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# Utilization of a Receptor Reserve for Effective Amplification of Mitogenic Signaling by an Epidermal Growth Factor Mutant Deficient in Receptor Activation

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**Abstract** The idea of a receptor reserve in mediating cellular function is well known but direct biochemical evidence has not been easy to obtain. This study stems from our results showing that L15 of epidermal growth factor (EGF) is important in both EGF receptor (EGFR) binding and activation, and the L15A analog of human EGF (hEGF) partially uncouples EGFR binding from EGFR activation (Nandagopal et al., [1996] Protein Engng 9:781-788). We address the cellular mechanism of mitogenic signal amplification by EGFR tyrosine kinase in response to L15A hEGF. L15A is partially impaired in receptor dimerization, shown by chemical cross-linking and allosteric activation of EGFR in a substrate phosphorylation assay. Immunoprecipitation experiments reveal, however, that L15A can induce EGFR autophosphorylation in intact murine keratinocytes by utilizing spare receptors, the ratio of total phosphotyrosine content per receptor being significantly lower than that elicited by wild-type. This direct biochemical evidence, based on function, of utilization of a receptor reserve for kinase stimulation suggests that an EGF variant can activate varying receptor numbers to generate the same effective response. L15A-activated receptors can stimulate mitogen-activated protein kinase (MAPK) that is important for mitogenesis. The lack of linear correlation between levels of receptor dimerization, autophosphorylation, and MAPK activation suggests that signal amplification is mediated by cooperative effects. Flow cytometric analyses show that 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 1 nM wild-type, indicating that MAPK activation alone is insufficient for maximal stimulation of mitogenesis. Higher concentrations of L15A reverse this effect, indicating that L15A and wild-type differ in the number of receptors each activates to induce the threshold response, which may be attained by cooperative activation of receptor dimers/oligomers by van der Waal's weak forces of attraction. The maintenance of a receptor reserve underscores an effective strategy in cell survival. J. Cell. Biochem. 83: 326–341, 2001. Published 2001 Wiley-Liss, Inc.†

**Key words:** cell proliferation; EGF mutagenesis; ligand–receptor interaction; partial agonist; receptor reserve; signal amplification; tyrosine kinase

The mitogenic response to the epidermal growth factor (EGF) ligand is mediated by the EGF receptor (EGFR) tyrosine kinase, a 170-kDa transmembrane glycoprotein expressed in epithelial and mesenchymal cell types [Carpenter and Cohen, 1990]. Ligand-induced EGFR dimerization coincides with the activation of the receptor's tyrosine kinase, leading to receptor autophosphorylation [Ullrich and Schlessinger, 1990]. This renders the receptor competent to interact with and phosphorylate intracellular substrates which transmit signals via modular SH2, SH3, or PH domains [for reviews, see van der Geer et al., 1994; Pawson, 1995]. The gamut of biochemical responses elicited in EGF-treated cells includes a rise in intracellular pH and $Ca^{2+}$ levels, increased rates of glycolysis, DNA, RNA, and protein syntheses, and a host of physiological changes

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[reviewed by Carpenter and Cohen, 1990; Carpenter and Wahl, 1990; Zwick et al., 1999; Hunter, 2000]. Given the pleiotropic nature of the response to EGF, it is remarkable how cells sort signals from background 'noise' in order to integrate physiological processes in terms of specificity and redundancy, as well as regulation in time and space [for reviews, see Zwick et al., 1999; Hunter, 2000].

Transformation of an EGF-dependent stimulus into a cellular response involves energy transduction, receptor activation and resultant signal amplification [Schlessinger and Ullrich, 1992; Hunter, 1995]. The nature and extent of amplification in this temporal sequence remain poorly understood, partly because the relative abundance and stoichiometry of interactions between effector components in a given signaling pathway may vary among different cell types. Furthermore, early biochemical events in a pathway are better defined than late events. Nonetheless, the concept of signal amplification best explains why the ultimate biological response, e.g., mitogenesis, saturates at ligand concentrations which do not reflect saturation of the primary response, e.g., receptor activation due to ligand binding. If activated growth factor-receptor complexes merely integrate input/output signals via multiple pathways, would there be any need for amplification? Perhaps more intriguing, what is the nature of the biological consequence, if any, when amplification is impaired in response to receptor stimulation?

This study was prompted by previous results from our laboratory demonstrating that the L15A human EGF (hEGF) mutant partially uncouples receptor binding from receptor activation in a substrate phosphorylation assay and its reduced binding affinity (2.4% relative to wild-type hEGF) and partial agonist activity are not due to gross structural perturbations as shown by $^{1}$H-NMR spectroscopy [Nandagopal et al., 1996]. Other studies have confirmed the importance of L15 of hEGF in ligand–receptor interaction [Neelam et al., 1996; Mullenbach et al., 1998] and receptor dimerization [Neelam et al., 1996]. We have proceeded to determine the cellular mechanism(s) of signaling by the L15A mutant and thereby elucidate the physiological consequence(s) thereof. L15A was compared to wild-type hEGF in stimulating receptor dimerization/oligomerization, receptor autophosphorylation, MAPK activation and cell proliferation. The results show that the L15A hEGF mutant, in spite of its deficiency in receptor activation as revealed by dimerization, autophosphorylation, and substrate phosphorylation studies, is nonetheless able to amplify threshold signals for mitogenesis by recruitment of enough suboptimally activated receptors from a receptor reserve to mediate the mitogenic response. The compensatory effects mediated by the receptor reserve point to the biological importance of the existence and maintenance of such a reserve in cell survival, as previously implicated by other investigators [Carpenter and Cohen, 1976; Aharonov et al., 1978; Carpenter and Wahl, 1990; Zhu, 1993; Soler et al., 1994a], but not in the context of an altered ligand–receptor interaction, as in the case of our studies with L15A. The present studies offer new insight into the possible design of receptor agonists and antagonists with therapeutic potential.

## MATERIALS AND METHODS

### Cell Culture

Balb/MK, an EGF-dependent murine epithelial keratinocyte cell line [Weissman and Aaronson, 1983], was a gift from Dr. Stuart Aaronson (Mt. Sinai, New York). Cell stocks were maintained at 37°C in a humidified atmosphere of 5% $CO_2$, as rapidly growing cultures in low $Ca^{2+}$ Eagle's minimal essential medium (EMEM) (Biofluids Inc.) containing 0.05 mM $CaCl_2$, 0.02 mM calcium pantothenate, 2 mM L-glutamine and 100 U/ml Penicillin-G. The medium was supplemented with 10% dialyzed FBS (GIBCO) and 5 ng/ml recombinant hEGF (complete medium). A431 (human epidermoid carcinoma) cells, grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, served as the source of EGFR.

### Recombinant hEGF Purification

Wild-type and L15A mutant hEGF were purified as previously described [Nandagopal et al., 1996]. Lyophilized protein samples were stored at −80°C till further use.

### Ligand-Induced Receptor Tyrosine Kinase Activation and Receptor Dimerization Assays

Ligand-dependent activation of EGFR was measured after solubilization of A431cell membranes and partial purification of the receptor

kinase by wheat-germ agglutinin affinity chromatography. The conditions were similar to those of Akiyama et al. [1985] with modifications described by Koland and Cerione [1988]. 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, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 250 mM $(NH_4)_2SO_4$, 100 mM $Na_3VO_4$, 5% glycerol, and 0.05% Triton X-100. The kinase reaction was initiated by the addition of [$\gamma$-$^{32}$P] ATP (1.35 Ci/mmol) and $(Glu_4,Tyr_1)_n$ (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% trichloroacetic acid (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 $^{32}$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 <2% of the total activity. Curve fitting was performed with the Deltagraph 4.01 software.

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 was previously described by Canals [1992]. 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 the addition of 80 mM glutaraldehyde (Polysciences Inc.) and after 1 min at room temperature, quenched by the addition of 2 M glycine, pH 9, in a final reaction volume of 50 μl. 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 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto Immobilon-P membranes (Millipore) and processed for immunoblotting with anti-EGFR monoclonal antibody (mAb) IgG1 (Transduction Labs), as per manufacturer's protocol. Membranes were blocked with 3% bovine serum albumin (BSA) (Sigma). Secondary goat-anti-mouse polyclonal antibody conjugated to horse radish peroxidase (HRP) was obtained from Transduction Labs Enhanced chemiluminescence (ECL) detection reagent was from Amersham.

Dimerization of the EGFR in intact A431 cells was examined by the protocol of Cochet et al. [1988] with modifications. Cells were grown in 35 mm plates to 80% confluence in DMEM supplemented with 10% fetal bovine serum (FBS). They were then incubated in DMEM containing 0.5% FBS for 48 h. Wild-type or mutant hEGF protein was diluted in DMEM to the desired concentrations and 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 phosphate buffered saline (PBS) and cross-linking was initiated with 15 mM 1-ethyl-3-[3(dimethylamino)propyl] carbodiimide (EDC) (Pierce Chem. Co.) buffered with 100 mM MES, pH 6.0. After incubation for 7 min at 37°C, the EDC solution was aspirated and the reaction neutralized by the addition of 1 M 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*g* for 5 min at 4°C, the protein contents of the supernatants were determined by the bichinchonic acid (BCA) assay (Pierce Chem. Co.) [Brown et al., 1989]. Aliquots containing 15 μg protein were treated with equal volumes of Laemmli buffer containing 0.7 M β-mercaptoethanol and denatured at 95°C for 3 min. 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/cm$^2$ and grown to ~70% confluence in 60 mm culture dishes. The monolayers were rinsed with PBS, pH 7.4, and then incubated for 48 h in complete medium lacking hEGF. Wild-type or mutant hEGF protein was 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 adding lysis buffer (100 mM Tris-Cl, pH 7.6, containing 1% SDS). Extracts were vortexed and denatured at 95°C for 3 min to reduce sample viscosity. After centrifugation at 12,000*g* for 5 min at 4°C, supernatants were assayed for protein content with the BCA reagent. Aliquots of the supernatant (750 μg) were incubated with either 4 μg anti-EGFR mAb or 4 μg normal rabbit serum protein (Sigma) for 2 h at 4°C as per manufacturer's protocol. Two micrograms rabbit-anti-mouse IgG (Sigma) were 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) overnight and centrifuged at 12,000*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) as per manufacturer's protocol. Receptor autophosphorylation was independently verified by immunoprecipitating tyrosyl-phosphorylated proteins from the soluble supernatant (750 μg) of denatured cell lysates with the PY20 mAb, resolving them on 7.5% gels by SDS–PAGE and probing the blots with anti-EGFR mAb. Secondary goat-anti-mouse polyclonal antibody conjugated to HRP and the ECL detection reagent were utilized for visualization of bands. Scanning densitometry was performed with the model 300A computing densitometer and the ImageQuant (version 3.15) software system (Molecular Dynamics).

### MAPK Activation Assays

Balb/MK cells were plated at a density of $10^4$ cells/cm$^2$ 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 protein was diluted in the same medium and added to the cells at 37°C. Growth factor concentrations and durations of induction were as indicated in Figures 3 and 4. 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). Extracts were vortexed and denatured at 95°C for 3 min to reduce sample viscosity. After centrifugation at 12, 000*g* at 4°C for 5 min, protein content of the supernatants was determined by the BCA assay. Samples (750 μg) were incubated with either 4 μg anti-ERK2 mAb (IgG2b) (Transduction Labs) or 4 μg normal rabbit serum protein for 2 h at 4°C as per manufacturer's protocol. Two micrograms 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 as per manufacturer's protocol. Secondary antibody incubation, ECL detection procedures, and densitometric scanning were carried out as described above.

### 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 5 and 6. Cells from triplicate dishes were trypsinized, pooled, centrifuged (1,000 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 $Ca^{2+}$/$Mg^{2+}$-free PBS and incubated with 50 μg/ml RNaseA (Worthington Inc.) for 30 min at 37°C with rotary shaking. Propidium iodide (PI) (Sigma) 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) equipped with a 488 nm argon laser. PI fluorescence was detected using a 585/42 nm dichroic filter. Chicken erythrocytes and calf thymus nuclei (Becton Dickinson) were used as standards to ensure the quality of DNA distribution patterns. Events/sample ($10^4$) were acquired at a flow rate of ~100 events/sec. All parameters including pulse width and 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 vs. 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) which is based on the analysis of DNA distributions as described by Dean and Jett [1974]. 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 $\chi^2$ value of $\leq 10$ was deemed acceptable, while a value $\leq 5$ was considered an excellent fit of the assumed model to the experimental data when tested for statistical significance.

## RESULTS

### Apparent Positive Cooperativity of Receptor Activation and Status of Ligand-Induced Receptor Dimerization

Tyrosine kinase activity of the solubilized receptor was measured as a function of ligand concentration using $(Glu_4,Tyr_1)_n$ as substrate. Kinase data pertaining to L15A and wild-type hEGF [Nandagopal et al., 1996] were re-evaluated using the Hill equation. Analysis of the dose response curve for wild-type hEGF, shown in Figure 1A, reveals apparent positive cooperativity with $n_{app} = 2.5$ and $EC_{50} = 0.2$ μM. This suggests that the increase in tyrosine kinase activity is due to ligand-induced receptor dimerization/oligomerization. The non-integral value of $n_{app}$ is probably due to contributions by receptor molecules that were only partially activated, i.e., the allosteric transition was incomplete in these cases. The slope of the curve indicates that a 4-fold variation in concentration of the activating ligand (0.08–0.32 μM) shifts the velocity from 16.7 to 83.3% $V_{max\ app}$ 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. Therefore, it could not be analyzed by the Hill equation. To compensate for its reduced receptor affinity, L15A was tested at concentrations 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 (~1 nM), it does not induce the same maximal response as wild-type hEGF [Nandagopal et al., 1996]. This suggests that induction of the effective response is dependent on allosteric activation of the receptor, a process impaired by the binding of L15A to the receptor.

EGF-induced dimerization of EGFRs [e.g., Böni-Schnetzler and Pilch, 1987; Yarden and Schlessinger, 1987a,b; Cochet et al., 1988; Fanger et al., 1989; Sherrill and Kyte, 1996]. Some studies have implicated the monomeric EGFR as the active kinase [Biswas et al., 1985; Koland and Cerione, 1988; Northwood and Davis, 1988; Carraway and Cerione, 1993] while others have attributed the activity to the dimer [Yarden and Schlessinger, 1987a,b; Cochet et al., 1988; Canals, 1992; Sherrill and Kyte, 1996; Neelam et al., 1998]. However, these studies examined the involvement of receptor aggregation without resolving the role(s) of ligand-receptor interactions in tyrosine kinase activation.

Ligand-induced dimerization of solubilized EGFR was examined by glutaraldehyde cross-linking. The anti-EGFR immunoblot in Figure 1B 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 range of 0.1–0.4 μM, similar to the $EC_{50}$ value obtained in the $(Glu_4,Tyr_1)_n$ phosphorylation assay (Fig. 1A). The band intensities of receptor dimers trapped at 0.1 and 0.4 μM wild-type are similar to those at 5 and 10 μM L15A, respectively. L15A can thus induce soluble EGFR dimerization at concentrations that are relatively ineffective in stimulating $(Glu_4,Tyr_1)_n$ phosphorylation (Fig. 1A). The ability of ligand-bound receptor dimers to attain the kinase-active conformation may additionally involve an allosteric transition due to ligand binding.

The status of EGFR dimerization was also examined in intact A431 cells when the receptor was stimulated by either wild-type hEGF or L15A. In contrast to wild-type hEGF, L15A is severely impaired in mediating receptor dimerization (Fig. 1C). This confirms the importance of L15 of hEGF in mediating receptor dimeriza-

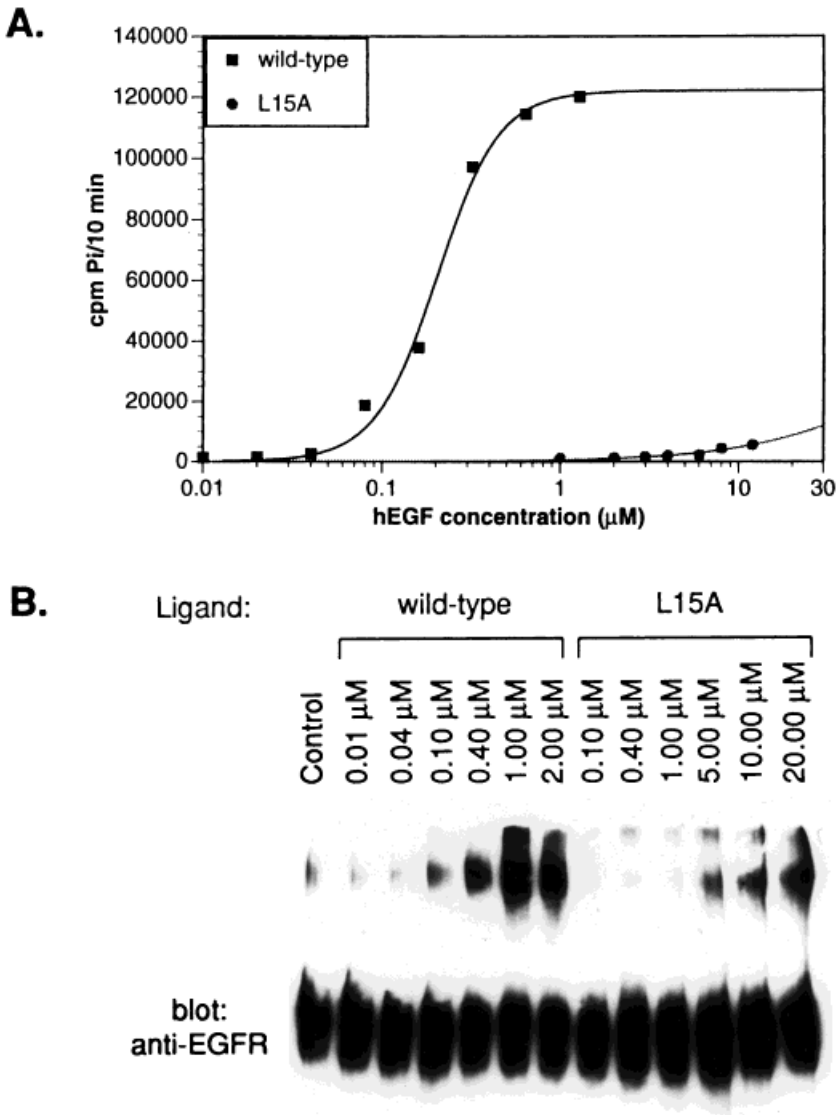


**Fig. 1.** Apparent positive cooperativity of receptor activation and the status of ligand-induced receptor dimerization. **A:** EGF-dependent phosphorylation of the $(Glu_4,Tyr_1)_n$ substrate by partially purified EGFR tyrosine kinase. **B:** Glutaraldehyde cross-linking of EGFR dimers in response to ligand binding and under conditions similar to the in vitro kinase assay. **C:** Ligand-induced EGFR dimerization in intact cells as determined by EDC cross-linking experiments. Data shown are representative of at least two independent experiments.

tion, thereby extending the observations of Neelam et al. [1996] to intact cells in culture. Whether no dimers were formed or they were unstable during the course of the assay cannot be distinguished unequivocally. The observed differences in the glutaraldehyde and EDC cross-linking assays may reflect the reaction conditions which differ in receptor microenvironment, ionic strength, temperature, incubation time, and chemistry of the cross-linking reaction. The propensity of the receptor to undergo more extensive dimerization in cell-free systems than in intact membranes has also been reported by other investigators [Cochet et al., 1988; Carraway and Cerione, 1993].

### Role of a Receptor Reserve in EGFR Autophosphorylation

The reduced potency of L15A in $(Glu_4,Tyr_1)_n$ substrate phosphorylation and EGFR dimerization assays prompted an investigation of receptor activation in intact cells. Dose–response parameters for ligand-induced receptor autophosphorylation were established in non-transformed murine epidermal keratinocytes (Balb/MK), which are EGF-dependent for growth [Weissman and Aaronson, 1983]. The robust response to wild-type hEGF stimulation (30 sec induction at 37°C) is shown in Figure 2A and B. Published $k_D$ values for receptor subclasses in Balb/MK cells suggest that the exquisite sensitivity of the response is mediated through the high-affinity subclass which has a $k_D = 0.2$ nM and ~4,500 sites/cell under conditions of receptor upregulation [Zendegui et al., 1988]. The kinase data presented here indicate that the low-affinity receptor subclass, which has a $k_D = 30$ nM and ~18,000 sites/cell under conditions of receptor upregulation [Zendegui et al., 1988], constitutes a receptor reserve/spare receptor pool of ~1 μM, the presence of which

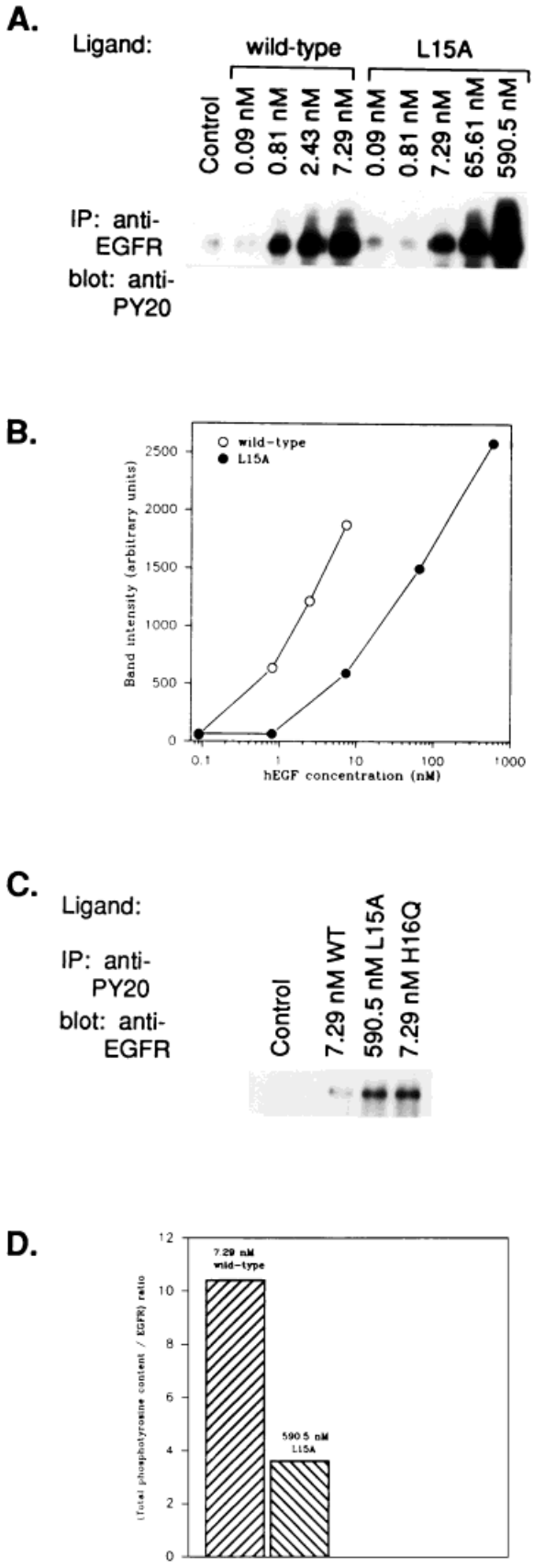


**Fig. 2.** Comparison of the ability of wild-type hEGF and L15A to promote EGFR autophosphorylation in intact Balb/MK cells. The dose–reponse curve in panel **(B)** is derived from densitometric scanning of band intensities in panel **(A)**. **(C)** Verification of agonist activity of 7.29 nM wild-type hEGF relative to 590.5 nM L15A by immunoprecipitating autophosphorylated EGFRs with PY20 and immunoblotting with an anti-EGFR mAb. H16Q hEGF, which binds EGFR with ~2.7-fold higher affinity relative to wild-type hEGF, was used as a positive control. **(D)** Functional evidence for a receptor reserve, as determined by the phosphotyrosine content/receptor ratio obtained from scanning the corresponding signal intensities in panels (A) and (C). Data are representative of two independent experiments.

steepens the dose–response curve for wild-type-induced autophosphorylation of EGFR. The activity profile of L15A, shown in Figure 2A and B, reveals two interesting features: (i) although the curve is 'right-shifted' the slope in the 7.29 (45.21 ng/ml) to 590.5 nM (3.67 μg/ml) concentration range almost parallels that of the wild-type stimulated curve and (ii) it thus appears that partial agonists like L15A may elicit maximal effects (similar to full agonists) in the presence of spare receptors. For both wild-type hEGF and L15A, the evoked response (autophosphorylation) is more senstitive than dimerization ability (compare Fig. 1C and 2A) at the doses examined with intact cells in culture.

Since partial agonists require a receptor reserve to induce maximal effects [Pollet and Levey, 1980; Ehlert, 1988; Zhu, 1993], it was of interest to assess potential differences in the phosphotyrosine content of receptors activated by L15A vs. wild-type hEGF. The agonist activity of 7.29 nM wild-type hEGF relative to 590.5 nM L15A was therefore verified by immunoprecipitating autophosphorylated EGFRs with an mAb to phosphotyrosine (PY20) 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 [Nandagopal et al., 1999], was also tested at a dose of 7.29 nM and served as a positive control. We reasoned that H16Q would engage more EGFRs and hence be more efficacious than wild-type in inducing receptor autophosphorylation at the same dose. Figure 2C shows that 590.5 nM L15A induces tyrosine phosphorylation of a larger fraction of receptor molecules compared to 7.29 nM wild-type hEGF and, as shown in Figure 2D, the ratio of total phosphotyrosine content per receptor is ~2.8-fold lower under these conditions. Furthermore, as predicted from the Furchgott equation [Furchgott and Bursztyn, 1968], 7.29 nM H16Q is equipotent relative to 590.5 nM L15A in this assay (Fig. 2C). Thus, based on direct biochemical evidence, we infer that *these ligands (i.e., wild-type, H16Q and L15A) activate different numbers of receptors to generate the same effective response.* This becomes possible since there exists a spare receptor pool/receptor reserve for kinase stimulation and the interpretation is consistent with the results presented earlier in Figure 1A.

Protein G-Sepharose/Agarose binds with similar affinity to Abs of the IgG2b (PY20) and IgG1 (anti-EGFR) isotypes. Since the high-affinity Abs were used at concentrations in far excess of Ags being immunoprecipitated, it is reasonable to assume that Ag–Ab complexes, once formed, will be pulled down with similar efficiency. Following transfer onto PVDF membranes, the Ag is concentrated in a very small area. Binding of the HRP-conjugated secondary Ab (goat anti-mouse) to the primary Ab–Ag complex on the membrane is very efficient. The HRP-catalyzed signal detection (ECL) is also highly sensitive, precluding significant differences in immunoblotting efficiencies. These considerations thus permitted a ratiometric estimation of signal intensities as depicted in Figure 2D.

### Partial Agonist (L15A) is Capable of Activating p42 MAPK

To ascertain whether EGFR autophosphorylation induced by L15A translates into biochemical responses in signaling pathways downstream of the EGFR, we investigated the activation of MAPK ($p42^{mapk}$) in Balb/MK cells. MAPKs are involved in the mitogenic response to growth factors [Rossomando et al., 1989; Haystead et al., 1992; Marshall, 1995; Meloche, 1995] and also implicated in the regulation of more proximal aspects of EGFR signaling [Heisermann et al., 1990; Northwood et al., 1991; Buday et al., 1995; Rozakis-Adcock et al., 1995] by feedback mechanisms. Furthermore, the stimulus–response curve for MAPK exhibits ultrasensitivity, a feature which promotes an all-or-none, switch-like response [Huang and Ferrell, 1996; Ferrell and Machleder, 1998].

The response to either L15A or wild-type hEGF was investigated in immunoprecipitation experiments using an anti-ERK2/MAP-2 mAb and probing immunoblots with an mAb to phosphotyrosine. As shown in Figure 3, both wild-type and L15A at 7.29 nM concentration, elicit a clear response of similar magnitude in this assay. There is a lack of linear correlation to the levels of ligand-inducible receptor autophosphorylation at these concentrations (Fig. 2). The results also demonstrate that EGFRs activated by L15A are indeed capable of transducing biochemical signals important for mitogenesis due to signal amplification. Since we have found the abundance of $p42^{mapk}$ protein to be greater relative to the EGFR in Balb/MK cells (data not shown), the results suggest that the efficacy of signaling is influenced by the $k_D$ to

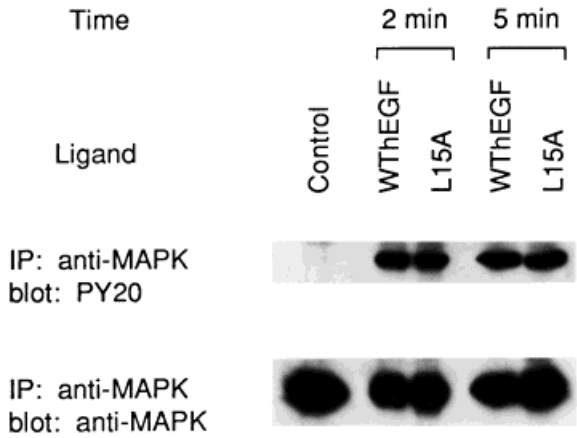


**Fig. 3.** Determination of EGF-inducible tyrosine phosphorylation of p42 MAP kinase by immunoprecipitation assay as described in Materials and Methods. Data obtained with equivalent concentrations (7.29 nM) of wild-type or mutant ligand are representative of at least two independent experiments.

metabolite ratio, rather than absolute concentrations of available ligand or receptor. Further studies are needed to assess the contribution of cooperative EGFR activation and the ultrasensitive response of MAPK to EGF stimulation in Balb/MK cells.

Having established that MAPK is indeed activated, we decided to address the nature of the correspondence between EGFR activation and MAPK activation. The kinetics of $p42^{mapk}$ activation/inactivation in response to equivalent concentrations (7.29 nM) of H16Q and wild-type hEGF was compared to 590.5 nM of L15A. As shown in Figure 4A and B, under these conditions L15A and H16Q induce a response that is only ~2-fold 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

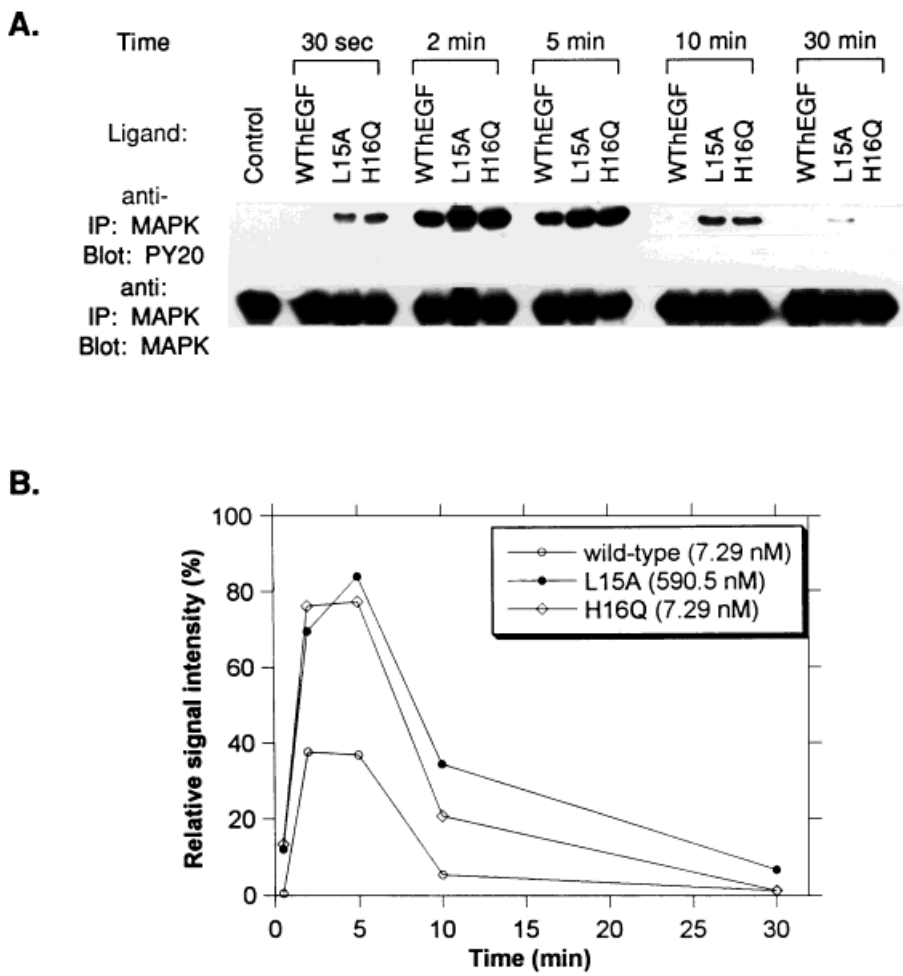


**Fig. 4.** Comparison of p42 MAP kinase activation/inactivation kinetics in Balb/MK cells in response to hEGF analogues. Normalized signal intensities in panel **(B)** are derived from densitometric scanning of band intensities in panel **(A)**. Data shown are representative of at least two independent experiments.

interval. Although the phosphorylation of $p42^{mapk}$ is transient, levels of $p42^{mapk}$ protein remain constant during the entire assay. The slopes of the curves depicted in Figure 4B suggest that EGFRs activated by the hEGF variants may dictate changes in relative intensity but not necessarily the duration of the response under the assay conditions. Cells attenuate the stimulus when the effective response exceeds the optimum, 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 [Carpenter and Cohen, 1979; Sand and Christoffersen, 1987; Pardee, 1989]. 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. (i) 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 pre-replicative phase of the present cycle and whether or not cells were deprived of hEGF/serum. (ii) 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. (iii) The overall distribution/shape of the subpopulation in S phase which is governed by the rate of DNA synthesis and any synchrony present.

Figure 5A 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 (Fig. 5B and 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 that 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 (Fig. 5A and B). There is no appreciable difference in the rate of exit from G0/G1 when the concentration of inducing

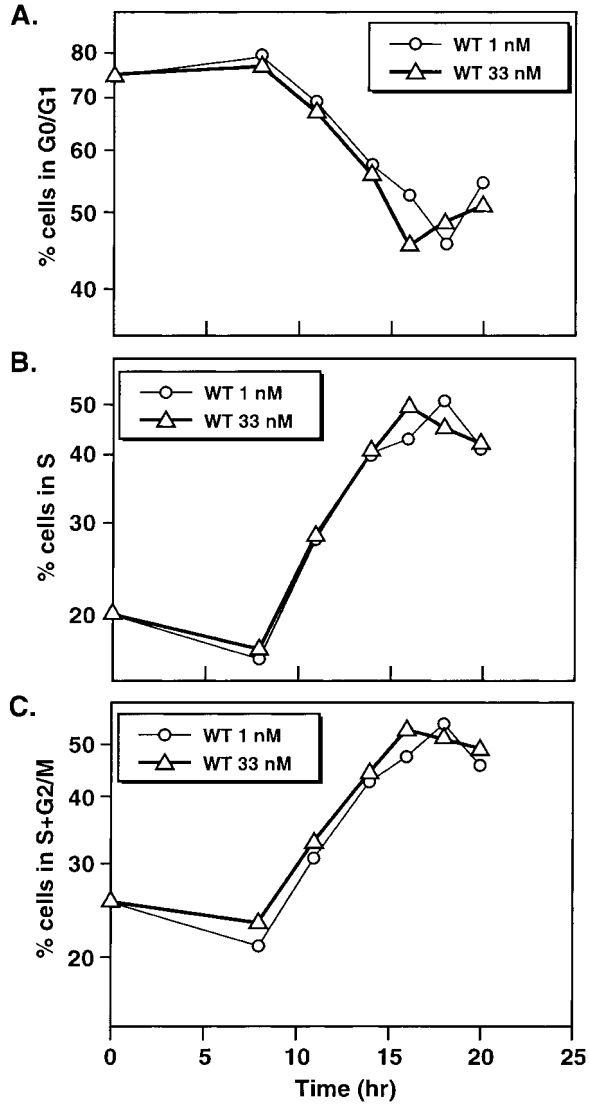


**Fig. 5.** Kinetic analysis of Balb/MK cell proliferation in response to saturating concentrations of wild-type hEGF: determination of lag, rate of entry into S-phase, and overall distribution of the subpopulation in S-phase. DNA content was monitored by flow cytometry and histograms were analyzed as described in Materials and Methods. The semi-logarithmic plots are representative of average data points from three independent experiments.

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. Also, the concentration mediating the threshold response is lower than 1 nM wild-type hEGF. Assuming the threshold response is proportional to receptor occupancy, these observations also indicate that spare receptors are available for interaction with 'excess' ligand present in the culture medium. Thus, both EGFR kinase stimulation

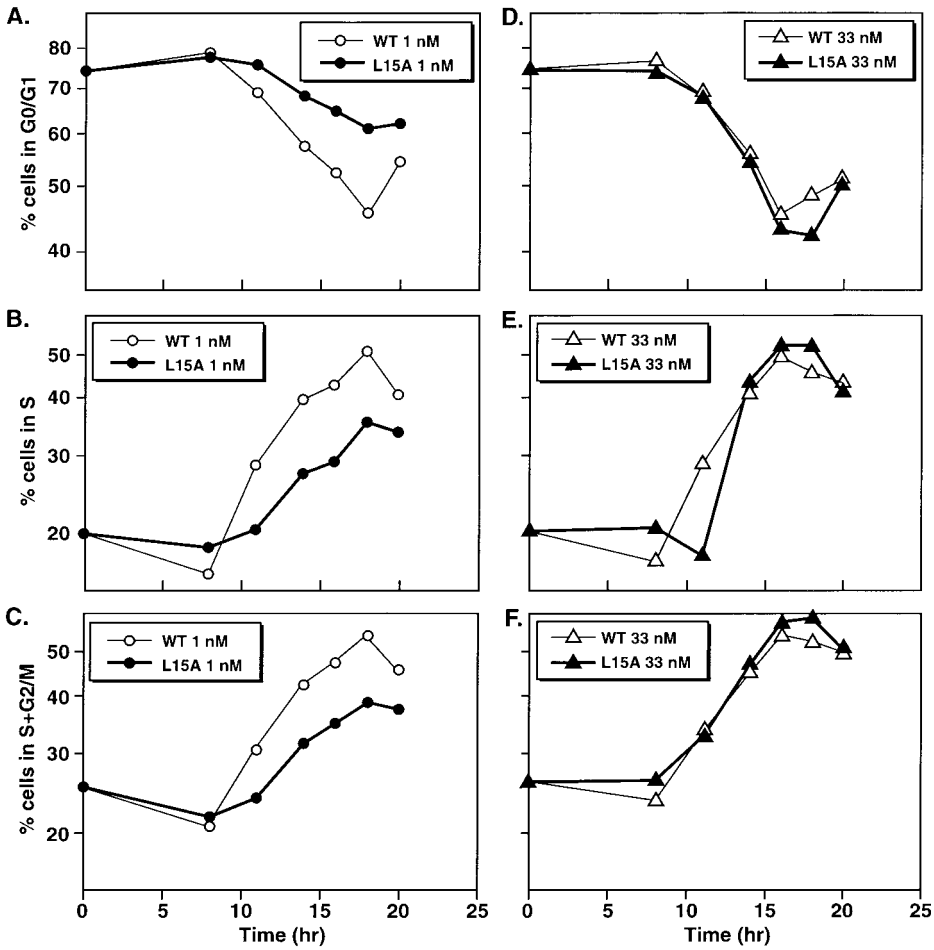


**Fig. 6.** Differential mitogenic response of Balb/MK cells to wild-type hEGF and L15A (panels **A, B, & C**): dependence of the rate of entry into S-phase and overall distribution of subpopulation in S-phase on attainment of thresholds. Data represent the average from three independent experiments. Dose-dependence of the threshold effect in EGF-mediated cell cycle progression (panels **D, E, & F**): functional evidence for amplification of threshold signals by a receptor reserve to stimulate the maximal mitogenic response. Data shown represent the average from three independent experiments.

(a short term response) and cell proliferation (a long term response) rely on the presence of a receptor reserve to transduce the stimulus due to EGF.

The transition of cells through the S phase is characterized by a progressive increase in cellular DNA content due to replicative DNA synthesis. Consequently the fluorescent signal increases in proportion to the PI bound stoichiometrically to DNA. The proliferating fraction, which consists of cells in S and G2/M, is depicted in Figure 5C. The distribution of the subpopulation in various stages of S phase is similar for both 1 and 33 nM wild-type stimulated curves (Fig. 5B), 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 consistent with the observations of van Hooijdonk et al. [1993].

The graphs in (Figure 6A, B, and 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 (Fig. 6A). However, a larger number of wild-type stimulated cells enter S phase more rapidly than mutant stimulated cells (Fig. 6A and 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. This idea is consistent with the results presented earlier in (Fig. 2C and D). 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 (Fig. 6B). 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 (Fig. 6D, E, and F). 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 (Fig. 6D). 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 in Figure 6E and F indicates that at the 11 h interval, ~15% 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 difference are unclear and merit further study. The distribution of cells in transit through the cell cycle is otherwise similar for wild-type and L15A stimulated curves.

## DISCUSSION

This study addresses the link between reception and relay of mitogenic signals by the EGFR and shows that L15 of hEGF engages the receptor via cooperative interactions that are critical for mitogenic signal transduction. The cooperative binding of hEGF to the EGFR under the in vitro kinase (functional) assay conditions is consistent with the sedimentation analyses of Sherrill and Kyte [1996] who reported that the 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 [Lauffenburger and Linderman, 1993]. 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.

Kinase and chemical cross-linking data presented in Figure 1A and B strongly suggest that the apparent cooperativity between ligand binding and receptor activation is due to ligand-induced receptor dimerization. This interpretation is supported by previous reports showing the specific activity of monomeric EGFR as a kinase to be lower than that of the dimeric species [Canals, 1992; Sorokin et al., 1994]. The activity profiles of the L15A mutant suggest an obligatory requirement for allosteric conformational change(s) in addition to receptor dimerization, which is plausible both energetically and mechanistically [Bormann and Engelman, 1992; Gadella and Jovin, 1995]. Thus, EGFRs which pre-exist as oligomers [Gadella and Jovin, 1995], covalently-linked homodimers [Sorokin et al., 1994], or are co-expressed as heterodimers in intact cells [Qian et al., 1994], bind ligand and function as kinases only when they also undergo the necessary allosteric transition. One should note recent findings [Ferguson et al., 2000] indicating that extracellular domains drive homo- but not heterodimerization of receptors of the erbB family of which erbB1 (EGFR) is the prototype. This is in agreement with suggestions made by other groups [Gamett et al., 1997; Huang et al., 1998] and appears to contradict the simple heterodimerization model that seem to be dominant in the recent literature. The precise mechanism of ligand-induced erbB hetero-oligomerization needs further study.

Time-resolved fluorescence imaging microscopy has shown that the high-affinity EGFRs in intact cells are present in a pre-dimerized/oligomerized state and stereochemical reorientation may be necessary for receptor activation [Gadella and Jovin, 1995]. Studies of the stoichiometry of EGF-EGFR binding have shown that 1:1 [Weber et al., 1984] or 2:2 [Lemmon et al., 1997] complexes can be formed. Given that high-affinity receptors in intact cells pre-exist as dimers/oligomers [Gadella and Jovin, 1995], 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 the signal intensity dictates choice of cell fate in vivo [Katz et al., 1995; Lillien, 1995]. It has recently been shown that a dimerization mechanism enables growth hormone binding affinity for its receptor to surpass the requirements for cellular activity [Pearce et al., 1999]. However, that study does not implicate a receptor reserve as being essential for signaling. 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.

The present studies, indicating a receptor reserve for EGFR activation and its importance in mediating threshold responses to EGF stimulation, extend earlier observations showing potent SHC tyrosine phosphorylation in EGF-treated MK cells at low receptor density [Soler et al., 1994a]. MAP kinase activation in response to threshold ligand concentrations probably proceeds along this pathway. L15A hEGF is not a partial agonist for mitogenesis, although it is so for receptor autophosphorylation. How might this be possible? Interestingly, mutation of all the major autophosphorylation sites in the receptor (Y→F) does not abrogate the mitogenic response to wild-type EGF [Decker, 1993; Gotoh et al., 1994; Li et al., 1994]. Furthermore, there is no stringent requirement for any of the autophosphorylation sites in EGFR-mediated signaling [Soler et al., 1994b]. Assuming that receptor autophosphorylation 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*. On the other hand, the data of Zendegui et al. [1988] suggest that maximal and sustained cell proliferation of Balb/MK cells by wild-type hEGF can be regulated by only ~500 high-affinity EGFRs per cell!

Studies with fibroblasts have shown that spare receptors exist for the mitogenic response [Aharonov et al., 1978] and receptor internalization [Carpenter and Cohen, 1976]. EGFR upregulation under conditions of growth factor deprivation, overexpression in oncogenesis and vaccinia virus growth factor-EGFR signaling in host-parasite interaction [Carpenter and Wahl, 1990] are all illustrative of the adaptive cellular response which exploits a receptor reserve to ensure cell survival. It is therefore of considerable interest to investigate temporal changes in the receptor reserve in future experiments. That spare receptors are an important determinant of observed intrinsic activity could be examined by "receptor titration" using pharmacological agents that block function by irreversible binding to the receptor. Physiological controls may operate at the levels of EGFR protein half-life, mRNA stability and/or transcription [Carpenter and Wahl, 1990], thereby offering new targets for therapeutic intervention in disease with small-molecule antagonists of EGFR.

From Figures 5 and 6, the lag (first 8 h) remains the same for wild-type or L15A at the doses they were tested, suggesting that ligand depletion and receptor trafficking [Reddy et al., 1996] are not the only determinants of the observed effects. Furthermore, superior receptor trafficking was obtained by Reddy et al. [1996] with Y13G, a low-affinity EGF analog with a wild-type $V_{max}$ for receptor activation [Tadaki and Niyogi, 1993] unlike L15A which is a low-affinity *and* low-$V_{max}$ analog. We have observed with clonogenic assays (a measure of the reproductive integrity of cells), using a range of low to high affinity hEGF mutants [Nandagopal et al., 1999], that additional factors may be involved in controlling the kinetics (perhaps equally important as equilibrium parameters) of cell proliferation. We are not suggesting that endocytic trafficking processes are not important, rather, we are invoking an important role for the receptor reserve in augmenting cellular growth when faced with suboptimal growth stimulation.

It is important to point out what this manuscript is telling us that we did not know already:

(a) To our knowledge, we present (utilizing the partial agonist activity of L15A) the first direct biochemical evidence for a receptor reserve based on function, viz., receptor autophosphorylation/activation and receptor activity/substrate phosphorylation, that do not rely just on biological end functions, e.g., thymidine uptake.

(b) We confirm previous observations of others [Carpenter and Cohen, 1976; Aharonov et al., 1978] by assaying biological functions (Figs. 5 and 6) in physiologically relevant cells that have not been genetically engineered (e.g., fibroblasts, which are easy to manipulate experimentally but are physiologically irrelevant to EGF signaling) to overexpress the EGFR. Furthermore, we extend the idea to biochemical functions more proximal to EGF-EGFR signaling by documenting the disjunction/disparity/divergence between dose responses for EGF binding, EGFR dimerization and tyrosine kinase activation in vitro and in vivo. We also provide evidence for signal amplification in the transmission of biochemical and biological responses by L15A.

(c) Although the present studies were performed with L15A, these findings may be extended to other low-affinity/"low $V_{max}$" mutants of EGF [Matsunami et al., 1990, Matsunami et al., 1991] or low-affinity naturally occuring ligands such as amphiregulin [Neelam et al., 1998] to explain how they manage to transduce a biochemical/biological response in vivo even though their binding and agonist activities are weak.

(d) Amplification at the level of receptor dimerization (high-affinity binding/low kD; higher$V_{max}$/more efficient) may not be essential if spare receptors are present. Alternatively, receptors (including the so-called "spare" ones) undergoing dimerization in response to ligand binding may present the cell with a kinetic advantage due to cooperativity. This is important for a biological response which needs to be instantaneous- not a gradual/graded accumulation of events. Results presented in Figure 5 indicate that L15A is not able to drive the same number of cells at the same rate as wild-type towards DNA synthesis. L15A displays lack of cooperativity (Hill coefficient cannot even be determined) and is deficient in inducing EGFR dimerization (Fig. 1). In other words, *spare receptors seem essential for DNA synthesis / survival whereas dimerization is not!*

(e) Signal amplication may occur at the level of each enzyme involved sequentially in the signaling pathway, e.g., EGFR (Tyr kinase), Ras (GTPase), Raf (Ser/Thr kinase), and MAP (Ser/Thr kinase). It may also occur at the level of differential recruitment of adapter proteins (Shc vs. Grb2) [Soler et al., 1994b] or at the level of heterodimerization (unpublished results). The subject merits further investigation.

## ADDENDUM

After this manuscript was completed, a report [Verveer et al., 2000] appeared that provided evidence (based on quantitative imaging of protein reaction states in cells) for rapid and extensive ligand-independent lateral propagation of erb1 receptor (EGFR) phosphorylation over the entire cell after focal stimulation with EGF. The production of this signaling wave at the plasma membrane leads to the full activation of all erbB receptors. If this finding is confirmed, one could perhaps speculate that spare receptors may act as integral components in this novel mechanism of signal transduction by EGF. This, of course, remains to be seen.

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