# Resistance to Receptor-Mediated Degradation of a Murine Epidermal Growth Factor Analogue (EGF-Val-47) Potentiates Its Mitogenic Activity†

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ABSTRACT: In most cell types two classes of epidermal growth factor (EGF) receptors can be found: a major class that binds EGF with relatively low affinity and a minor class that binds with very high affinity. Structure–function studies have shown that mutations at amino acid 47 in the EGF molecule severely reduce its affinity for the EGF receptor but do not cause preferential binding to one or the other subclass of receptors. Using three EGF derivatives with a mutation at amino acid 47 (Ser-47, Leu-37-Tyr-47, and Val-47), we have investigated the relative contribution of the two receptor subclasses to the EGF-dependent mitogenic response. We show that mitogenicity correlates exclusively with occupancy of the high-affinity receptor and that full occupancy of this subclass is required for maximal stimulation. In addition we demonstrate that for the EGF-Val-47 analogue this requirement can be abrogated and half-maximal biological activity reached with a high-affinity receptor occupancy of only 8%. While the rate of internalization did not significantly differ between EGF-Val-47 and native mEGF, the analogue was much more resistant to degradation by cellular proteases and, after binding and receptor-mediated internalization, was released into the medium predominantly in an intact form. We propose that the increased mitogenicity of EGF-Val-47 is due to its prolonged half-life, resulting in continued occupancy of the high-affinity EGF receptor.

Structure–function studies on proteins often direct their attention to residues which are required for enzymic activity, for correct folding, or for interactions with other proteins. There is a tendency to overlook the structural requirements for precursor processing (Steiner & Oyer, 1967) or the structures required to initiate inactivation of the hormone or growth factor. In some hormone systems, processing signals such as Lys-Arg or Arg-Arg have been recognized (Bell et al., 1983), although the discriminatory specificity of the processing enzymes is still somewhat of a mystery. For mitogens such as epidermal growth factor (EGF) and transforming growth factor $\alpha$ (TGF$\alpha$) (Burgess, 1989) we have hardly even started to understand the significance, the enzymic basis, or the sequence motifs in the precursor (Gray et al., 1983; Scott et al., 1983) which determine the initial cleavage patterns. In many biological signaling processes it is important to inactivate the potent signals, and this can occur by at least two mechanisms: pharmacological clearance (via the liver and/or kidney) and/or receptor-mediated degradation (Pastan & Willingham, 1981).

Both conservation across species and conservation between EGF and TGF$\alpha$, as well as site-directed modification of EGF, have indicated that the leucine at position 47 of murine mEGF and the equivalent leucine in TGF$\alpha$ are important for receptor binding and mitogenic activity (Defeo-Jones et al., 1988; Burgess et al., 1988; Simpson et al., 1985; Ray et al., 1988; Moy et al., 1989).

Although substitutions of Leu-47 decrease the activity of most analogues (Engler et al., 1988; Moy et al., 1989), there is often residual receptor binding, tyrosine kinase activation, and mitogenic activity. Removal of Leu-47 reduces both the binding affinity and mitogenic potency by more than 100-fold; however, while substitution by valine reduces the binding by 100-fold, its mitogenic potency is almost equivalent to that of wild-type mEGF.

We have determined that the unexpectedly high potency of EGF-Val-47 is associated with a lower rate of receptor-mediated degradation. These results highlight an aspect of growth factor biochemistry which is often neglected. Growth factors are extremely potent, and it appears that the rate of clearance and/or receptor-mediated degradation is a critical determinant in controlling their biological function.

If site-directed mutagenesis could be employed to modulate the rate of destruction, it might be easier to design more effective EGF or TGF$\alpha$ agonists and/or antagonists.

## Experimental Procedures

*Reagents.* Murine epidermal growth factor (mEGF) was purified to homogeneity from mouse submaxillary glands as previously described (Burgess et al., 1983). The production of recombinant EGF analogues with amino acid substitution at position 47 (Ser-47, Leu-37-Tyr-47, and Val-47) has been described in full elsewhere (Ray et al., 1988). The concentration of the mEGF and the analogues in our stock solutions was determined by amino acid analysis.

Iodinated mEGF and EGF-Val-47 were prepared by the Iodogen method (Fraker & Speck, 1978), except that the free iodine was removed with a Sep-Pac $C_{18}$ cartridge (Waters Associates, Milford, MA). The specific radioactivity of the radioiodinated EGFs varied between 5 and 8 $10^5$ dpm/pmol. Phenylarsine oxide was purchased from Sigma (St. Louis, MO).

*Mitogenic Assay.* Balb/c 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium (DME), supplemented

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Table I: Binding Constants for $mEGF_{47}$ Mutants[a]

| ligand | $K_{d1}$ (pM) | $K_{d2}$ (nM) |
|---|---|---|
| mEGF | 7 | 3 |
| Ser-47 | 800 | 140 |
| Leu-37-Tyr-47 | 1000 | 220 |
| Val-47 | 700 | 20 |

[a] Confluent cultures of Balb/c 3T3 were incubated with a constant amount of $^{125}$I-EGF and increasing amounts of unlabeled ligands (20 pM to 16 nM) for 45 min at 37 °C. Binding data were analyzed with the LIGAND program (Munson, 1981).

Table II: Relative Potency of $mEGF_{47}$ Mutants[a]

| ligand | $EC_{50}$ (pM) | receptors occupied at $EC_{50}$ | |
|---|---|---|---|
| | | high affinity | low affinity |
| mEGF | 20 | 2000 | 100 |
| Ser-47 | 1500 | 2500 | 100 |
| Leu-37-Tyr-47 | 600 | 2500 | 100 |
| Val-47 | 60 | 300 | 50 |

[a] Half-maximal effective concentration ($EC_{50}$) and receptor numbers were derived from the plots presented in Figure 1. Similar results were obtained in three independent experiments.

with 5% fetal calf serum. Mitogenic activity of mEGF and its derivatives was determined by [$^3$H]thymidine incorporation, as previously described (Burgess et al., 1983). Briefly, confluent, serum-starved Balb/c 3T3 were cultured for 24 h in the presence of the EGF derivatives. [$^3$H]Thymidine (0.5 $\mu$Ci/mL) was added for the last 4 h, prior to harvesting of the cells.

*Receptor Binding Studies.* All binding studies were carried out on confluent cultures of Balb/c 3T3 fibroblasts. Since serum starvation did not alter the number of EGF receptors, cells were not serum starved prior to the binding experiments, and the binding was performed in DME containing 1% (w/v) bovine serum albumin (BSA) at 37 °C for the times indicated. All receptor assays were performed in triplicate, and nonspecific binding was determined by addition of a 100-fold excess of EGF or EGF analogue.

The relative affinity of EGF analogues for the EGF receptor was determined by adding to the cells a standard amount of $^{125}$I-EGF (100 pM) together with increasing amounts (20 pM to 16 nM) of the unlabeled analogues and incubating at 37 °C for 45 min. At the end of the incubation, the unbound ligands were removed by two washes in phosphate-buffered saline (PBS), and the cell monolayer was solubilized in 1 M NaOH for counting in a Packard counter. The binding data were plotted and analyzed with the LIGAND program (Munson, 1981). Surface-associated and internalized ligands were determined by the acid-stripping technique (Heigler et al., 1979). Specific ligand degradation was determined by TCA precipitation [10% (w/v) TCA at 4 °C for 1 h] and was corrected for degradation which occurred in the presence of a 100-fold excess of unlabeled ligand. Release of unbound ligand in the absence of internalization was determined by pretreating the cells with phenylarsine oxide ($10^{-5}$ M) for 10 min at room temperature (Wiley & Cunningham, 1981).

## RESULTS

*Mutations at Position 47 Decrease the Affinity of mEGF for Its Receptor.* Three mEGF analogues with mutations at position 47 (Ser-47, Leu-37-Tyr-47, and Val-47) (Ray et al., 1988) were assayed quantitatively for their ability to compete with the binding of $^{125}$I-EGF to its receptor on human (A431) and mouse (Balb/c 3T3) cell lines. We observed that mouse EGF binds with a 2-fold higher affinity to human cells than to mouse cells. The relative affinities of each analogue were comparable whether binding to the mouse or to the human EGF receptor, but all three analogues displayed reduced binding compared to the native mEGF. The estimated affinity constants for the murine EGF receptor are shown in Table I. The displacement binding kinetics for all analogues were curvilinear and were best represented by two binding components. Even when the relative affinities were shifted by more than 2 orders of magnitude, the two affinity states of the EGF receptor are detectable.

*Complete Occupancy of the High-Affinity EGF Receptor Is Sufficient for Inducing a Full Mitogenic Response.* Given the large difference in their affinity for the EGF receptor, the EGF analogues described in Table I provide an opportunity to investigate the relationship between receptor occupancy and the subsequent biological response. In particular, we have been able to analyze the relative contribution of high- and low-affinity receptors to the transmission of the mitogenic signal.

After purification, the concentrations of mEGF and its analogues (Ser-47, Leu-37-Tyr-47, and Val-47) in the stock solutions were determined by amino acid analysis; the solutions were diluted serially in Dulbecco's modified Eagle's medium containing 1% (w/v) bovine serum albumin and tested for either mitogenic activity or inhibition of $^{125}$I-EGF binding to a confluent monolayer of Balb/c 3T3 cells.

The fractional occupancy of the two receptor subclasses (calculated from the binding kinetics) was compared to [$^3$H]thymidine incorporation at each concentration of ligand (Figure 1). It is evident from these data that occupancy of only the high-affinity EGF receptor is required for mitogenic activity. The mitogenic response and high-affinity receptor occupancy increase concomitantly (Figure 1), reaching maximum levels at approximately the same concentration. This suggests that full occupancy of the high-affinity receptor is required for the maximal mitogenic response.

Given the large difference between the number of high- and low-affinity receptors (3000 vs 30000), we calculated the actual numbers of receptors occupied at each concentration of EGF. Again, mitogenic activity was present when only 10 low-affinity receptors were occupied, compared to 500 high-affinity receptors (data not shown). The relationship between half-maximal mitogenic dose and numbers of receptors occupied is shown in Table II. The close agreement between the number of high-affinity receptors occupied and the mitogenic stimulus (Table II) does not, however, apply to the EGF-Val-47 analogue, which gave half-maximal mitogenic activity with a high-affinity receptor occupancy of only 10%. We did not attempt to calculate the total number of receptors that would be triggered under the conditions of the mitogenic assay (i.e., a 24-h period). However, if our estimates of the relative affinities of EGF-Val-47 and mEGF are correct, we can expect a significantly lower number of receptors to be occupied by EGF-Val-47 than by mEGF at any time point.

This discrepancy could be caused by an erroneous interpretation of the Scatchard plots, due to a failure to reach equilibrium conditions or to a true difference in the behavior of the EGF-Val-47–receptor complexes. To discriminate between these alternatives, we radiolabeled the EGF-Val-47 analogue and analyzed its binding to the EGF receptor and its fate during the mitogenic signaling process.

*Kinetics of $^{125}$I-EGF-Val-47 Binding to and Internalization by 3T3 Cells.* The binding of iodinated EGF-Val-47 and of mEGF to Balb/c 3T3 cells was compared directly. Due to the difference in apparent affinity between the two ligands, we expected that at a given ligand concentration there would be a higher receptor occupancy with mEGF than with EGF-Val-47. Pilot experiments were therefore performed with a

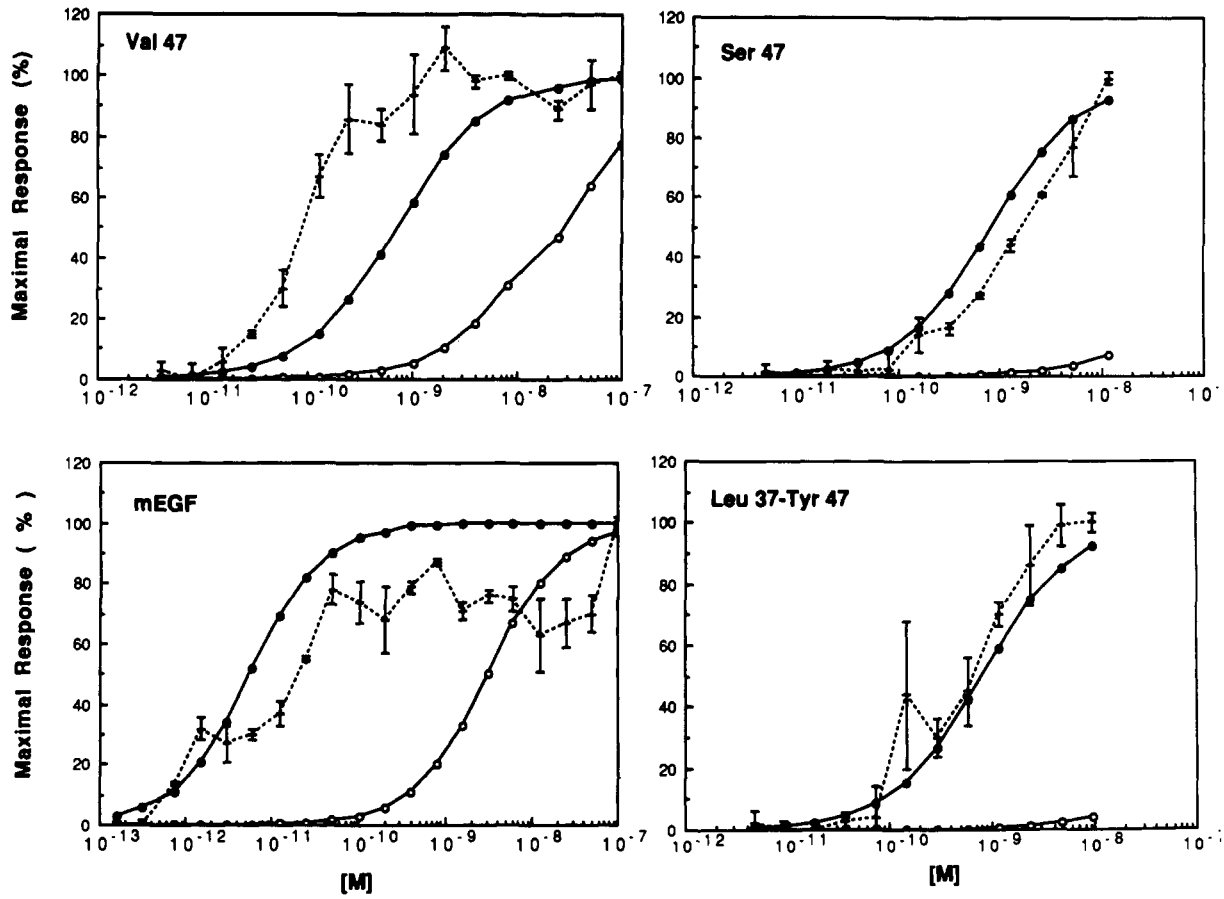


FIGURE 1: EGF-Ser-47, EGF-Leu-37-Tyr-47, and EGF-Val-47: receptor occupancy and mitogenic response. Serial dilutions of mEGF or the analogues were assayed in parallel for their ability to compete for $^{125}$I-mEGF binding and for their mitogenic activity on confluent monolayers of Balb/c 3T3 cells. Competition data were analyzed with the LIGAND program (Munson, 1981) and receptor occupancy curves generated from the estimated affinity constants. Results are expressed as a percentage of maximum values for both the mitogenic activity and the fractional receptor occupancy. (---) mitogenic response; (●) high-affinity receptor occupancy; (○) low-affinity receptor occupancy. Maximum thymidine incorporation: Ser-47, 10852 cpm; mEGF, 10314 cpm; Tyr-47, 9914 cpm; Val-47, 10291 cpm.

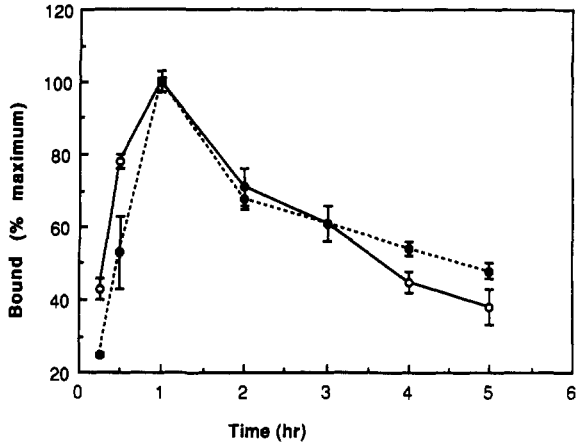


FIGURE 2: Kinetics of binding of $^{125}$I-mEGF and $^{125}$I-EGF-Val-47. Confluent monolayers of Balb/c 3T3 cells were treated with $^{125}$I-EGF at 200 pM (○) or $^{125}$I-EGF-Val-47 at 1 nM (●) for the times indicated, and the amount of ligand bound specifically was determined. Maximum specific binding was 6230 ± 219 cpm for $^{125}$I-EGF and 3099 ± 106 cpm for $^{125}$I-EGF-Val-47.

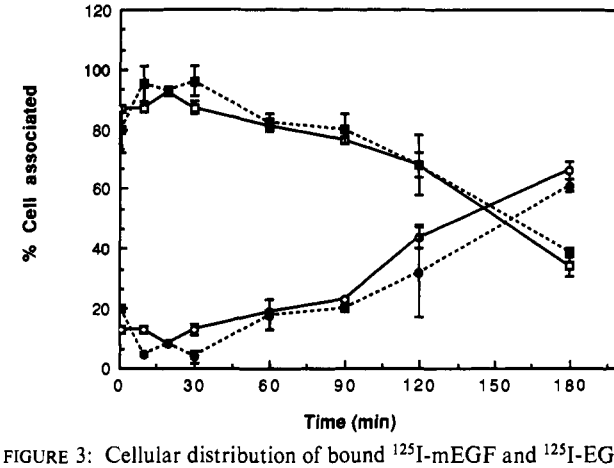


FIGURE 3: Cellular distribution of bound $^{125}$I-mEGF and $^{125}$I-EGF-Val-47. Internalized and surface-bound ligands were discriminated by the acid wash technique (Haigler et al., 1979). (□) Internalized $^{125}$I-EGF; (■) internalized $^{125}$I-EGF-Val-47; (○) surface-bound $^{125}$I-mEGF; (●) surface-bound $^{125}$I-EGF-Val-47.

50-fold or with a 5-fold higher concentration of $^{125}$I-EGF-Val-47 than of mEGF. Since there was no difference between the two sets of experiments when the results were plotted as a percentage of maximum values, the data presented were all obtained with a ratio of $^{125}$I-EGF-Val-47 to mEGF of 5:1. The kinetics of binding at 37 °C for the two ligands are shown in Figure 2. There appeared to be a slight but significant delay in the binding of EGF-Val-47, but by 1 h there was no detectable difference in the binding of the two ligands. The cellular distribution (i.e., internal and surface associated) of the two ligands was also very similar (Figure 3). The slightly higher proportion of internalized EGF-Val-47 was probably due to the higher $k_{off}$ and consequently to a systematic loss of the surface-bound ligand.

These results led us to believe that the difference in the apparent affinity constant between mEGF and EGF-Val-47 was due to a significant difference in the $k_{off}$ rates of the

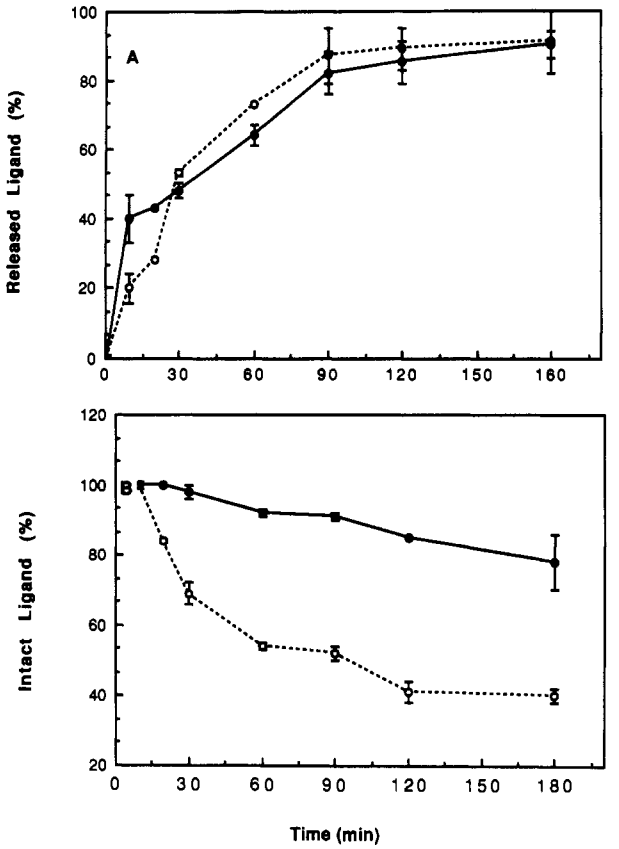


FIGURE 4: Release and degradation of $^{125}$I-mEGF and $^{125}$I-EGF-Val-47. Balb/c 3T3 cells were treated with $^{125}$I-mEGF or $^{125}$I-EGF-Val-47 for 40 min at 37 °C. The cells were then washed, placed in fresh medium, and monitored for ligand release (A) and degradation (B). Both sets of data are expressed as a percentage of maximum specific values. (○) $^{125}$I-mEGF; (●) $^{125}$I-EGF-Val-47.

ligand–receptor complex and that the explanation for the unexpectedly high mitogenicity of EGF-Val-47 was associated with an altered signaling response or with changes in the processing of the ligand.

*Processing of EGF-Val-47 and mEGF.* $^{125}$I-EGF-Val-47 (1 nM) and mEGF (200 pM) were allowed to bind to confluent monolayers of Balb/c 3T3 cells for 40 min at 37 °C; the unbound ligand was removed; the cells were washed and incubated in fresh medium (in the absence of ligand) for up to 3 h. At given time points the medium was removed, and the proportion of intact ligand released by the cells was determined by TCA precipitation (Figure 4). This protocol allowed us to determine the fate of receptor-bound ligand and to minimize the problems associated with different rates of internalization and differential receptor occupancy.

Again, at the earliest times $^{125}$I-EGF-Val-47 was released into the medium faster than native EGF, but this difference was barely significant (Figure 4A). However, the degradation rates of the two were quite different, and the proportion of intact $^{125}$I-mEGF released into the medium decreased steadily, while the $^{125}$I-EGF-Val-47 released was predominantly intact (Figure 4B). Since we followed only the ligand which had been bound and internalized via the EGF receptor, the degradation rates of $^{125}$I-EGF-Val-47 and $^{125}$I-mEGF must represent specific, receptor-mediated processing of the two ligands. We ruled out a differential sensitivity to proteases present in the assay medium by correcting all data for nonspecific degradation, i.e., the degradation occurring in the presence of a 100-fold excess of unlabeled mEGF. Even after 3 h, nonspecific degradation amounted to less than 10% for both $^{125}$I-EGF-Val-47 and $^{125}$I-mEGF.

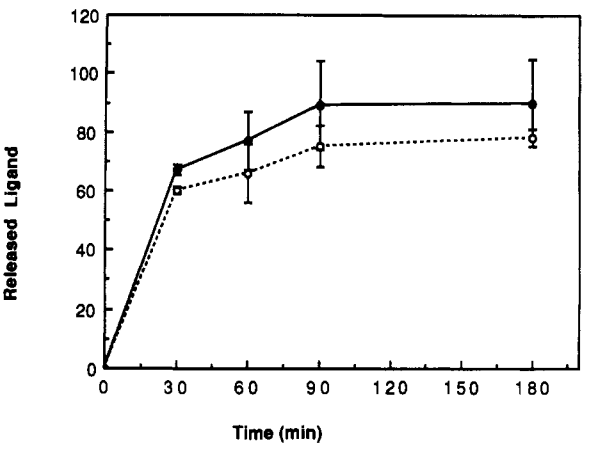


FIGURE 5: Ligand release in cells treated with phenylarsine oxide. Cells treated with phenylarsine oxide ($10^{-5}$ M, 10 min at room temperature) were incubated at 37 °C for 40 min with either $^{125}$I-EGF (200 pM) or $^{125}$I-EGF-Val-47 (1 nM). The unbound ligand was removed, and the cells were transferred to fresh medium. At the times indicated, the radioactivity released was measured, and degradation was determined by TCA precipitation. More than 90% of the cell-associated radioactivity was on the cell surface at the start of the second incubation. By the end of the incubation, less than 10% of the iodinated ligand released from the cell surface had been degraded. (○) EGF; (●) EGF-Val-47.

To confirm that degradation of mEGF was dependent upon internalization, we performed the same experiment with cells treated with phenylarsine oxide [i.e., rendered incapable of endocytosis (Wiley & Cunningham, 1982)]. As expected, the release of $^{125}$I-EGF-Val-47 is faster than the release of $^{125}$I-mEGF (Figure 5), but neither ligand was degraded significantly (after 3 h at 37 °C specific TCA precipitability of $^{125}$I-mEGF was 100% compared with 40% for $^{125}$I-mEGF from untreated cells).

Thus it appears that mutation of Leu-47 to Val-47 not only influences receptor binding but also affects intracellular processing and that a prolonged half-life of the EGF-Val-47 analogue is responsible for its increased potency as a mitogen.

## Discussion

Although it has long been recognized that EGF binding to its receptor comprises a high- and a low-affinity component, the relative role of the two sites is still a matter of some discussion. It has been postulated that the high-affinity receptor might be selectively involved in mitogenesis (Schechter et al., 1978; Kawamoto et al., 1983), and it has been shown that manipulating the affinity state of the EGF receptor leads to changes in its tyrosine kinase activity, with the high-affinity state being more active than the low-affinity state (Davis et al., 1988; Davis & Czech, 1985). However, the physiological role of the two affinity states has been questioned and the apparent high-affinity component attributed to cellular processing of the ligand–receptor complexes (Knauer et al., 1983; Wiley, 1989).

One of the major problems encountered in determining a role for the two affinity states of the EGF receptor is the different time frames between assays: 30–60 min for equilibrium binding and 24 h for mitogenicity. This can be complicated further by the finding that EGF is needed continuously for over 8 h for maximal stimulation of an EGF-dependent epithelial cell line (Sizeland et al., 1989).

Although the steady-state model of the ligand–receptor interaction described by Wiley and Cunningham (1981) and by Knauer et al. (1983) takes into account the interplay of ligand and receptor internalization, degradation, and recycling,

and in some respects should be a better description than the equilibrium model of the complex events leading to mitogenesis, it does not take into account the high-affinity binding component, since it is virtually undetectable under steady-state conditions, and fails to recognize the "immediate-early" effects that EGF has on its receptor.

Our present results directly address this controversy, since they are comparable with the data obtained by Knauer et al. (1983). In both studies a linear relationship between receptor occupancy and mitogenic response was found, but in our study the estimated affinity of the receptor is nearly 3 orders of magnitude higher, and the correlation between mitogenic response and high-affinity receptor occupancy is close ($r = 0.98$ for mEGF). The role of the high-affinity EGF receptor in EGF signal transduction has been recently confirmed by Defize et al. (1989) and Bellot et al. (1990). By using antibodies which selectively block either the low- or the high-affinity binding, these authors have shown that triggering of the high-affinity receptor is sufficient to elicit the early cellular responses to EGF, including receptor autophosphorylation, increase in intracellular pH, and induction of expression of the nuclear protein, c-fos.

If the high-affinity component is all that is required for mitogensis, we are left with the puzzle that the great majority of EGF receptors in any given cell type are redundant or have other functions. One possible role for these receptors could be to internalize and degrade excess ligand and hence inactivate the mitogenic signal. Alternatively, the low-affinity EGF receptor could initiate a separate set of responses associated with differentiation or activation rather than mitogensis (Pignataro & Ascoli, 1990).

It is interesting in this context to note that most of the reported metabolic effects of EGF, such as the increase in intracellular calcium levels and phosphoinositide turnover (Muldoon et al., 1988) or activation of phospholipase C (Wahl & Carpenter, 1988; Wahl et al., 1989), are elicited at supramitogenic concentrations of EGF which correlate with a high fractional occupancy of the low-affinity EGF receptor.

We have shown that the half-life of EGF in the medium is an important parameter in the assessment of its mitogenic activity and that sustained occupancy of a small number of "high-affinity receptors" (HAR) is as effective as short-term triggering of a larger number of HAR.

It has been recently shown that internalization of the EGF–EGF receptor complex is not required for its mitogenic activity (Clark et al., 1988); on the contrary, cells bearing internalization-defective EGF receptor mutants exhibit an increased ability to respond to EGF. The inability of these cells to endocytose the EGF–HAR complex results in persistently elevated levels of occupied receptor (Chen et al., 1989) and enhanced responsiveness to EGF (Wells et al., 1990). A similar link between increased half-life of the EGF receptor in the presence of the bovine papilloma virus protein E5 and enhanced responsiveness to EGF has been noted by Martin et al. (1989). In many respects EGF-Val-47 parallels functionally the situation in which the receptor is not internalized and degraded. In both systems, the net effect is a prolongation of existence for the cell surface receptor–ligand complex, which in turn results in an enhanced mitogenic response.

It is not clear why a substitution of valine for leucine at position 47 in the EGF molecule should alter its susceptibility to cellular proteases. It should be possible to produce an even more potent form of mEGF by altering the $Asp_{46}$–$Leu_{47}$ peptide bond to a thioamide. This should preserve the conformational characteristics of the Leu-47 side chain but increase the resistance of the growth factor receptor mediated degradation.

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# Ligand Exclusion on Acetylcholinesterase†

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ABSTRACT: This paper examines covalent reactivity of AchE with respect to *cationic* and *uncharged* methylphosphonates and substrates in the absence and presence of cationic ligands selective for the active center and the peripheral anionic site. The organophosphorus inhibitors are enantiomeric alkyl methylphosphonothioates (**1–5**) containing cycloheptyl and isopropyl phosphono ester groups and *S*-methyl, *S*-*n*-pentyl, and *S*-[β-(trimethylammonio)ethyl] leaving groups; these agents differ in their configuration about phosphorus and their steric, hydrophobic, and electrostatic characteristics. The synthetic substrates examined are acetylthiocholine, *p*-nitrophenyl acetate, and 7-acetoxy-4-methylcoumarin (7AMC). Antagonism of the methylphosphonothioate reaction by cationic ligands is strongly dependent on the nature of both the cation and the methylphosphonate but independent of the configuration about phosphorus. While all cations cause *linear* mixed inhibition of acetylthiocholine hydrolysis, there are observed a variety of inhibition patterns of 7AMC and *p*-nitrophenyl acetate hydrolysis that are distinctly *nonlinear*, as well as patterns in which the reciprocal plots intersect in the upper right quadrant. Strong antagonism of cationic (methylphosphonyl)thiocholines correlates very well with linear inhibition of acetylthiocholine. Ligands that cause only negligible antagonism of the uncharged methylphosphonates display nonlinear inhibition of uncharged substrates. These relationships, since they are most pronounced for peripheral site ligands and are strongly dependent on the charge carried by the reactant, suggest that the peripheral anionic site alters enzyme reactivity through an electrostatic interaction with the net negative active center. Such behavior indicates a potential role for the peripheral anionic site in conserving AchE catalytic efficiency within a narrow range of values.

Acetylcholinesterase (AchE)[1] plays a central role in neuromuscular transmission by hydrolyzing acetylcholine released following depolarization of the presynaptic nerve terminal. The enzyme exists in nerve and muscle as a polymorphic family of molecular forms associated with the basal lamina and plasma membranes (Rotundo, 1987; Toutant & Massoulie, 1988). Since inhibition of AchE leads initially to a prolongation of end-plate currents and eventually to blockade of neuromuscular transmission (Katz & Miledi, 1973; Hartzell et al., 1975), removal of $Ach^+$ is essential to efficient neuromuscular transmission.

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[1] Abbreviations: AchE, acetylcholinesterase; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; NMA, *N*-methylacridinium; decyl-TMA, decyltrimethylammonium; hexyl-TMA, hexyltrimethylammonium; PTMA, phenyltrimethylammonium; 7AMC, 7-acetoxy-4-methylcoumarin; 7HMC, 7-hydroxy-4-methylcoumarin; M7C, *N*-methyl-7-[(dimethylcarbamoyl)oxy]quinolinium iodide; $AcSch^+$, acetylthiocholine; $Ach^+$, acetylcholine.

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