curves for H16 hEGF mutants. The rate of EGF-dependent phosphorylation of exogenously added $(\text{Glu}_4, \text{Tyr}_1)_n$ substrate by partially purified EGF receptor was measured as described under **Materials and methods**. All curves depicted represent the best fit through the average data points of duplicate experiments.

Table II-2. Clonogenic efficiencies of H16 hEGF mutants.

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

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

Discussion

Biological responses to EGF and TGF α are not always identical (17) and some studies have suggested that the EGFR discriminates between bound EGF and bound TGF α (18-22). Furthermore, pH sensitivity of the ligand-receptor interaction dictates intracellular trafficking (internalization / recycling / degradation) of the EGF/TGF α superfamily (23) and may influence long term responses to growth factor stimulation (24). Structure-function analyses are thus essential for distinguishing between *common* and *specific* modes of receptor recognition and activation.

Site-directed mutagenesis of H16 does not indicate an intrinsic structural requirement for the imidazole functionality. The H16Q and H16N mutants exhibit increased relative binding affinities and agonist activities *in vitro*, unlike the H16R mutant which is impaired in receptor recognition. At physiological pH, the R side-chain acts only as hydrogen-bond donor in contrast to the Q and N side-chains which may engage in hydrogen-bond donor/acceptor interactions and thereby compensate for normal H16 function. This interpretation is consistent with NMR analyses of hEGF solution structure, which predict the following hydrogen-bonding interactions: V34-H16(CO), H16(H $^{\delta}$)-Y44(CO), and C δ / C ϵ protons of H16 with the C ϵ protons of Y37 (1, 25). The results from this study suggest that this intricate network reinforces a flexible loop contributing to hEGF-receptor recognition and activation.

The role of H16 in hEGF structure-function is revealed by the following:

- (i) The pK $_a$ value of H16 in hEGF is similar to that of H18 in hTGF α suggesting *common* modes of EGFR recognition.
- (ii) The binding data indicate a requirement for a polar, isosteric side-chain at position 16 of hEGF that functions as both hydrogen-bond donor and acceptor.
- (iii) Since the mutant proteins are similar to wild-type hEGF in their native conformation, as determined by immunoblotting experiments, the observed loss or gain of function arises from the differential ability of these mutant ligands to alter critical contacts essential for optimal receptor activation. This study confirms predictions based on NMR (1,2) and clarifies the biochemical properties of H16 involved in ligand-receptor interactions.

References

1. Hommel, U., Harvey, T. S., Driscoll, P. C. and Campbell, I. D. (1992) *J. Mol. Biol.*, **227**, 271-282
2. Li, Y-C. and Montelione, G. T. (1995) *Biochemistry*, **34**, 2408-2423
3. Tappin, M. J., Cooke, R. M., Fitton, J. E. and Campbell, I. D. (1989) *Eur. J. Biochem.*, **179**, 629-637
4. Murray, M. B., Lamerdin, J. A., Nandagopal, K., Niyogi, S. K. and Serpersu, E. H. (1996) *FASEB J.*, **10**, A1400
5. Shiah, H-S., Chen, T-Y., Chang, C-M., Chow, J. T., Kung, H-S. and Hwang, J. (1992) *J. Biol.Chem.*, **267**, 24034-24040
6. Defeo-Jones, D., Tai, J. Y., Vuocolo, G. A., Wegrzyn, R. J., Schofield, T. L., Riemen, M. A. and Oliff, A. (1989) *Mol. Cell. Biol.*, **9**, 4083-4086
7. Feild, J. A., Reid, R. H., Rieman, D. J., Kline, T. P., Sathe, G., Greig, R. G. and Anzano, M. A. (1992) *Biochem. J.*, **283**, 91-98
8. Helmsley, A., Arnheim, N., Toney, M. D., Cortopassi, G. and Galas, D. J. (1989) *Nucleic Acids Res.*, **17**, 6545-6551
9. Hanahan, D. (1985) in Glover, D. M. (ed), *DNA Cloning*, IRL Press, Oxford, Vol. **1**, pp. 109-135
10. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA*, **55**, 5463-5467
11. Akiyama, T., Kadooka, T. and Ogawara, H. (1985) *Biochem. Biophys. Res. Commun.*, **131**, 442-448
12. Campion, S. R., Matsunami, R. K., Engler, D. A. and Niyogi, S. K. (1990) *Biochemistry*, **29**, 9988-9993
13. Carpenter, G. (1985) *Methods Enzymol.*, **109**, 107-108
14. Hunter, W. M. and Greenwood, F. C. (1962) *Nature*, **194**, 495-496
15. Koland, J. G. And Cerione, R. A. (1988) *J. Biol. Chem.*, **263**, 2230-2237
16. Campion, S. R., Biamonte, C., Montelione, G. T., and Niyogi, S. K. (1993) *Protein*

Engng., **6**, 651-659

17. Derynck, R. (1992) *Adv. Cancer Res.*, **58**, 27-52
18. Lax, I., Johnson, A., Howk, R., Sap, J., Bellot, F., Winkler, M., Ullrich, A., Vennstrom, B., Schlessinger, J. and Givol, D. (1988) *Mol. Cell. Biol.*, **8**, 1970-1978
19. Richter, A., Drummond, D. R., MacGarvie, J., Puddicombe, S. M., Chamberlin, S. G. and Davies, D. E. (1995) *J. Biol. Chem.*, **270**, 1612-1616
20. van de Poll, M. L. M., Lenferink, A. E. G., van Vugt, M. J. H., Jacobs, J. J. L., Janssen, J. W. H., Joldersma, M. and van Zoelen, E. J. J. (1995) *J. Biol. Chem.*, **270**, 22337-22343
21. Puddicombe, S. M., Chamberlin, S. G., MacGarvie, J., Richter, A., Drummond, D. R., Collins, J., Wood, L. and Davies, D. E. (1996) *J. Biol. Chem.*, **271**, 15367-15372
22. Summerfield, A. E., Hudnall, A. K., Lukas, T. J., Guyer, C. A. and Staros, J. V. (1996) *J. Biol. Chem.*, **271**, 19656-19659
23. French, A. R., Tadaki, D. K., Niyogi, S. K. and Lauffenburger, D. A. (1995) *J. Biol. Chem.*, **270**, 4334-4340
24. Reddy, C. C., Niyogi, S. K., Wells, A., Wiley, H. S. and Lauffenburger, D. A. (1996) *Nature Biotech.*, **14**, 1696-1699
25. Hommel, U., Dudgeon, T. J., Fallon, A., Edwards, R. M. and Campbell, I. D. (1991) *Biochemistry*, **30**, 8891-8898

Chapter III

FUNCTIONAL IMPORTANCE OF LEUCINE 15 OF HUMAN EPIDERMAL GROWTH FACTOR IN RECEPTOR RECOGNITION AND ACTIVATION

Introduction

High-resolution analyses of three-dimensional structures of hEGF (1), mEGF (2, 3) and hTGF α (4), derived from two-dimensional NMR, predict that the crucial R41 residue of hEGF (5) lies in a hydrophobic pocket, which includes L15 and Y13, near the domain-domain interface of hEGF (see Figure III-1). The hydrophobicity of Y13 of hEGF is required for receptor binding (6). Thus it was of interest to define the putative receptor-recognition epitope involving Y13, L15 and R41 of hEGF.

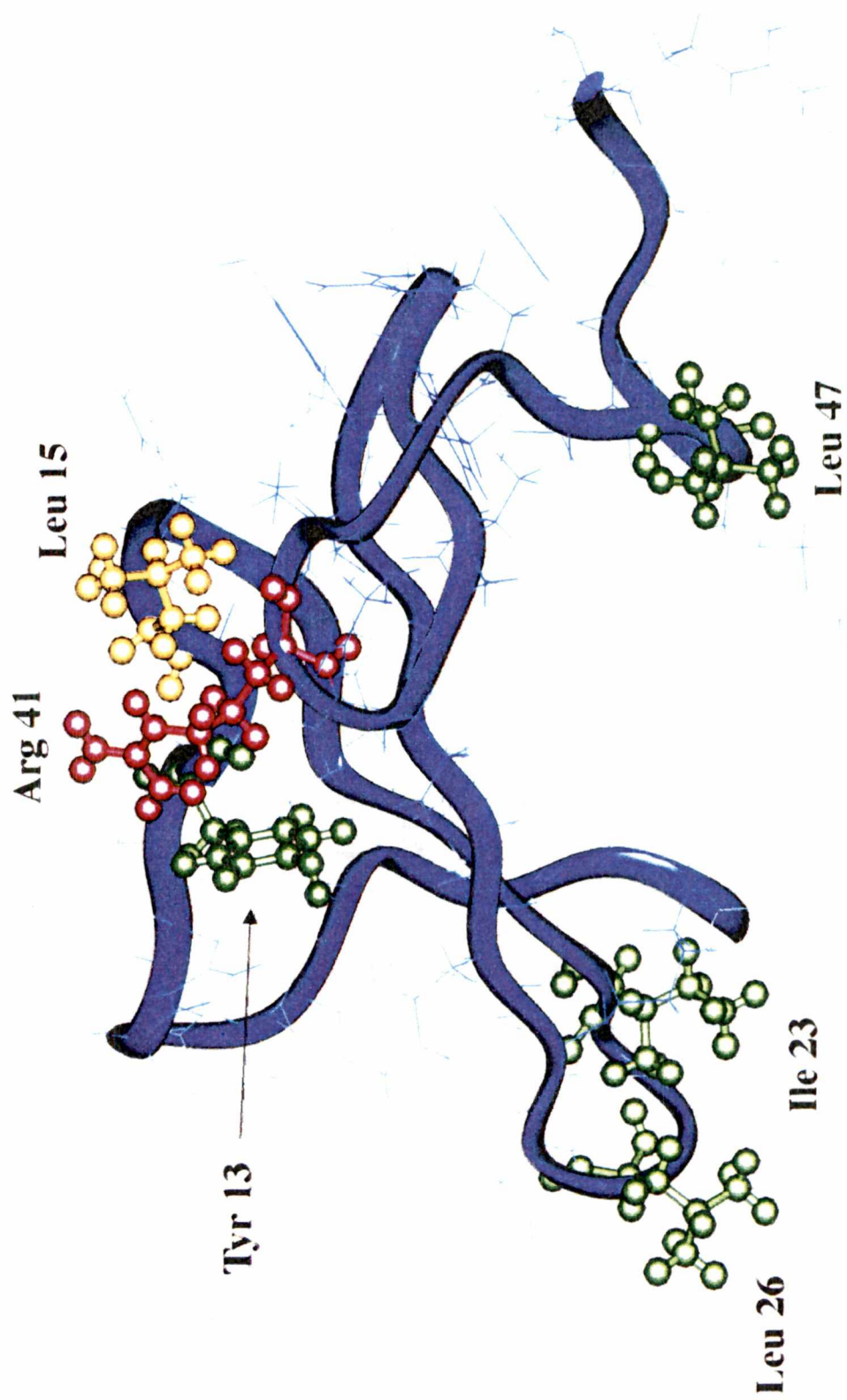
Sequence alignments reveal that the residue at position 15 of EGF is conserved as a leucine in different species and as a phenylalanine for the corresponding residue (F17) in hTGF α . This suggests that a large hydrophobic side-chain mediates receptor binding. The potential role of size and hydrophobicity of L15 in hEGF structure-function was therefore evaluated by site-directed mutagenesis. The relative affinity and agonist activity of mutant hEGF proteins were determined by radioreceptor competition and receptor tyrosine kinase stimulation assays, respectively. Selected mutants were examined by one-dimensional NMR spectroscopy for possible structural perturbations introduced by specific amino acid substitutions. These studies permit evaluation of the role of L15 of hEGF in ligand-receptor interactions.

Materials and Methods

Oligonucleotide-directed site-specific mutagenesis

Deoxyoligonucleotides containing the desired mutations were synthesized in a Milligen / Biosearch Cyclone Plus DNA synthesizer. The sequences of the oligonucleotides, with the mutated codons underlined and the mismatches in boldface, are as follows: L15A, 5'-CTGCGCGCCACGACGGTGTGTTG-3'; L15E, 5'-CTGCGGAGCACGACGGTGTGTTG-3'; L15F, 5'-CTGCTTTCCACGACGGTGTGTTGC-3'; L15R, 5'-CTGCCGGACACGACGGTGTGTTGC-3'; L15V, 5'-CTGCGTGCCACGACGGTGTGTTG-3'; and L15W, 5'-CTGCTTGGCACGACGGTGTGTTGC-3'. A PCR strategy (7) was utilized to

Figure III-1. Computer model depicting the Y13-R41-L15 recognition unit in hEGF. It also shows the position of L15 relative to the positions of other residues in hEGF found to be important for receptor recognition. NMR coordinates were provided by Dr. G. T. Montelione (Rutgers University). The model was generated by Dr. John A. Lamerdin using MOLSCRIPT software (Kraulis, P. J. (1991) *J. Appl. Cryst.*, **24**, 946-950).



generate site-directed mutants of hEGF with high efficiency. Double stranded DNA of the expression vector, pEGF1, containing the *hEGF* insert, was used as the template. Each deoxyoligonucleotide primer bearing the mutation was utilized in a 'back to back' configuration with the wild-type reverse primer 5'-TACCCGTCGTGAGACAGCGG-3'. The conditions for PCR amplification and isolation of mutants have been described in Nandagopal *et al.* (8) and the Materials and Methods section of Chapter II. All engineered mutations and the absence of any undesired genetic alterations were confirmed by sequencing the entire *hEGF* gene.

Expression and purification of recombinant hEGF proteins

Both mutant and wild-type hEGF proteins were expressed in *E. coli* and purified to homogeneity as described in Nandagopal *et al.* (8) and the Materials and Methods section of Chapter II. The fractions containing each hEGF protein were pooled, then lyophilized and stored at -80 °C.

Radioreceptor competition assay

The relative binding affinities of the hEGF variants were determined by a radioreceptor competition binding assay as described in Nandagopal *et al.*, (8) and the Materials and Methods section of Chapter II.

Receptor tyrosine kinase stimulation assay

The ability of wild-type and mutant hEGF proteins to stimulate the protein-tyrosine kinase activity of the EGF receptor was determined by measuring the incorporation of ^{32}P from [γ - ^{32}P]ATP into the synthetic polypeptide substrate (Glu₄,Tyr₁)_n as a function of growth factor concentration. The assay conditions have been described in Nandagopal *et al.* (8) and the Materials and Methods section of Chapter II.

NMR spectroscopy

Purified protein samples were prepared for NMR experiments by repeated lyophilization and dissolution in $^2\text{H}_2\text{O}$ to a concentration of ~0.96 mM in a final volume of 500 μl . All NMR spectra were obtained on a wide-bore Bruker AMX 400 MHz spectrometer at 27 °C. Samples were titrated first to pH 3.4 and then pH 7.2 (without correction for isotope effect); spectral data were obtained at both acidic and neutral pH. One-dimensional data sets were

collected over a spectral width of 4807 Hz with an acquisition time of 3.4 sec. Quadrature phase detection and a 90° observation pulse (6.7-7 μ sec) were used. Thirty two thousand data points of 64-128 transients were collected. The residual $^2\text{HO}^1\text{H}$ signal was saturated with a low-power pulse during the relaxation delay of 3.0 sec. Data were processed with 0.5-1 Hz line broadening before Fourier transformation. Chemical shifts were referenced to 2,2-dimethyl-2-sila-pentane-5-sulfonate (DSS).

Results

Purification and electrophoresis of hEGF mutants

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

Appropriate fractions of each mutant hEGF protein, obtained after purification by reversed-phase HPLC, were examined by native gel electrophoresis. The results confirm the homogeneity of the hEGF mutant proteins and their purity is estimated to be ~ 93 - 97% , based on Coomassie staining of native gels (data not shown). The observed electrophoretic mobilities agree with the mutations introduced. For example, the altered migration pattern of the L15R or L15E mutant can be ascribed to the additional positive or negative charge introduced into the hEGF molecule (data not shown).

Determination of relative affinities of hEGF mutants

The competition binding curves, depicted in Figure III-2, were generated by measuring the amount of receptor-bound ^{125}I -hEGF remaining on the filters, after equilibrium binding in the presence of increasing concentrations of competing hEGF species. The concentration of wild-type or mutant hEGF protein required for 50% competition of wild-type ^{125}I -hEGF (IC_{50}) was thus determined graphically for each hEGF variant. The ratio of wild-type to mutant IC_{50} values provided an index of the relative receptor affinity of each hEGF mutant.

As shown in Table III-1, the relative binding affinities of the hEGF analogues range from 45% to 1.6% of wild-type hEGF. The most conservative change to a valine lowers the binding to 18%; when the side-chain is shortened to an alanine, the binding diminishes to 2.4%. The charged amino-acid substitutions, L15R and L15E, are the least tolerated at this site. While the receptor affinity of the L15R ligand is found to be 1.6% of wild-type, no appreciable binding was detected in the case of the L15E mutant. Replacement of leucine with tryptophan or phenylalanine, which are both bulky and hydrophobic, results in relative binding affinities that are 26% and 45%, respectively.

Determination of relative agonist activities of hEGF mutants

The mitogenic effect of EGF is mediated through the stimulation of the receptor's intrinsic tyrosine kinase activity. The kinase stimulation curves, depicted in Figure III-3(A & B), represent the velocity of the EGF-dependent kinase reaction measured as a function of the concentration of the hEGF species present in the assay. Protein concentrations required to elicit half-maximal stimulation of the protein-tyrosine kinase activity of the receptor (EC_{50}) were determined for wild-type hEGF and the mutants from Figure III-3(B). The ratio of the EC_{50} value of wild-type compared with that for each mutant was used to assess the relative agonist activities of the hEGF variants.

Data presented in Table III-1 show that in general the order in which the L15F, L15W, L15V and L15A mutants stimulate the tyrosine kinase activity follows the same order as observed in the radioreceptor competition assay. At saturating ligand concentrations, the maximal levels of kinase stimulation by the L15F, L15W and L15V variants are similar to the level (V_{max}) by wild-type hEGF. Saturating concentrations of the L15A mutant activate

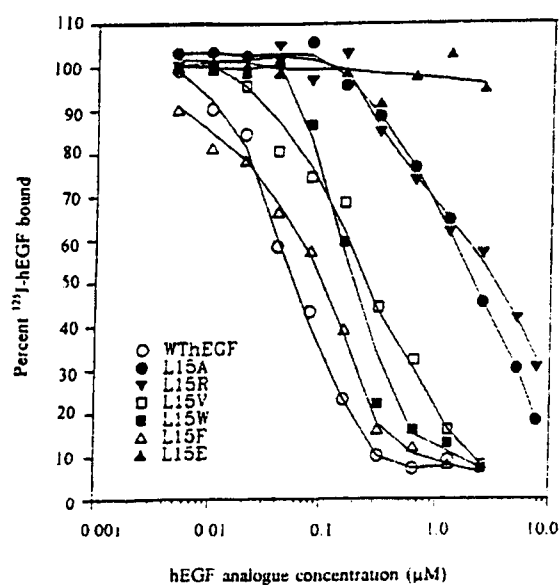


Figure III-2. Radioreceptor competition binding curves for L15 hEGF mutants. Varying concentrations of each mutant were incubated at 37 °C for 30 min with ^{125}I -labeled wild-type hEGF (~150,000 cpm/pmol) and EGF receptor-containing membrane fraction (20 $\mu\text{g}/\text{ml}$ total protein) in 20 mM HEPES (pH 7.4) and 0.1% BSA. All curves shown represent the best fit through the average data points of duplicate experiments.

Table III-1. Biochemical properties of L15 hEGF mutants

hEGF species	Relative receptor affinity ^a	Relative agonist activity ^b	V _{max} cpm x 10 ⁻³	Relative maximal stimulation
Wild-type	100	100	120	100
L15F	45	27	123	103
L15W	26	22	120	100
L15V	18	12	119	100
L15A	2.4	nd	5.6	4.6
L15R	1.6	4.0	64	53
L15E	nd	nd	nd	nd

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

^bRelative agonist activity = $EC_{50}(\text{wild-type})/EC_{50}(\text{mutant}) \times 100\%$

^cRelative maximal stimulation = $V_{\max}(\text{mutant})/V_{\max}(\text{wild-type}) \times 100\%$

^dnd = not determined. See text under **Results** describing the determination of IC_{50} and EC_{50} values.

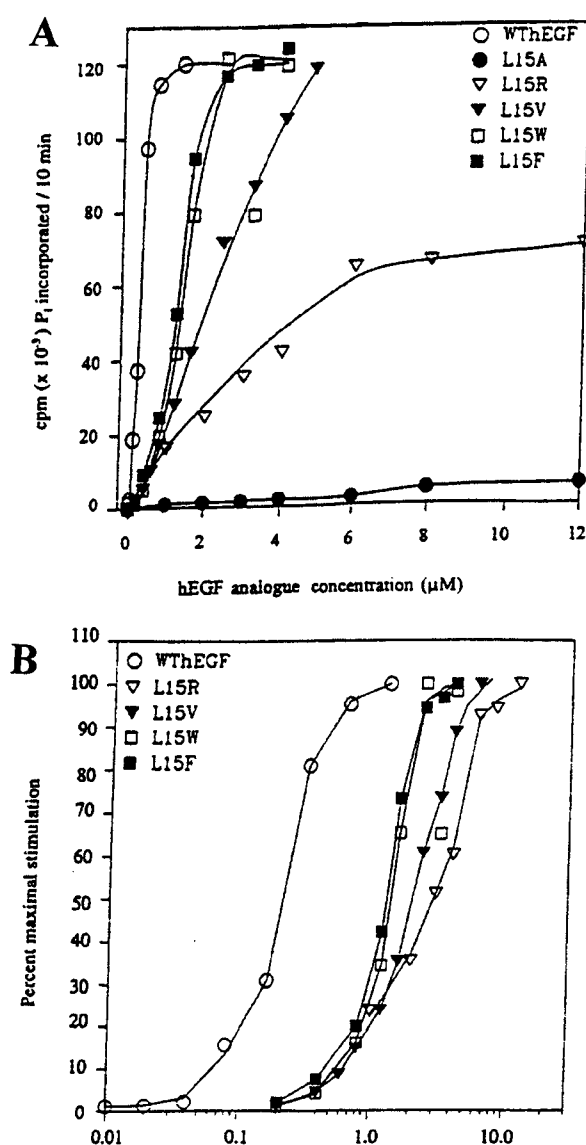


Figure III-3. Receptor tyrosine kinase stimulation curves for L15 hEGF mutants. The rate of EGF-dependent phosphorylation of exogenously added $(\text{Glu}_4, \text{Tyr}_{11})_n$ substrate by partially purified EGF receptor was measured as described under **Materials and methods**. (B) depicts a semi-logarithmic plot of the data presented in (A). All curves depicted represent the best fit through the average data points of duplicate experiments.

the receptor poorly (4.6% of wild-type $V_{\max}$) and preclude estimation of an EC_{50} value for this variant. This is in marked contrast to the activity profile for the L15R mutant which elicits a significant response in terms of kinase stimulation (53% of wild-type $V_{\max}$) at saturating concentrations of the ligand. The L15E mutant could not be characterized in this assay due to its very low affinity for the receptor; the kinase reaction is unreliable at protein concentrations above 15-20 μ M. Differences observed in the relative receptor affinities and agonist activities for some mutants may reflect the assay conditions which differ in receptor microenvironment, ionic strength, temperature and incubation time.

NMR spectra of selected hEGF mutants

NMR spectroscopy was utilized to evaluate potential structural changes in hEGF resulting from site-directed mutagenesis. Aromatic residues are spread throughout the hEGF molecule in its three-dimensional structure and the resonance frequencies and intensities of these aromatic ring protons are sensitive to molecular conformation and solvent accessibility. Therefore, comparisons of these parameters between wild-type and mutant proteins provide a convenient approach for distinguishing correctly from incorrectly folded mutants (9-11).

The L15A and L15R hEGF mutants were chosen for NMR studies on the basis of their differences in side-chain size and hydrophobic character and because of their extremely low biochemical activity as compared to wild-type hEGF. The L15E variant was not examined by NMR because of its poor yield. The aromatic regions of the 1-D ^{1} H-NMR spectra from 5.5-9.0 ppm are shown in Figure III- 4A (pH 7.2) and Figure III-4B (pH 3.4) for wild-type, L15A, and L15R hEGF variants. The sequence-specific resonance assignments for the wild-type protein shown in Figure III-5 are derived from previously published data (10).

As most chemical shifts of wild-type and mutant hEGF proteins are very similar at pH 3.4 (Figure III-5), it was possible to extend resonance assignments from wild-type hEGF to the mutant proteins. With the exception of minor shifts of the W49C2H, Y13E/Y37D resonances ($\sim$ 6.8 ppm region) and the W50C4H/W49C7H/W49C6H resonances ($\sim$ 7.3-7.4 p.p.m. region) in the case of the L15A variant, other minor perturbations that could be characterized were very similar in the spectra of both mutants (see Table III-2). The spectral shifts that are common to both L15R and L15A mutants as compared to wild-type hEGF,

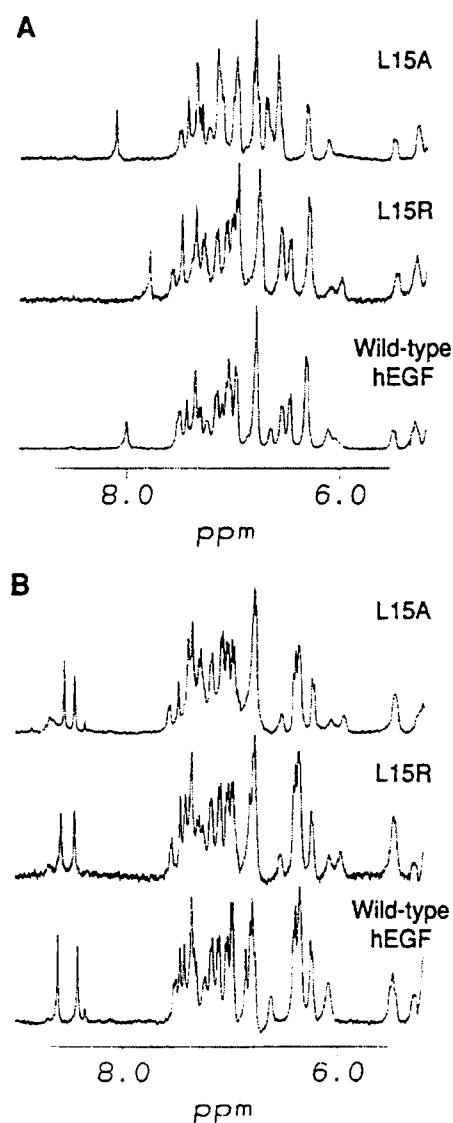


Figure III-4. ^1H -NMR spectra of aromatic regions in wild-type and L15 hEGF mutants at (A) pH 7.2 and (B) pH 3.4. The samples were prepared in $^2\text{H}_2\text{O}$ at a final concentration of ~ 0.96 mM. Spectra were acquired at 27°C over a spectral width of 4807 Hz; 64-128 transients were collected.

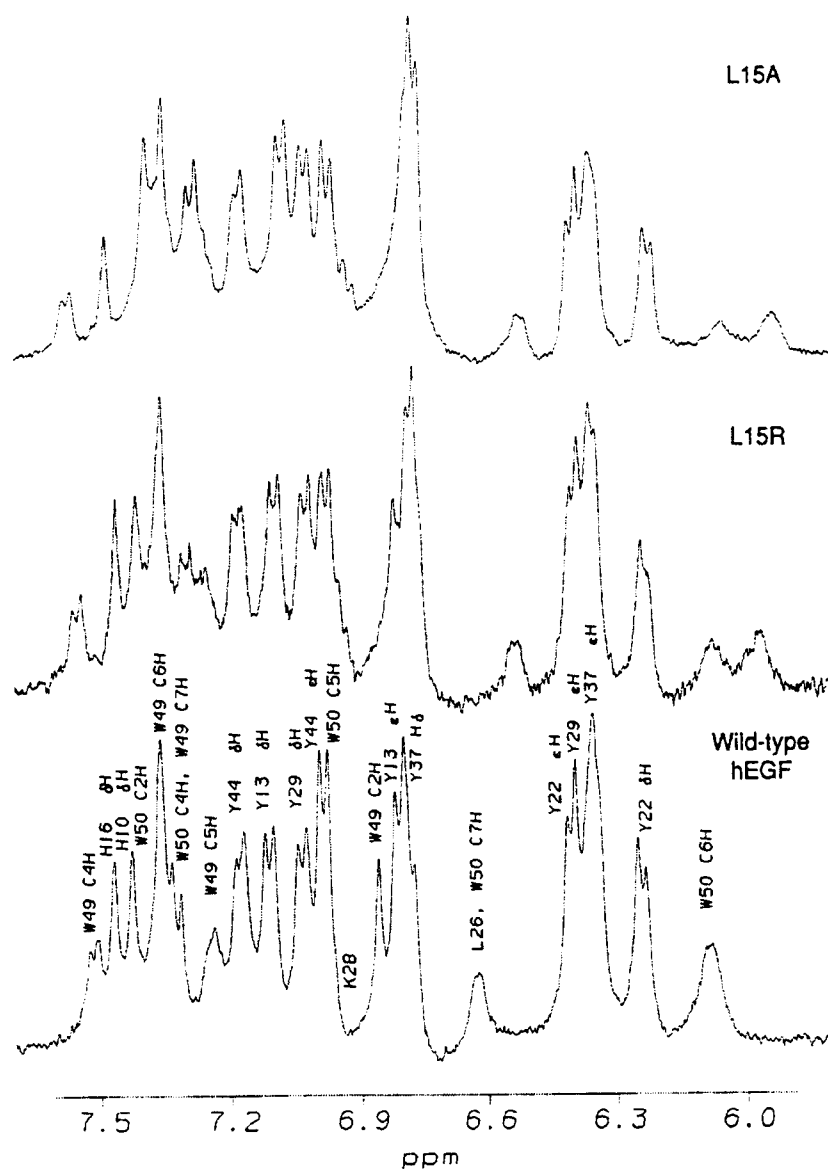


Figure III-5. Expansion of the ^1H -NMR spectra of wild-type and L15 mutants in the 6.0-7.5 p.p.m. region. Sequence-specific assignments for wild-type hEGF are shown in order to permit comparison with the L15R and L15A variants.

Table III-2. Listing of proton resonances exhibiting minor changes ($\Delta\delta \leq 75$ p.p.b.) in chemical shifts between wild-type and mutant hEGF molecules at pH 3.4

L15A	L15R
W50C7H/L26HN ^{a,b}	W50C7H/L26HN ^{a,b}
W50C5H	W50C5H
W49C5H	W50C4H/W49C7H /W49C6H ^{a,b}
W49C4H	W49C5H
W49C2H	W49C4H
Y37 ϵ H/Y29 ϵ H ^a	W49C2H
Y37 δ H/Y13 ϵ H ^a	Y37 δ H/Y13 ϵ H ^a
Y13 δ H	Y13 δ H

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

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

involve residues dispersed throughout these molecules. Therefore, the spectral differences between the L15R and L15A mutants are relatively minor and are not localized in the structures of these mutant proteins. Spectral data obtained at pH 7.2 (Figure III-4A) reveal that the L15A mutant protein, unlike L15R, displays an "altered" NMR profile when compared to wild-type hEGF. Several peaks with small chemical shift differences and some peak broadening can be observed (see **Discussion**). Caution was exercised with regard to assigning the few resonances with comparatively large chemical shifts (≥ 75 p.p.b.), changes in lineshape or intensity relative to the corresponding resonances in wild-type hEGF.

Discussion

The importance of L15 of hEGF in ligand-receptor interactions is revealed by the following:

- (i) Both the geometry of the amino acid side-chain and hydrophobic character of the residue at position 15 in hEGF are important in receptor binding.
- (ii) Although the L15A and L15R mutants have comparable relative receptor affinities, the low $V_{\max}$ phenotype observed with the L15A mutant indicates that it is a partial agonist and suggests a partial uncoupling between receptor binding and activation. The kinase data thus implicate a functional role for L15 of hEGF in receptor activation.
- (iii) One-dimensional NMR analyses of the L15A and L15R mutants do not indicate the presence of gross structural perturbations that could possibly explain their drastically reduced biochemical activity compared to wild-type hEGF.

The changes observed in the relative affinities of L15 hEGF mutants lend credence to L15 being an active participant in receptor recognition along with Y13 (6), I23 (11,12), L26 (11, 13) and L47 (14, 15, 16) [see Figure III-1] by promoting hydrophobic interactions. The hydrophobic contribution of a given residue to the conformational free energy of a protein is proportional to the difference between the standard state accessibility and the mean solvent accessibility. In effect, this measures the area buried by the residue upon folding (17). Qualitative considerations of such thermodynamic parameters reveal that subtle variations in side-chain length have profound effects on mean fractional area loss and ΔG° , the Nozaki-Tanford free energy of transfer from an aqueous to a non-aqueous environment (17). Interestingly enough, a central hydrophobic region in the human growth hormone-receptor

complex accounts for more than three-quarters of the binding free energy required for optimal receptor-ligand interactions (18).

The amino group of L15 in the N-terminal domain is also in close proximity to the carbonyl group of R41 in the C-terminal domain (1). The non-conservative substitution of R at position 15 may either interfere with the inter-domain hydrogen-bonding capacity of R41 or affect the pK of the R41 "partner" on the receptor by altering the local charge distribution. This could lead to a drastically decreased receptor affinity. The substitution of Y13, which is located in the domain-domain interface of hEGF (1), with R also decreases the receptor affinity to only 5.5% of wild-type hEGF (6). It can be pointed out that the negative charge in the L15E mutant may neutralize the positive charge of R41, thereby preventing R41 from binding complementary residues on the extracellular surface of the EGF receptor. L15 of hEGF, in concert with Y13 (6), provides the guanidinium moiety of R41 (5) a unique orientation in a hydrophobic cleft and this configuration may be conducive to the formation of resonance-stabilized guanidinium-carboxylate ion pairs necessary for high-affinity binding to the EGF receptor. Alternatively, planar stacking interactions (19) between R41 and an aromatic side-chain in the receptor protein can be envisaged. Confirmation of these predictions awaits resolution of co-crystal structures of the active receptor-ligand complex.

The one-dimensional NMR spectra of L15A and L15R mutant proteins obtained at pH 3.4 are similar to the spectrum of wild-type hEGF with very few peaks exhibiting significant chemical shifts (≥ 75 p.p.b.) indicating no major structural changes in these mutants with respect to wild-type hEGF conformation. In interpreting the spectral data obtained at neutral pH, however, it is important to note that chemical shifts are extremely sensitive to even minor conformational changes. The changes may be due to the introduction or replacement of charged functional groups (*eg.*, L15R). If the size of the side-chain is decreased significantly (*eg.*, L15A), altered packing interactions could give rise to differences in the internal flexibility of the protein with resultant changes in chemical shifts. The relationships between chemical shifts and conformational changes cannot be easily interpreted in structural terms (20). The three-dimensional structure of EGF has been reported to be largely

independent of pH over the range 2-8 (3). Hence, the three-dimensional structures of the L15R and L15A mutants are considered to be similar to native hEGF structure.

Site-directed mutagenesis of L15 in the N-terminal domain and that of L47 in the C-terminal domain (14) indicate a stringent requirement for a γ -branched "leucine-type" residue at these positions in the hEGF molecule. Substitution with a β -branched amino-acid residue results in a relative binding affinity of only 18% (L15V) or 17% (L47I) compared to wild-type hEGF. Replacement with a methyl group (A for L) at either position further decreases the relative binding affinity to 2.4% (L15A) and 1.8% (L47A). The properties of the L15A mutant described in this study are consistent with the observations made by Shin *et al.* (21), who reported that chemically synthesized L15A had a drastically reduced mitogenic potency. The moderate loss of receptor affinity seen with the L15F and L15W mutants (present study) could be partially due to steric crowding; these mutants may not fit properly into the ligand-binding "pocket" of the EGF receptor, even though the mutations may not greatly disturb interactions within the hEGF molecule itself. Feild *et al.* (22) showed that the F17Y mutant of hTGF α retains ~8% of the relative binding affinity, although colony forming ability in a soft-agar transformation assay was not impaired. No comparable data are available with respect to the L47 site of hEGF.

Dose-response curves for *in vitro* agonist activity indicate that the L15A (present study), I23T, L26G (23) and L47A (14, 23) hEGF mutants exhibit a low $V_{\max}$ phenotype. The basis for this observation merits further investigation since it suggests partial uncoupling between the events of receptor recognition and kinase stimulation. The genetically engineered panel of hEGF mutants may thus be useful in deciphering the mechanism of EGFR activation. In turn, this could lead to the rational design of EGFR antagonists with therapeutic potential.

References

1. Hommel, U., Harvey, T. S., Driscoll, P. C., and Campbell, I. D. (1992) *J. Mol. Biol.*, **227**, 271-282
2. Montelione, G. T., Wuthrich, K., Burgess, A. W., Nice, E. C., Wagner, G., Gibson, K. D., and Scheraga, H. A. (1992) *Biochemistry* **31**, 236-249

3. Kohda, D., and Inagaki, F. (1992) *Biochemistry* **31**, 11928-11938
4. Moy, F. J., Li, Y., Rauenbuehler, P., Winkler, M. E., Scheraga, H. A., and Montelione, G. T. (1993) *Biochemistry* **32**, 7334-7353
5. Engler, D. A., Campion, S. R., Hauser, M. R., Cook, J. S., and Niyogi, S. K. (1992) *J. Biol. Chem.*, **267**, 2274-2281
6. Tadaki, D. K., and Niyogi, S. K. (1993) *J. Biol. Chem.*, **268**, 10114-10119
7. Helmsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J. (1989) *Nucl. Acids Res.*, **17**, 6545-6551
8. Nandagopal, K., Tadaki, D. K., Lamerdin, J. A., Serpersu, E. H., and Niyogi, S. K. (1996) *Protein Engng.*, **9**, 781-788
9. Engler, D. A., Montelione, G. T., and Niyogi, S. K. (1990) *FEBS Lett.*, **271**, 47-50
10. Campion, S. R., Biamonti, C., Montelione, G. T., and Niyogi, S. K. (1993) *Protein Engng.*, **6**, 651-659
11. Campion, S. R., Matsunami, R. K., Engler, D. A. and Niyogi, S. K. (1990) *Biochemistry* **29**, 9988-9993
12. Koide, H., Muto, Y., Kasai, H., Kohri, K., Hoshi, K., Takahashi, S., Tsukumo, K., Sasaki, T., Oka, T., Miyake, T., Fuwa, T., Kohda, D., Inagaki, F., Miyazawa, T. and Yokoyama, S. (1992) *Biochim. Biophys. Acta* **1120**, 257-261
13. Campion, S. R., Geck, M. K. and Niyogi, S. K. (1993) *J. Biol. Chem.* **268**, 1742-1748
14. Matsunami, R. K., Yette, M. L., Stevens, A., and Niyogi, S. K. (1991) *J. Cell. Biochem.*, **46**, 242-249
15. Moy, F. J., Scheraga, H. A., Liu, J., Wu, R. and Montelione, G. T. (1989) *Proc. Natl. Acad. Sci., USA*, **86**, 9836-9840
16. Dudgeon, T. J., Cooke, R. M., Baron, M., Campbell, I. D., Edwards, R. M. and Fallon, A. (1990) *FEBS Lett.*, **261**, 392-396
17. Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H. and Zehfus, M. H. (1985) *Science* **229**, 834-838
18. Clackson, T. and Wells, J. A. (1995) *Science* **267**, 383-386

19. Flocco, M. M. and Mowbray, S. L. (1994) *J. Mol. Biol.*, **235**, 709-717
20. Redfield, C. and Dobson, C. M. (1990) *Biochemistry* **29**, 7201-7214
21. Shin, S. Y., Watanabe, M., Kako, K., Ohtaki, T., and Munekata, E. (1994) *Life Sci.*, **55**, 131-139
22. Feild, J. A., Reid, R. H., Rieman, D. J., Page Kline, T., Sathe, G., Greig, R. G., and Anzano, M. A. (1992) *Biochem. J.*, **283**, 91-98
23. Matsunami, R. K., Campion, S. R., Niyogi, S. K., and Stevens, A. (1990) *FEBS Lett.*, **264**, 105-108

Chapter IV

THE L15A HUMAN EPIDERMAL GROWTH FACTOR MUTANT PARTIALLY IMPAIRS ALLOSTERIC ACTIVATION OF THE RECEPTOR : ATTAINMENT OF THRESHOLDS IN THE PRESENCE OF A RECEPTOR RESERVE

Introduction

The γ -branched aliphatic side-chain of L15 of hEGF is important in the formation of a catalytically active ligand-receptor complex (1), as revealed by the following observations:

- (i) The L15A mutant displays a binding affinity of 2.4% relative to wild-type hEGF.
- (ii) A partial uncoupling between receptor recognition and activation is suggested by the partial agonist activity of the L15A mutant in an *in vitro* kinase assay.
- (iii) The L15A mutant exhibits only minor structural alterations, as discerned from its ^1H -NMR spectrum relative to wild-type hEGF (1).

The underlying basis for the observed partial agonist activity has been examined both in 'cell-free' systems and in cell culture. Results presented here lead to the conclusion that L15 of hEGF engages the receptor *via* cooperative interactions which are critical for attaining thresholds during mitogenic signal transduction.

Materials and Methods

Cell culture

Balb/MK, an EGF-dependent murine epidermal keratinocyte cell line (2) was obtained from Dr. Stuart Aaronson (NIH). Cell stocks were maintained at 37 °C in a humidified atmosphere of 5% CO₂, as proliferating cultures in low Ca²⁺ EMEM (Biofluids Inc., Rockville, MD) containing 0.05 mM CaCl₂, 0.02 mM calcium pantothenate, 2 mM L-glutamine and 100 units/ml Penicillin-G. The medium was supplemented with 10% dialyzed FBS (GIBCO, NY) and 5 ng/ml recombinant hEGF (complete medium). A431, a human epidermoid carcinoma cell line, was obtained from the American Type Culture Collection. Cells stocks were maintained at 37 °C in a humidified atmosphere of 5% CO₂, as proliferating cultures in DMEM supplemented with 10% FBS (GIBCO, NY).

Recombinant hEGF purification

Wild-type and L15A mutant hEGF were purified as described in Nandagopal *et al.* (1) and

in the Materials and Methods section of Chapter II. Lyophilized protein samples were reconstituted in phosphate-buffered saline (PBS, pH 7.4) and stored at - 80 °C.

Receptor tyrosine kinase activation and ligand-induced receptor dimerization assays

Ligand-dependent activation of EGFR was measured after solubilization of A431 cell membranes and partial purification of the kinase by wheat-germ agglutinin affinity chromatography. The conditions, described in the Materials and Methods section of Chapter II, were similar to those of Akiyama *et al.* (3), with modifications by Koland and Cerione (4). Aliquots of EGFR preparations (1 µg total protein) were preincubated with varying concentration of wild-type or mutant hEGF for 15 min at room temperature in buffer containing 20 mM HEPES, pH 7.2, 250 mM NaCl, 2 mM MnCl₂, 10 mM MgCl₂, 1 mM DTT, 250 mM (NH₄)₂SO₄, 100 µM Na₃VO₄, 5% glycerol and 0.05% Triton X-100. The kinase reaction was initiated by the addition of [γ -³²P]ATP (1.35 Ci/mmol) and (Glu₄,Tyr₁)_n [Sigma Chemical Co.] substrates to final concentrations of 75 µM and 0.5 mg/ml, respectively, in a total volume of 100 µl. After incubation for 10 min at room temperature, the reaction was terminated by the addition of 5% TCA containing 10 mM Na-pyrophosphate. The acid-insoluble material was collected on 25 mm Millipore HAWP filters, washed extensively with 5% TCA containing 10 mM Na-pyrophosphate, dried and processed for liquid scintillation spectrometry. The radioactivity incorporated in the absence of hEGF was subtracted from hEGF-stimulated values. The kinase activities reported here include the incorporation of ³²P into both the polypeptide substrate and the receptor. The contribution of the latter, as determined by assaying the receptor in the absence of the polypeptide substrate, was < 2% of the total activity. Curve fitting was performed with the Deltagraph 4.01 software (Deltapoint Inc., Monterey, CA).

Chemical cross-linking experiments were designed to examine the ability of wild-type and mutant hEGF proteins to induce receptor dimerization. The protocol for cross-linking has been previously described by Canals (5). Briefly, solubilized and partially purified EGFR (2 µg total protein) from A431 cell membranes was preincubated with varying ligand concentration in the kinase assay buffer (described above) for 15 min at room temperature. The cross-linking reaction was initiated by addition of 80 mM glutaraldehyde (Polysciences

Inc., Warrington, PA) and after 1 min at room temperature, quenched by addition of 2 M glycine, pH 9, the final reaction volume being 50 μ l. An equal volume of 2X Laemmli buffer containing 0.7 M β -mercaptoethanol was added to the samples which were then denatured at 95 °C for 3 min. The samples were resolved on 5% gels by SDS-PAGE, transferred to Immobilon-P membranes (Millipore, MA) and processed for immunoblotting with anti-EGFR monoclonal antibody (mAb) [Transduction Labs.], as per manufacturer's protocol. Membranes were blocked with 3% BSA (Sigma Chemical Co.). Secondary goat-anti-mouse polyclonal antibody conjugated to HRP was obtained from Transduction Labs., KY. The ECL detection reagent was purchased from Amersham, IL.

Dimerization of the EGFR in intact A431 cells was examined by the protocol described in Cochet *et al.* (6) with modifications. Cells were grown in 35 mm dishes to 80% confluence in DMEM supplemented with 10% FBS. They were subsequently incubated in DMEM containing 0.5% FBS for 48 h. Wild-type or mutant hEGF proteins were diluted in DMEM to the desired concentrations and then added to the cells for 5 min at 37 °C. Control incubations were carried out in the absence of inducing ligand. The cells were rapidly rinsed with PBS and cross-linking was initiated with 15 mM EDC (Pierce Chemical Co.), buffered with 100 mM MES, pH 6. After incubation for 7 min at 37 °C, the EDC solution was aspirated and the reaction neutralized by addition of 1 M Tris-Cl, pH 7.6, for 5 min at 37 °C. The monolayers were rinsed with PBS and dissolved in 100 mM Tris-Cl, pH 7.6, containing 1% SDS. The extracts were vortexed and denatured at 95 °C for 3 min to reduce sample viscosity. After centrifugation at 12000 x g for 5 min at 4 °C, protein content of the supernatants was determined by the BCA assay (Pierce Chemical Co.). Fifteen μ g aliquots were treated with Laemmli buffer containing 0.35 M β -mercaptoethanol and denatured at 95 °C for 3 min. The samples were resolved on 5% gels by SDS-PAGE and processed for immunoblotting with anti-EGFR mAb as described above.

Receptor autophosphorylation assays

Balb/MK cells were plated at a density of 10^4 cells/cm² and grown to ~70% confluence in 60 mm dishes. The monolayers were then rinsed with PBS and incubated for 48 h in complete medium lacking hEGF. Wild-type or mutant hEGF proteins were serially diluted

in the same medium to the desired concentrations and added to the cells for 30 sec at 37 °C. The experiments were terminated by aspirating the medium, rapidly rinsing the monolayer with PBS and addition of lysis buffer (100 mM Tris-Cl, pH 7.6 and 1% SDS). The extracts were vortexed and denatured (95 °C for 3 min) to reduce sample viscosity. After centrifugation at 12,000 x g for 5 min at 4 °C, protein content of the supernatants was determined by the BCA assay. The samples (750 µg) were incubated with either 4 µg anti-EGFR mAb [IgG1] (Transduction Labs) or 4 µg normal rabbit serum protein (Sigma Chemical Co.) for 2 h at 4 °C as per manufacturer's protocol. Two µg rabbit-anti-mouse IgG (Sigma Chemical Co.) was added to each sample and incubated for 1 h at 4 °C with end-on shaking. Immune complexes were collected on protein G-Sepharose beads (Sigma Chemical Co.) overnight and centrifuged at 12,000 x g for 15 min at 4 °C. The beads were washed with PBS and solubilized in reducing Laemmli buffer at 95 °C for 3 min. Samples were resolved on 7.5% gels by SDS-PAGE and processed for immunoblotting using an anti-phosphotyrosine mAb (PY20; IgG2b)[Transduction Labs., KY] as per manufacturer's protocol. Receptor autophosphorylation was independently verified by immunoprecipitating tyrosyl-phosphorylated proteins in the soluble supernatant (750 µg) of denatured cell lysates with the PY20 mAb. Samples were resolved on 7.5 % gels by SDS-PAGE and blots were probed with the anti-EGFR mAb. Secondary antibody incubation and ECL detection procedures were carried out as described previously.

MAP kinase assays

Balb/MK cells were plated at a density of 10^4 cells / cm² and grown to ~ 70% confluence in 60 mm dishes. The monolayers were rinsed with PBS and incubated for 48 h in complete medium lacking hEGF. Wild-type or mutant hEGF proteins were diluted to the desired concentrations in the same medium and added to the cells for defined intervals at 37 °C. The experiments were terminated by aspirating the medium, rapidly rinsing the monolayer with PBS and adding lysis buffer (100 mM Tris-Cl, pH 7.6, containing 1 % SDS). The extracts were vortexed and denatured at 95 °C for 3 min to reduce sample viscosity. After centrifugation at 12, 000 x g at 4 °C for 5 min, protein content of the supernatants was determined by the BCA assay. Samples (750 µg) were incubated with 4 µg anti-MAP-2

kinase mAb [IgG2b] (Transduction Labs., KY) for 2 h at 4 °C as per manufacturer's protocol. Two µg rabbit-anti-mouse IgG was added to each sample and incubated for 1 h at 4 °C with end-on shaking. Immune complexes were collected on Protein G-Sepharose beads overnight, centrifuged and washed thoroughly with PBS. The beads were solubilized in reducing Laemmli buffer at 95 °C for 3 min. Resultant supernatants were resolved on 10% gels by SDS-PAGE and processed for immunoblotting using the PY20 mAb. Secondary antibody incubation and ECL detection procedures were carried out as described previously.

Flow cytometry - sample preparation, measurements and data analysis

Balb/MK cells were seeded at a density of 10^4 cells per 60 mm dish in complete medium and grown to 70% confluence. Cells were then deprived of hEGF for a period of 36 h to suspend DNA synthesis. The resultant cultures were not, however, synchronous with respect to point of arrest in the cell cycle. Subsequently, quiescent cultures were stimulated with wild-type or mutant hEGF diluted in the conditioned medium and harvested at various time intervals as indicated in Figures 4-6. Cells from triplicate dishes were trypsinized, pooled, centrifuged (1000 rpm, 5 min, 4 °C) and fixed in ice-cold 70% ethanol. Samples were stored at 4 °C until further use. For staining of DNA, samples were first resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and incubated with 50 µg/ml RNaseA for 30 min at 37 °C with rotary shaking. Propidium iodide was then added to a final concentration of 50 µg/ml per sample. Prior to analysis, the samples were filtered through gauze (mesh 56 µm).

DNA content was determined using a FACStar^{PLUS} flow cytometer (Becton Dickinson Systems, CA) equipped with a 488 nm argon laser. Propidium iodide fluorescence was detected using a 585 / 42 nm dichroic filter. Chicken erythrocytes and calf thymus nuclei (Becton Dickinson, CA) were used as standards to ensure the quality of DNA distribution patterns. 10^4 events / sample were acquired at a flow rate of ~100 events / sec. All parameters including pulse width and pulse height were recorded in list mode. Doublet discrimination was used to identify artefacts due to doublets of diploid cells and single tetraploid cells by plotting the pulse area versus pulse width on dot plots. The fractions in G0/G1, S and G2/M phases of the cell cycle at each time point were calculated using the Multicycle software program (Phoenix Flow Systems, San Diego, CA) which is based on the

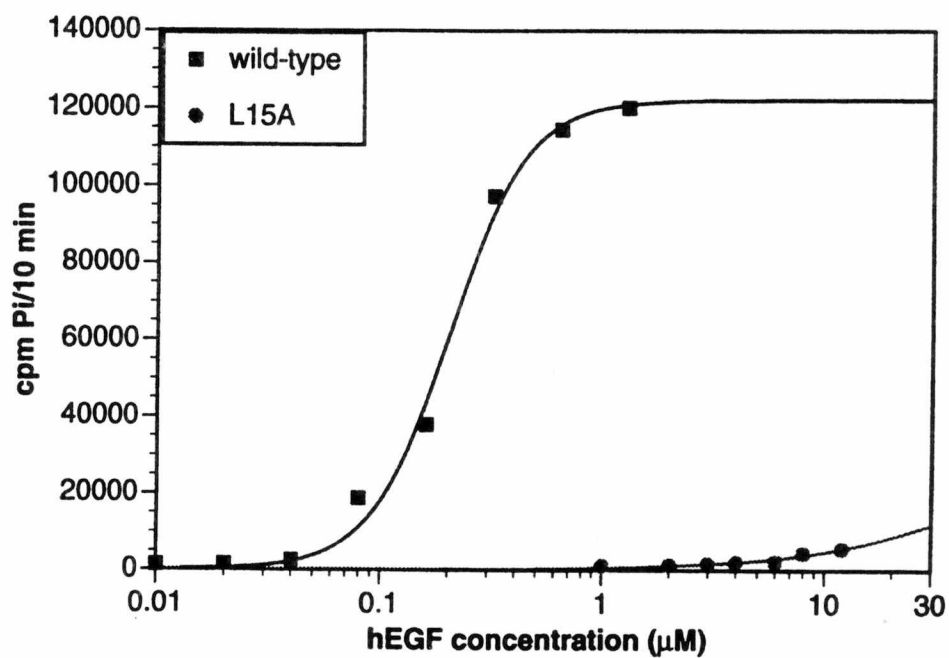
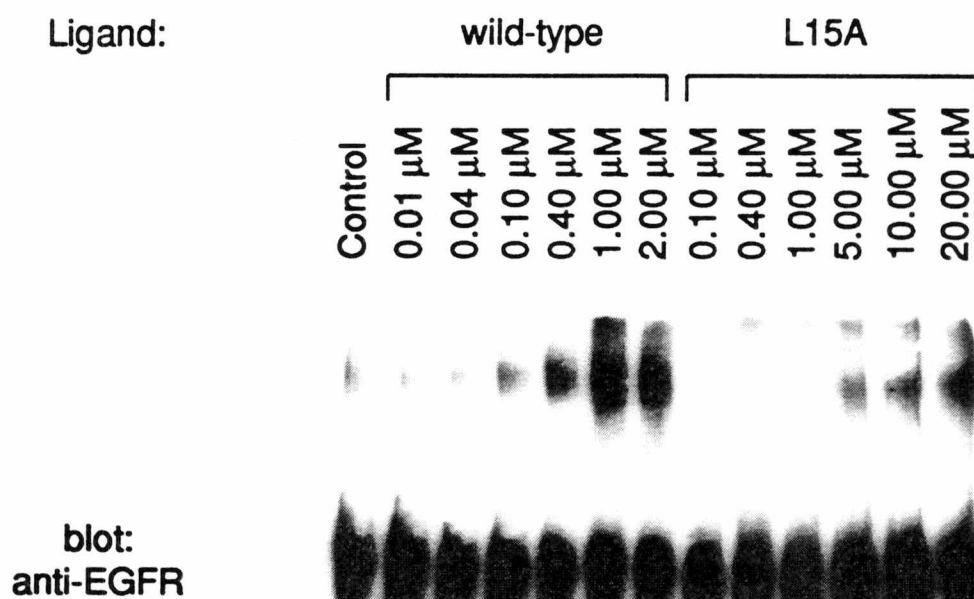
analysis of DNA distributions as described in Dean and Jett (7). DNA content histograms were generated by fixing the ratio of G2 : G1 peak channels at 1.97, constraining the coefficient of variation (CV) in G1 and G2 to be equal, using a first order S and incorporating a background correction for the exclusion of debris, clumping and sliced nuclei. A χ^2 value of ≤ 10 was deemed acceptable, while a value ≤ 5 was considered an excellent fit of the assumed model to the experimental data when tested for statistical significance.

Results

Apparent positive co-operativity of receptor activation and status of ligand-induced receptor dimerization

Tyrosine kinase activity of the solubilized EGFR was measured as a function of ligand concentration using the exogenous substrate $(\text{Glu}_4, \text{Tyr}_1)_n$. Kinase data pertaining to the L15A mutant and wild-type hEGF (1) were re-evaluated in terms of the Hill equation. Analysis of the dose-response curve for wild-type hEGF, shown in Figure IV-1(A), reveals positive cooperativity with $n_{\text{app}} = 2.5$ and $\text{EC}_{50} = 0.2 \mu\text{M}$. This suggests that the increase in tyrosine kinase activity is due to ligand-induced receptor dimerization. Non-integral values of n_{app} may arise from contributions by receptor molecules that have been only partially activated i.e., the allosteric transition is incomplete in such cases. The slope of the curve indicates that a 4-fold variation in concentration of activating ligand ($0.08 - 0.32 \mu\text{M}$) shifts the velocity from 16.7% to 83.3% V_{max} , thus providing exquisite control over the reaction velocity at fixed concentration of available substrate. The activity profile of the L15A mutant indicates lack of rate saturation with respect to $(\text{Glu}_4, \text{Tyr}_1)_n$ phosphorylation. Therefore it could not be analyzed by the Hill equation. To compensate for its reduced receptor binding affinity, L15A was previously tested at concentrations which were 50-fold greater than the EC_{50} value for wild-type hEGF. However, an EC_{50} value cannot be estimated for L15A since it functions as a partial agonist; even when it binds all the receptor molecules available ($\sim 1 \text{ nM}$), it does not induce the same maximal response as wild-type hEGF (1). This analysis indicates that induction of the effective response is dependent on allosteric activation of the receptor, a process impaired by the binding of L15A to the EGFR.

Figure IV-1. Apparent positive cooperativity of receptor activation and the status of ligand-induced receptor dimerization. Panel (A) depicts the relative agonist activities in a (Glu₄,Tyr₁)_n substrate phosphorylation assay. (B) Glutaraldehyde cross-linking of solubilized EGFR dimers. (C) Status of EGFR dimerization in intact cells. Data are representative of at least two independent experiments.

A.**B.**

C.

Ligand

	WT			L15A		
Control	nM	nM	nM	nM	nM	nM
	5	50	500	5	50	500

Blot: anti-
EGFR

Ligand-induced dimerization of solubilized EGFR was examined by glutaraldehyde cross-linking. The anti-EGFR immunoblot in Figure IV-1(B) shows that wild-type hEGF exhibits dose-dependent stabilization of a dimeric receptor conformation that can be trapped covalently by the cross-linker and present the receptor epitope necessary for antibody recognition. The concentration required for half-maximal receptor dimerization is in the 0.1 - 0.4 μ M range, which is similar to the EC_{50} value obtained for wild-type hEGF in the (Glu₄,Tyr₁)_n phosphorylation assay. The band intensities of receptor dimers trapped at 0.1 and 0.4 μ M wild-type are similar to those obtained at 5 and 10 μ M L15A, respectively. The L15A mutant can thus induce soluble EGFR dimerization at concentrations that are relatively ineffective in stimulating (Glu₄,Tyr₁)_n phosphorylation [Figure IV-1(A)]. The ability of ligated receptor dimers to attain the kinase-active conformation may additionally involve an allosteric transition due to ligand binding.

Figure IV-1(C) reveals the status of EGFR dimerization in intact A431 cells when the receptor is stimulated by either wild-type hEGF or the L15A mutant. The results show that in contrast to wild-type hEGF, L15A is severely impaired in mediating receptor dimerization. Whether no dimers were formed or if they were unstable during the course of the assay cannot be distinguished unequivocally. Qualitatively, the finding is consistent with data presented in Figure IV-1(B). The observed differences in the glutaraldehyde and EDC cross-linking assays may reflect the assay conditions which differ in receptor microenvironment, ionic strength, temperature, incubation time and chemistry of the cross-linking reaction.

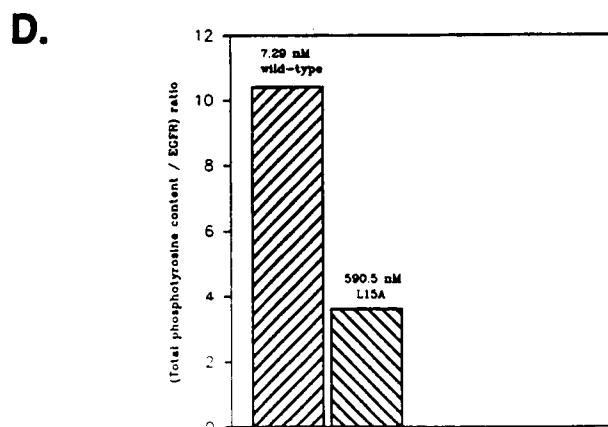
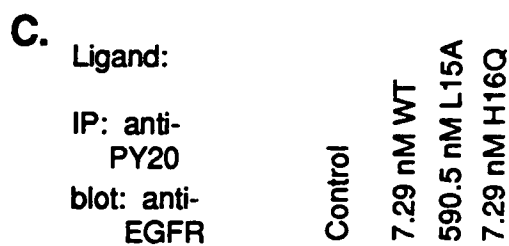
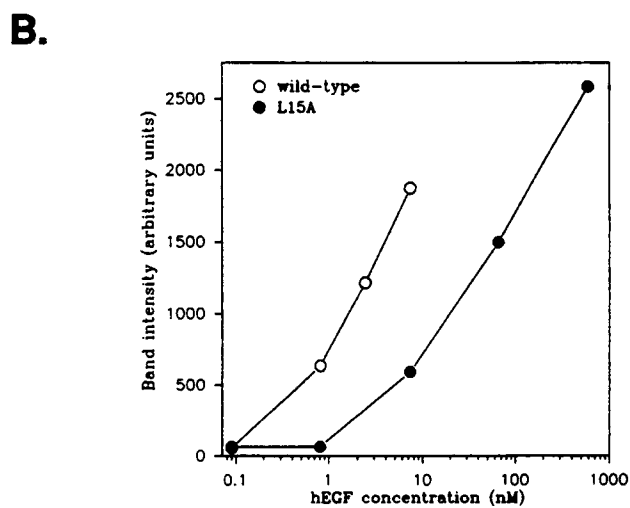
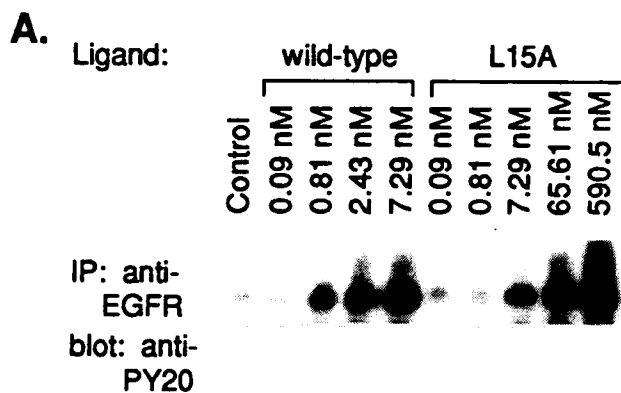
Tyrosine phosphorylation of the EGFR in living cells

The reduced potency of the L15A mutant in the (Glu₄,Tyr₁)_n substrate phosphorylation assay prompted investigation of receptor activation in intact cells. Solubilized EGFRs may adopt 'non-native' conformations in micelles or expose sites that may undergo spurious phosphorylation. Such a situation is less likely in the case of EGFRs which are anchored in the membranes of living cells. Dose-response parameters for ligand-inducible autophosphorylation of the EGFR were therefore established in Balb/MK cells.

The robust response to wild-type hEGF stimulation (30 sec induction at 37 °C) is shown in Figure IV-2(A & B). Published k_D values for receptor subclasses in Balb/MK cells suggest that the exquisite sensitivity of EGFR tyrosine phosphorylation is mediated mainly through the high affinity subclass which has a $k_D = 0.2$ nM and ~ 4500 sites / cell under conditions of receptor upregulation (8). The kinase data presented here indicate that the low affinity receptor subclass ($k_D = 30$ nM; ~ 18000 sites / cell under conditions of receptor upregulation [8]) constitutes a receptor reserve/spare receptor pool of ~ 1 μ M, the presence of which steepens the dose-response curve for wild-type-induced tyrosine phosphorylation of the EGFR. The activity profile of the L15A mutant, shown in Figure IV-2(A & B), reveals several interesting features: (i) The requirement for a higher dose is consistent with its low affinity. (ii) L15A induces significant tyrosine phosphorylation of the EGFR in the 7.29 nM (45.21 ng/ml) to 590.5 nM (3.67 μ g/ml) concentration range. (iii) Partial agonists may elicit maximal effects in the presence of spare receptors. The mutant phenotype observed in the (Glu₄,Tyr₁)_n phosphorylation assay [Figure IV-1(A)] may be due to artificial depletion of the receptor reserve.

Since partial agonists require spare receptors to induce maximal effects, it was of interest to assess differences in the phosphotyrosine content of receptors activated by L15A and wild-type hEGF. The agonist activity of 7.29 nM wild-type hEGF relative to 590.5 nM L15A was therefore verified by immunoprecipitation of activated EGFRs with a mAb (PY20) to phosphotyrosine and immunoblotting with an anti-EGFR mAb. The H16Q mutant which binds the EGFR with ~ 2.7 -fold higher affinity relative to wild-type hEGF (9) was also tested at a dose of 7.29 nM and served as a positive control in these experiments. Figure IV-2(C) shows that L15A induces tyrosine phosphorylation of a larger fraction of receptor molecules compared to wild-type hEGF and the ratio of total phosphotyrosine content / receptor is ~ 2.8 -fold lower (Figure IV-2[D]) under these conditions. By inference then, these ligands differ in the number of receptors that they must activate in order to generate the same effective response. This interpretation is also consistent with the findings presented earlier in Figure IV-1(A).

Figure IV-2. EGFR autophosphorylation in Balb/MK cells. The dose-response curve in panel (B) is derived from densitometric scanning of band intensities in (A). The phosphotyrosine content/receptor ratio in (D) is derived from scanning the corresponding signal intensities in panels (A) and (C). Data are representative of two independent experiments.



MAP kinase activation as a measure of downstream signal transduction

MAP kinases are involved in the mitogenic response to growth factors (10) and are also implicated in the regulation of more proximal aspects of EGFR signalling (11-14). The stimulus-response curve for MAP kinase suggests that it functions like a cooperative enzyme with a Hill coefficient of 4-5 (15). To ascertain whether or not EGFR phosphorylation by L15A translates into biochemical responses in signaling pathways downstream of the EGFR, the activation of p42 MAP kinase was investigated in Balb/MK cells.

The response to equivalent concentrations (7.29 nM) of either L15A or wild-type hEGF was evaluated in immunoprecipitation experiments using an anti-p42 MAP kinase mAb and probing blots with a mAb to phosphotyrosine (PY20). As seen in Figure IV-3, both wild-type and L15A elicit a response of similar magnitude in this assay. Clearly there is a lack of linear correlation to the levels of ligand-inducible autophosphorylation of the EGFR at these concentrations [Figure IV-2(A)].

The kinetics of MAP kinase activation in response to equivalent concentrations (7.29 nM) of H16Q and wild-type hEGF when compared to 590.5 nM of L15A is shown in Figure IV-4(A & B). Under these conditions, L15A and H16Q induce a response that is greater in magnitude compared to wild-type hEGF. The response peaks at 2-5 min, after which it rapidly declines to near basal levels during the 10-30 min interval. These results show that: (i) EGFRs activated by L15A are capable of genuine signal amplification. (ii) Cooperative effects are important for signal transduction in the presence of threshold ligand concentrations. (iii) When the induced response exceeds optimum, cells attenuate the stimulus, probably by dephosphorylation mechanisms.

Cell proliferation analysis by flow cytometry

EGF regulates G0/G1 transition and progression through G1 into S phase of the cell cycle in many cell types (16-18). The mitogenic response of Balb/MK cells to L15A or wild-type hEGF stimulation was monitored by flow cytometric analysis of cellular DNA content. Three parameters were examined:

(1) The lag / kinetic interval between the time of growth factor addition and onset of S phase; this depends on progress made in previous cycles, macromolecular syntheses related to the

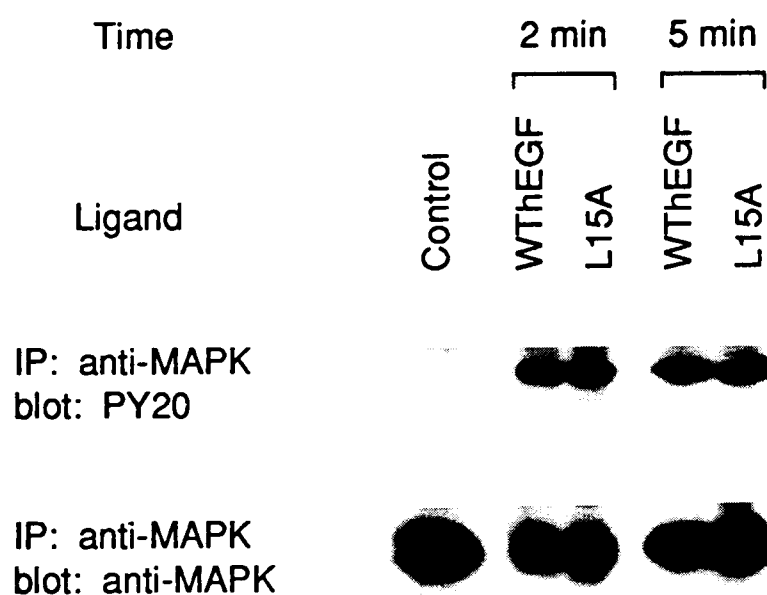


Figure IV-3. EGF-inducible tyrosine phosphorylation of p42 MAP kinase was determined as described in **Materials and Methods**. Data obtained with equivalent concentrations (7.29 nM) of ligand, are representative of at least two independent experiments.

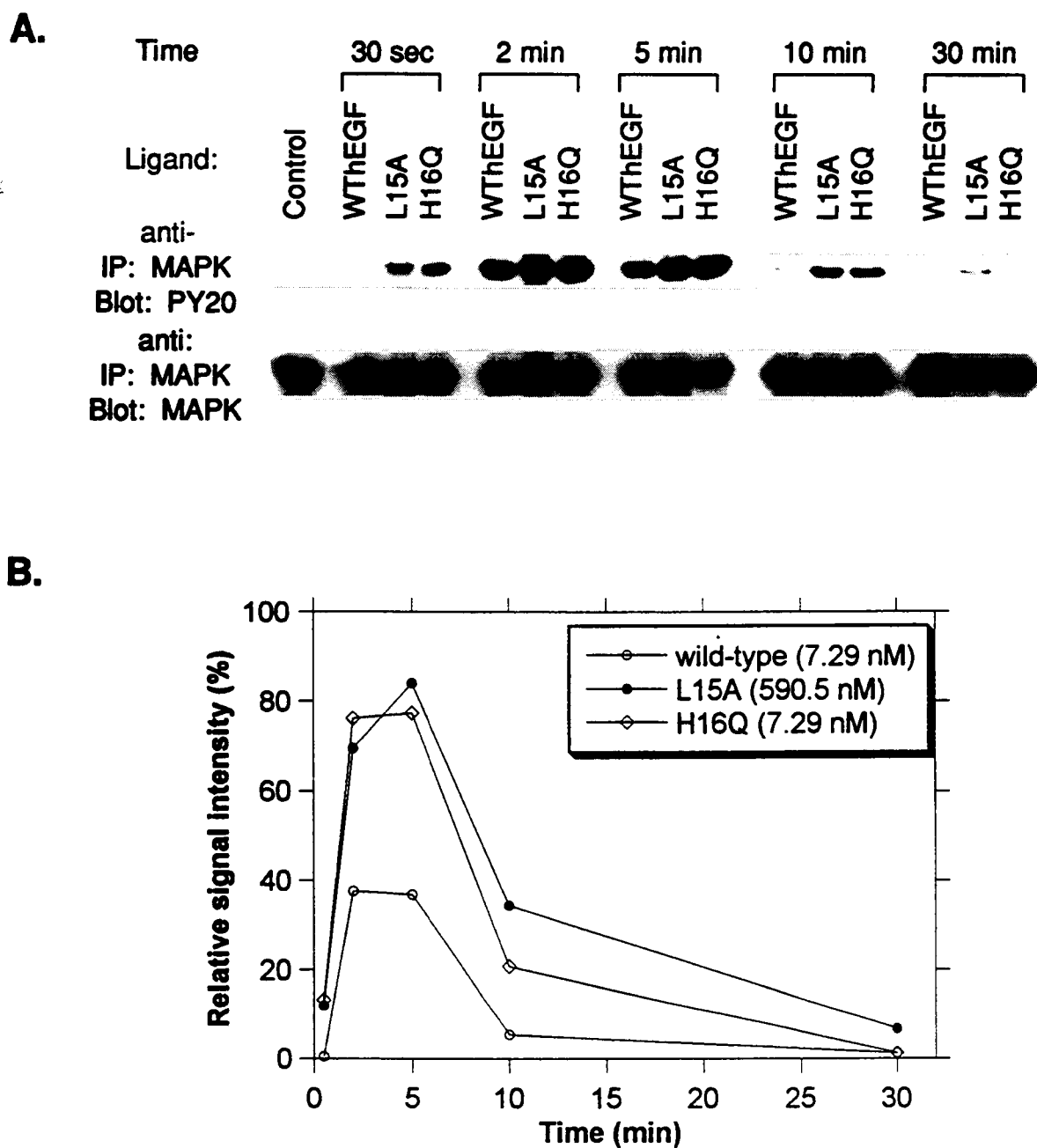


Figure IV-4. Kinetics of activation / inactivation of p42 MAP kinase in Balb/MK cells. Normalized signal intensities in panel (B) are derived from densitometric scanning of bands in panel (A). Data are representative of at least two independent experiments.

pre-replicative phase of the present cycle and whether or not cells were deprived of hEGF /serum.

(2) The rate of entry into S phase as indicated by the slope; this is determined by the fraction of the cell population which attains threshold at any given time.

(3) The overall distribution/shape of the subpopulation in S phase which is governed by the rate of DNA synthesis and any synchrony present.

Figure IV-5(A) shows that ~75% of the cell population accumulates in G0/G1 following hEGF withdrawal from the culture medium for 36 h. Stimulation with either 1 or 33 nM wild-type hEGF results in a lag of 8 h, during which time ~5% of the cells arrested in G2/M or possibly even late S of the previous cycle enter G0/G1. Cell numbers in the S and G2/M phases register a corresponding decrease during this interval [Figure IV-5(B & C)]. Thus, ~ 80% of the population is distributed through various stages of G0/G1 at the end of the 8 h lag. The minimum lag is independent of EGF concentration under these conditions.

Cells which attain threshold at the G1/S boundary proceed into S at ~ 8 h, maximal occupancy of the S phase compartment being observed at ~16-18 h [Figure IV-5(A & B)]. There is no appreciable difference in the rate of exit from G0/G1 when the concentration of inducing ligand is either 1 or 33 nM wild-type hEGF. This indicates that the threshold and response rate per cell are similar in both cases. Furthermore, the concentration mediating the threshold response is lower than 1 nM wild-type hEGF. Assuming that the threshold response is proportional to receptor occupancy, these observations also suggest that spare receptors are available for interaction with 'excess' ligand present in the culture medium.

The transition of cells through S phase is characterized by a progressive increase in cellular DNA content due to replicative DNA synthesis. The fluorescent signal thus increases in proportion to the propidium iodide bound stoichiometrically to DNA. The proliferating fraction, which consists of cells in S and G2/M, is depicted in Figure IV-5(C). The distribution of the subpopulation in various stages of S phase is similar for both 1 and 33 nM wild-type stimulated curves [Figure IV-5(B)], suggesting that the rates of DNA synthesis are also rather similar under these conditions. The average duration of S phase is estimated to be ~7 h, assuming that cells are in exponential growth under these conditions, a finding

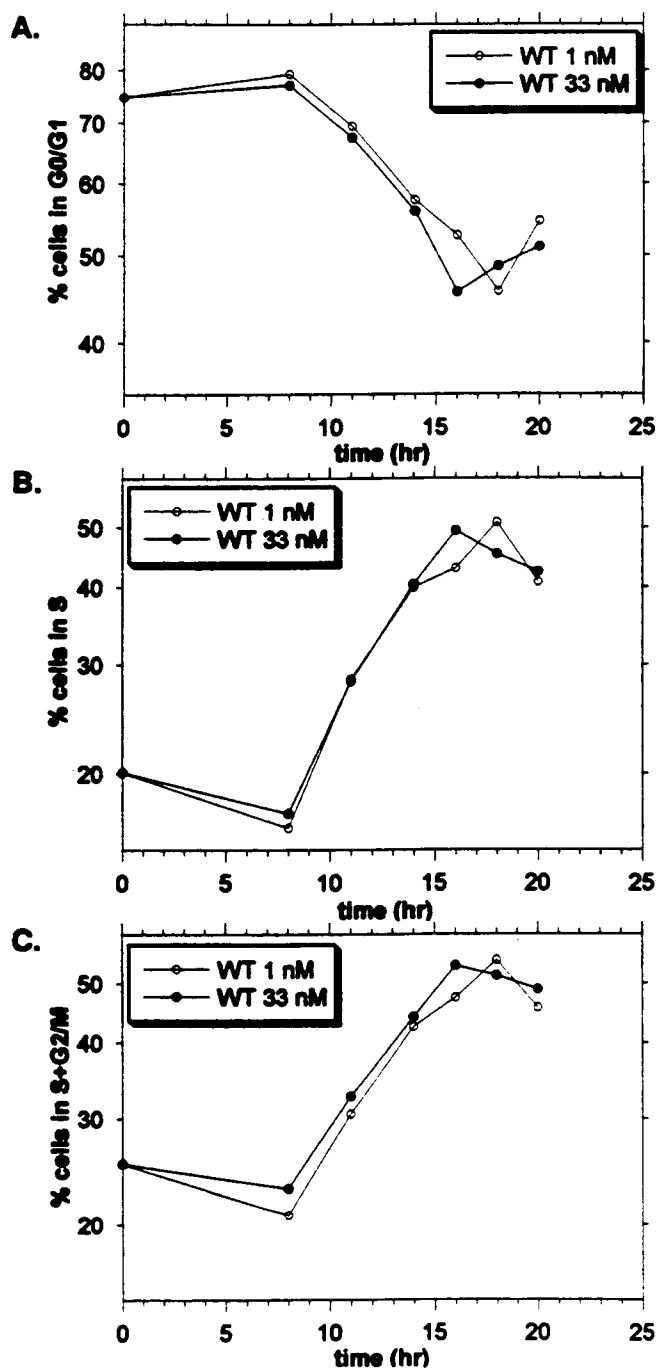


Figure IV-5. Cell proliferation in response to saturating concentrations of wild-type hEGF. DNA content was monitored by flow cytometry and histograms were analyzed as described in **Materials and Methods**. Semi-logarithmic plots are representative of average data points from three independent experiments.

consistent with the observations of van Hooijdonk *et al.* (19).

The graphs in Figure IV-6(A, B & C) depict the differences in cell cycle kinetics when the mitogenic response is elicited by wild-type or L15A hEGF at 1 nM concentration. In both cases, cells go through G0/G1 with the same minimum lag of 8 h, before reaching S phase [Figure IV-6(A)]. However, *a larger number* of wild-type stimulated cells enter S phase *more rapidly* than mutant stimulated cells [Figure IV-6(A & B)]. The difference in threshold is reflected in the slopes of the two curves. Clearly, the threshold response is not achieved readily by cells stimulated with L15A. Thus, the fraction of ligated receptors which transduce the threshold response are non-identical for L15A and wild-type hEGF. The steeper slope for the wild-type stimulated curve is indicative of more efficient signal amplification. That the response rates are different is evident from the distributions of the respective S phase subpopulations [Figure IV-6(B)]. Cells stimulated by subthreshold concentrations of L15A are less efficient than wild-type stimulated cells in the induction of DNA synthesis.

The mitogenic response to 33 nM wild-type or L15A illustrates the dose-dependence of the threshold effect [Figure IV-7(A, B & C)]. The slopes of the curves indicate that cells stimulated by mutant or wild-type hEGF exit G0/G1 at similar rates following the 8 h lag [Figure IV-7(A)]. The increased concentration of the mutant compensates for its reduced potency and thereby permits more cells in the population to attain thresholds. Consequently, the response rates are similar in cells stimulated by the L15A mutant and wild-type hEGF. Inspection of the curves shown in Figure IV-7(B & C) indicates that at the 11 h interval, ~13% of the population stimulated by L15A remains in the G2/M compartment of the previous cycle compared to ~5% of the cells stimulated by wild-type hEGF. Reasons for this observation are not clear and merit further investigation. The distribution of cells in transit through the cell cycle is otherwise similar for both wild-type and L15A stimulated curves.

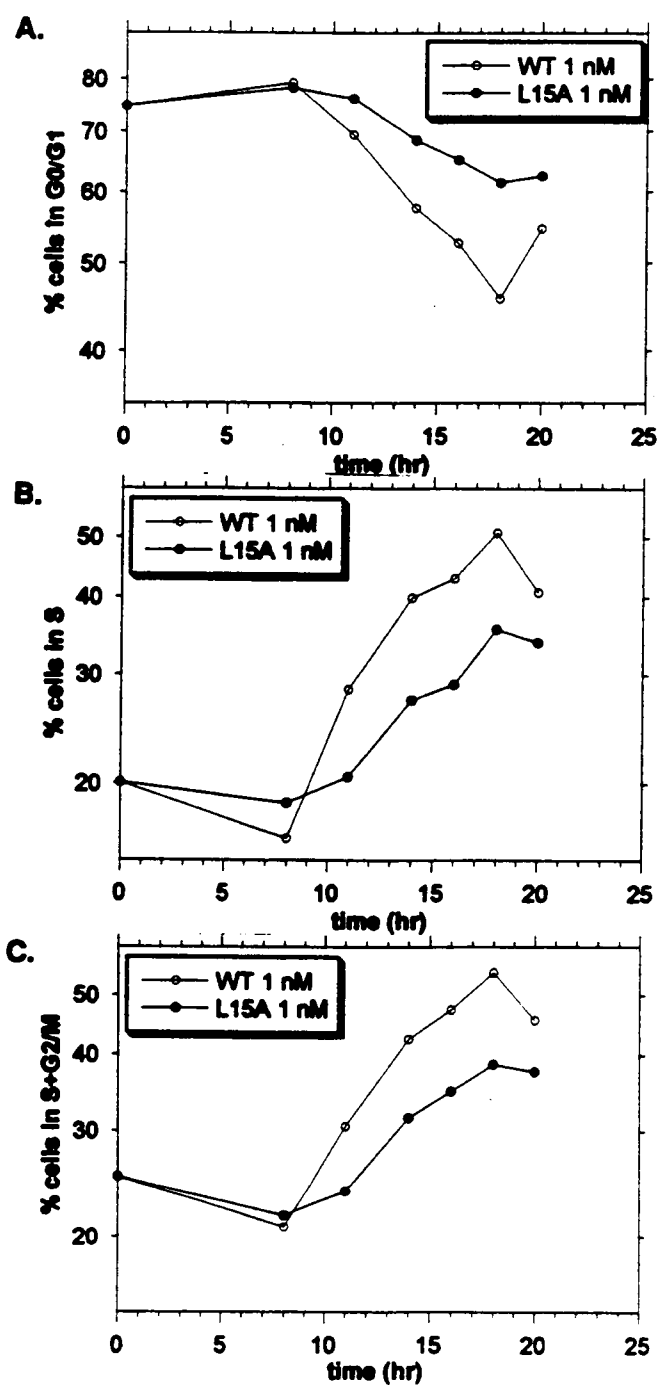


Figure IV-6. Dependence of the rate of entry into S phase on attainment of thresholds. DNA content was monitored by flow cytometry and histograms were analyzed as described in **Materials and Methods**. Semi-logarithmic plots are representative of average data points from three independent experiments.

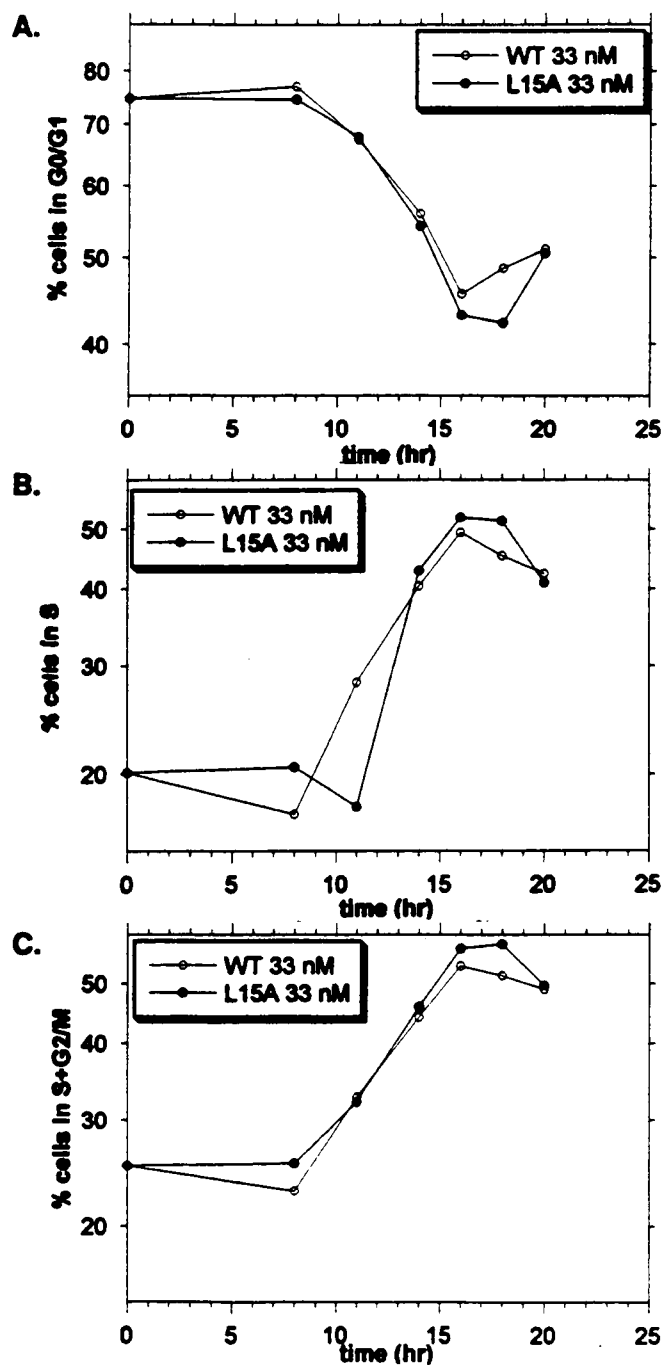


Figure IV-7. Dose-dependence of the threshold effect in EGF-mediated cell cycle progression. DNA content was monitored by flow cytometry and histograms were analyzed as described in **Materials and Methods**. Semi-logarithmic plots are representative of the average data points from three independent experiments.

Discussion

The aggregation state of cell surface receptors can regulate transmission of signals by various growth factors and cytokines (20, 21). For the EGF/EGFR paradigm, however, structural and functional components that mechanistically relate receptor aggregation to tyrosine kinase activation remain unresolved. This study addresses the role of hEGF in mediating this process and shows that L15 of hEGF engages the receptor *via* cooperative interactions that are critical for mitogenic signal transduction. The experimental findings in support of this conclusion are as follows:

- (i) Induction of the effective response is dependent on allosteric activation of the EGFR.
- (ii) The relationship between ligand binding and receptor activation in the $(\text{Glu}_4, \text{Tyr}_1)_n$ phosphorylation assay appears cooperative due to agonist-induced receptor dimerization / oligomerization.
- (iii) The L15A mutation in hEGF partially impairs dimerization and allosteric activation of the EGFR *in vitro*. L15A induces significant autophosphorylation of the EGFR in the presence of a receptor reserve.
- (iv) p42 MAP kinase activation, a measure of downstream signal amplification, does not reveal a linear correlation between receptor autophosphorylation and the effective response.
- (v) Wild-type hEGF and L15A differ in the threshold concentrations required for entry of cells into S phase following the exit from G0/G1 of the cell cycle, indicating that allosteric activation of pre-existing receptor dimers / oligomers may dictate the rate at which thresholds are attained.

The description of cooperative binding of hEGF to the EGFR under the *in vitro* kinase assay conditions is consistent with the sedimentation analyses of Sherrill and Kyte (22) who reported that binding of EGF to solubilized EGFR is sigmoidal ($n_H = 1.7$). The inability of the L15A mutant to maximally activate the EGFR in the substrate phosphorylation assay, provokes the question as to what promotes the allosteric transition. Stable receptor-ligand 'bonds' are formed by van der Waal's weak forces of attraction which are both non-covalent and saturable (23). Steric complementarity permits the γ -branched L15 side-chain of hEGF to form multiple van der Waal's bonds simultaneously and thereby confers specificity of

interaction during an allosteric transition. Such an opportunity would not present itself to the smaller side-chain of the L15A mutant and may account for its reduced potency as an agonist.

There have been numerous reports describing EGF-induced dimerization of EGFRs (5, 6, 22, 24-27). Some studies have implicated the monomeric EGFR as the active kinase (4, 28-30) while others have attributed the activity to the dimer (5, 6, 22, 24, 25). However, these studies have examined the involvement of receptor aggregation without resolving the role(s) of ligand-receptor interactions in tyrosine kinase activation.

Kinase and chemical cross-linking data presented in Figure IV-1(A & B) strongly suggest that the apparent cooperativity between ligand binding and receptor activation derives from ligand-induced receptor dimerization. This interpretation is supported by previous reports that have shown the specific activity of monomeric EGFR as a kinase to be lower than that of the dimeric species (5, 31). The activity profiles of the L15A mutant suggest an obligate requirement for allosteric conformational change(s) in addition to receptor dimerization, which is plausible both energetically and mechanistically (32). The statistical probability that membrane proteins *in vivo* would oligomerize, is a million-fold greater than the probability that these proteins would exist in isolated states (33). Thus, EGF receptors which pre-exist as oligomers (34), covalently-linked homodimers (31) or are co-expressed as heterodimers in intact cells (35), bind ligand and function as kinases only when they also undergo the necessary allosteric transition.

The demonstration of a receptor reserve for EGFR activation and its importance in mediating threshold responses to EGF stimulation, as described in this study, extends the earlier observations of Soler *et al.* (36), who demonstrated potent SHC tyrosine phosphorylation in EGF-treated MK cells at low receptor density. MAP kinase activation in response to threshold ligand concentrations probably proceeds along this pathway. The L15A hEGF mutant is not a partial agonist for mitogenesis, although it is so for receptor autophosphorylation. Interestingly, mutation of all the major autophosphorylation sites in the receptor (Y→F) does not abrogate the mitogenic response to wild-type EGF (37-39). Assuming that receptor autophosphorylation then reflects the extent of conformational

change induced by ligand binding, *the presence of a receptor reserve ensures that threshold concentrations of L15A can engage enough suboptimally activated receptors in order to stimulate the maximal mitogenic response*. The experimental data of Zendegui *et al.* (8), suggest that maximal and sustained cell proliferation of Balb/MK cells by wild-type hEGF is regulated by ~500 high affinity EGFRs per cell! Studies with fibroblasts have also shown that spare receptors exist for the mitogenic response and receptor down regulation (40, 41). Therefore, it is of interest to investigate temporal changes in receptor reserve in future experiments.

Given that high-affinity receptors in intact cells pre-exist as dimers / oligomers (34), allosteric activation of this receptor subpopulation presents several advantages for attaining biological thresholds as a result of cooperative effects. Fewer receptors with enhanced intrinsic kinase activity can engage the agonist for longer duration, especially if the ligand concentration is limiting or when signal intensity dictates choice of cell fate *in vivo* (42, 43). Existence of a receptor reserve combined with the flexibility afforded by internalization / recycling mechanisms may have been essential to the evolution of the EGF/EGFR superfamily of receptor-ligand systems in development. Survival strategies of this nature may be preferred over a mechanism that necessitated a selective growth factor for each type of target cell.

References

1. Nandagopal, K., Tadaki, D. K., Lamerdin, J. A., Serpersu, E. H., and Niyogi, S. K. (1996) *Protein Engng.*, **9**, 781-788
2. Weissman, B. E., and Aaronson, S. A. (1983) *Cell* **32**, 599-606
3. Akiyama, T., Kadooka, T., and Ogawara, H. (1985) *Biochem. Biophys. Res. Commun.*, **131**, 442-448
4. Koland, J. G., and Cerione, R. A. (1988) *J. Biol. Chem.*, **263**, 2230-2237
5. Canals, F. (1992) *Biochemistry* **31**, 4493-4501
6. Cochet, C., Kashles, O., Chambaz, E. M., Borrello, I., King, C. R., and Schlessinger, J. (1988) *J. Biol. Chem.*, **263**, 3290-3295

7. Dean, P. N., and Jett, J. H. (1974) *J. Cell Biol.*, **60**, 523-527
8. Zendegui, J. G., Inman, W. H., and Carpenter, G. (1988) *J. Cell. Physiol.*, **136**, 257-265
9. Nandagopal, K. and Niyogi, S. K. (1995) *FASEB J.*, **9**, A1415
10. Meloche, S. (1995) *J. Cell. Physiol.*, **163**, 577-588
11. Heisermann, G. J., and Gill, G. N. (1988) *J. Biol. Chem.*, **263**, 13152-13158
12. Countaway, J. L., McQuilkin, G. N., and Davis, R. J. (1990) *J. Biol. Chem.*, **265**, 3407-3416
13. Rozakis-Adcock, M., van der Geer, P., Mbamalu, G., and Pawson, T. (1995) *Oncogene* **11**, 1417-1426
14. Buday, L., Warne, P. H., and Downward, J. (1995) *Oncogene* **11**, 1327-1331
15. Huang, C-Y. F., and Ferrell Jr., J. E. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10078-10083
16. Sand, T-E., and Christoffersen, T. (1987) *J. Cell. Physiol.*, **131**, 141-148
17. Pardee, A. B. (1989) *Science* **246**, 603-608
18. Aaronson, S. A. (1993) In *The Harvey Lectures*, Series **87**, 17-34, Wiley-Liss, Inc.,
19. van Hooijdonk, C. A. E. M., van Erp, P. E. J., Freund, R. L., de Jongh, G. J., Schalkwijk, J., van de Kerkhof, P. C. M., and Mier, P. D. (1993) *Cell Prolif.*, **26**, 567-576
20. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61**, 203-212
21. Heldin, C. H. (1995) *Cell* **80**, 213-223
22. Sherrill, J. M., and Kyte, J. (1996) *Biochemistry* **35**, 5705-5718
23. Lauffenburger, D. A., and Linderman, J. J. (1993) In *Receptors*, Oxford University Press, Inc., New York, pp. 269
24. Yarden, Y., and Schlessinger, J. (1987 a) *Biochemistry* **26**, 1434-1442
25. Yarden, Y., and Schlessinger, J. (1987 b) *Biochemistry* **26**, 1443-1451
26. Boni-Schnetzler, M., and Pilch, P. F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7832-7836

27. Fanger, B., Stephens, J. E., and Staros, J. V. (1989) *FASEB J.*, **3**, 71-75
28. Biswas, R., Basu, M., Sen-Majumdar, A., and Das, M. (1985) *Biochemistry* **24**, 3795-3802
29. Northwood, I. C., and Davis, R. J. (1988) *J. Biol. Chem.*, **263**, 7450-7453
30. Carraway III, K. L., and Cerione, R. A. (1993) *J. Biol. Chem.*, **268**, 23860-23867
31. Sorokin, A., Lemmon, M. A., Ullrich, A. and Schlessinger, J. (1994) *J. Biol. Chem.*, **269**, 9752-9759
32. Bormann, B. J., and Engelman, D. M. (1992) *Annu. Rev. Biophys. Biomol. Struct.*, **21**, 223-242
33. Grasberger, B., Minton, A. P., DeLisi, C., and Metzger, H. (1986) *Proc. Natl. Acad. Sci., USA*, **83**, 6258-6262
34. Gadella Jr., T. W. J., and Jovin, T. M. (1995) *J. Cell Biol.*, **129**, 1543-1558
35. Qian, X., LeVea, C. M., Freeman, J. K., Dougall, W. C., and Greene, M. I. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 1500-1504
36. Soler, C., Alvarez, C. V., Beguinot, L., and Carpenter, G. (1994) *Oncogene* **9**, 2207-2215
37. Decker, S. J. (1993) *J. Biol. Chem.*, **268**, 9176-9179
38. Gotoh, N., Tojo, A., Muroya, K., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Yazaki, Y., and Shibuya, M. (1994) *Proc. Natl. Acad. Sci., USA*, **91**, 167-171
39. Li, N., Schlessinger, J., and Margolis, B. (1994) *Oncogene* **9**, 3457-3465
40. Aharanov, A., Pruss, R. M., and Herschman, H. R. (1978) *J. Biol. Chem.*, **253**, 3970-3977
41. Carpenter, G., and Cohen, S. (1976) *J. Cell Biol.*, **71**, 159-171
42. Lillien, L. (1995) *Nature* **377**, 158-162
43. Katz, W. S., Hill, R. J., Clandinin, T. R., and Sternberg, P. W. (1995) *Cell* **82**, 297-307

Chapter V

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The link between reception and relay of mitogenic signals by the EGF / EGFR system has been examined from a reductionist perspective. Nonetheless, tangible clues have emerged from the experimental results presented in this dissertation. While they offer new insights, they also pose new questions!

What controls the rate and extent of EGF action? Equilibrium analyses (e.g., Scatchard plots) have identified both high and low affinity receptor binding sites, suggesting that these are discrete entities. Little is known about EGFR densities in the membrane. However, there is evidence that in response to EGF, the high affinity receptor binding sites associate with the cytoskeleton through actin (1-3) and are sufficient for signal transmission (4,5). Since the high affinity sites represent ~2-20% of the total sites, their properties have not been easy to study. No information is available as to whether or not the system exists in two or more kinetically distinguishable states. Since it is not known if ligand binding is rapid relative to conformational change or vice versa, the possibility of binding to a preferred conformation rather than inducing a change in receptor conformation cannot be addressed at present.

Time-resolved fluorescence imaging microscopy has shown that the high affinity EGFRs in intact cells are present in a predimerized / oligomerized state and that stereochemical reorientation may be necessary for receptor activation (6). Studies on the stoichiometry of EGF-EGFR binding have shown that 1:1 (7) or 2:2 complexes (8) can be formed. The present study extends these earlier observations by suggesting that van der Waal's forces induce cooperative reorientation and activation of the EGFR in the context of pre-existing dimers / oligomers. In terms of membrane deformation, such a mechanism would involve far less expenditure of energy as compared to the disruption of alpha-helical structures or covalent bond scission / formation. It also provides a rationale as to why sensitivity, which is a local property, is maximal at threshold and decreases with an increase in the stimulus. This is consistent with findings which indicate that a receptor occupancy of < 1% triggers a reversible reduction in the affinity of cell surface EGFRs by 10 min ($t_{1/2} = 2-2.5$ min) and a maximal response requires only 5% occupancy (9). The surface EGFRs remain in the low

affinity state as long as EGF is present (9). It is not clear, however, whether the signals which mediate these transitions are independent of changes in receptor phosphorylation status, the increase in intracellular pH and/or Ca^{2+} levels.

Is the proposed model of EGFR activation applicable to receptor heterodimerization? Although the stoichiometry of EGF binding to other members of the erbB family is unresolved, betacellulin - an EGF homologue, is capable of binding both the erbB1 and erbB4 receptors (10). Cooperative interactions in the context of pre-existing heterodimers are yet to be demonstrated. The L15A hEGF mutant characterized in this study is being further investigated with regard to its role in receptor heterodimerization and signalling specificity by Dr. Mary B. Murray, a post-doctoral fellow in Dr. S. K. Niyogi's laboratory, in collaboration with Dr. Donna E. Davies at the CRC Cancer Research Institute, Southampton, UK.

The idea of a receptor reserve, as introduced in this thesis, helps explain the basis for the partial agonist activity of L15A and also the response efficacy of activated EGFRs in biochemical and biological contexts. It is of interest to know if the processes of EGFR overexpression and/or heterodimerization simply increase the receptor reserve for 'EGF-like' agonists. Spare receptors are known to influence response efficacy in diverse systems and may be a general feature of receptor biology. Some examples include the response to choline derivatives at the motor end-plate (11), production of cAMP and corticosterone by variants of adrenocorticotrophic hormone (12), α_2 / β -adrenergic receptor function in fat cells (13), prediction of the clinical utility of D1 dopamine receptor agonists (14) and the partial agonist activity of oxytocin at vasoconstrictor vasopressin receptors on human uterine arteries (15).

Based on computer simulations, it has also been suggested that ligand binding is augmented by 'a diffusive entrapment effect' (16). In this scenario, low affinity receptors or proteoglycan coreceptors are predicted to trap ligand molecules and 'funnel' them to the less abundant, spatially restricted high affinity receptors (17, 18). Though no direct evidence is as yet available to support this hypothesis, it presents an alternate view of the dynamics of receptor-ligand interactions.

The emergent theme in this dissertation is that of cooperative effects mediating the threshold response. In this regard, the Balb/MK model can be further utilized to investigate the status of tyrosyl phosphatases, e.g., SHPTP2 (19), tumor suppressors, e.g., p27KIP1 (20), cyclins, cyclin-dependent kinases and replication licensing proteins (21) in promoting EGF-dependent cell cycle progression. Furthermore, the effects of other signal transducers e.g., PI-3 kinase (22) can be examined and/or novel substrates of mitogenic signals may be identifiable. Also of interest is whether signalling components are compartmentalized in structures such as caveolae (23) in epithelial cells.

Structure-function analyses of hEGF suggest that hydrophobic interactions (favoured entropically) are the major driving force in receptor binding. Given the dynamics of receptor-ligand interactions, the importance of electrostatic forces, hydrogen-bonding interactions and van der Waal's forces derives from the formation of complexes of *small* net stability! Molecular determinants of these forces remain unresolved at the level of the receptor. Therefore, rational design of EGFR inhibitors, superagonists and receptor mutants by protein engineering will be feasible when co-crystal structures and algorithms for the computational chemistry of receptor-ligand interactions become available.

References

1. van Bergen en Henegouwen, P. M., Defize, L. H., de Kroon, J., van Damme, H., Verkleij, A. J., and Boonstra, J. (1989) *J. Cell. Biochem.*, **39**, 455-465
2. den Hartigh, J. C., van Bergen en Henegouwen, P. M., Verkleij, A. J., and Boonstra, J. (1992) *J. Cell Biol.*, **119**, 349-355
3. Rijken, P. J., Post, S. M., Hage, W. J., van Bergen en Henegouwen, P. M., Verkleij, A. J., and Boonstra, J. (1995) *Exp. Cell. Res.*, **218**, 223-232
4. Defize, L. H. K., Boonstra, J., Meisenhelder, J., Kruijer, W., Tertoolen, L. G. J., Tilly, B. C., Hunter, T., van Bergen en Hengouwen, P. M., Moolenaar, W. H., and de Laat, S. W. (1989) *J. Cell Biol.*, **109**, 2495-2507
5. Bellot, F., Moolenaar, W. H., Kris, R., Mirakhur, B., Verlaan, I., Ullrich, A., Schlessinger, J., and Felder, S. (1990) *J. Cell Biol.*, **110**, 491-502
6. Gadella Jr., T. W. J., and Jovin, T. M. (1995) *J. Cell Biol.*, **129**, 1543-1558

7. Weber, W., Bertics, P. J., and Gill, G. N. (1984) *J. Biol. Chem.*, **259**, 14631-14636
8. Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D. M., and Schlessinger, J. (1997) *EMBO J.*, **16**, 281-294
9. Wiley, H. S., Walsh, B. J., and Lund, K. A. (1989) *J. Biol. Chem.*, **264**, 18912-18920
10. Riese, D. J., Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996) *Oncogene* **12**, 345-353
11. del Castillo, J., and Katz, B. (1957) *Proc. Roy. Soc. London Ser. B.*, **146**, 369-381
12. Seelig, S., and Sayers, G. (1973) *Arch. Biochem. and Biophys.*, **154**, 230-239
13. Arner, P. (1992) *Am. J. Clin. Nutr.*, **55**, 228S-236S
14. Watts, V. J., Lawler, C. P., Gonzales, A. J., Zhou, Q. Y., Civelli, O., Nichols, D. E., and Mailman, R. B. (1995) *Synapse* **21**, 177-187
15. Jovanovic, A., Jovanovic, S., Tulic, I., and Grbovic, L. (1997) *Br. J. Pharmacol.*, **121**, 1468-1474
16. Northrup, S. H., and Erickson, H. P. (1992) *Proc. Natl. Acad. Sci., USA*, **89**, 3338-3342
17. Schlessinger, J., Lax, I., and Lemmon, M. A. (1995) *Cell* **83**, 357-360
18. Tzahar, E., Pinkas-Kramarski, R., Moyer, J. D., Klapper, L. N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B. J., Sela, M., Andrews, G. C., and Yarden, Y. (1997) *EMBO J.*, **16**, 4938-4950
19. Bennett, A. M., Hausdorff, S. F., O'Reilly, A. M., Freeman, R. M., and Neel, B. G. (1996) *Mol. Cell. Biol.*, **16**, 1189-1202
20. Wu, X., Rubin, M., Fan, Z., DeBlasio, T., Soos, T., Koff, A., and Mendelsohn, J. (1996) *Oncogene* **12**, 1397-1403
21. Assoian, R. K. (1997) *J. Cell Biol.*, **136**, 1-4
22. Marte, B. M., and Downward, J. (1997) *Trends Biochem. Sci.*, **22**, 355-358
23. Mineo, C., James, G. L., Smart, E. J., and Anderson, R. G. W. (1996) *J. Biol. Chem.*, **271**, 11930-11935

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