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# Internalization and Processing of the EGF Receptor in the Induction of DNA Synthesis in Cultured Fibroblasts: The Endocytic Activation Hypothesis

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The addition of EGF to cultured murine 3T3 cells produces a decrease in EGF binding activity with concomitant internalization and degradation of the initially bound EGF. When the EGF receptor on cultured 3T3 cells is affinity labeled with high specific activity $^{125}$I-EGF, and the fate of the affinity labeled EGF-receptor complex determined, the loss in binding activity was accounted for by receptor internalization and subsequent proteolytic processing of the EGF receptor molecules in the lysosomes. Studies of the effects of EGF concentration on EGF binding by cells, EGF-induced receptor internalization and EGF-induced stimulation of $^{3}$H-thymidine uptake into cellular DNA show that there is a direct correlation between EGF-induced receptor internalization and EGF-induced stimulation of DNA synthesis, but not between EGF binding and EGF-induced stimulation of DNA synthesis. This correlation is lost at high EGF concentrations, where stimulation of DNA synthesis is suboptimal. Optimal stimulation of DNA synthesis requires a minimum of 6 h of incubation of EGF with cells, and the suboptimal stimulation of DNA synthesis at high EGF concentration is intensified when the period of incubation of EGF with cells is less than 6 h. These data are consistent with a model of hormone signal transmission by Endocytic Activation, wherein the activation of EGF-induced processes requires constant EGF-induced internalization of receptor for a requisite 6–8 h period as an obligatory step in production of "second messenger" in the action of this hormone.

Epidermal growth factor (EGF) is one of an expanding class of characterized polypeptide hormones that effectively stimulate DNA synthesis when added to quiescent, but responsive, cultured cells [1–4]. In the initial purification of murine EGF by Cohen, EGF activity was assayed for its ability to induce premature incisor eruption and eye opening in mice [5]. Human EGF was also purified by Carpenter and Cohen [6], and a strikingly similar peptide was purified independently by Gregory and his colleagues from human urine and termed urogastrone, since the latter workers followed their purification by assay-

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ing for inhibition of gastric acid secretion [7]. Murine EGF and human urogastrone have extensive homology [7], and human EGF and urogastrone are probably identical [8]; human urogastrone and murine EGF both induce premature eye opening in mice, and both inhibit gastric acid secretion [8]. In addition to its ability to induce short range effects in cultured cells, such as DNA synthesis, EGF substantially extended the cellular reproductive range of cultured human epidermal keratinocytes from 50 to 150 generations [9]. In spite of this impressive array of biological responses, the mechanism(s) by which EGF stimulates DNA synthesis and mitogenesis in cultured cells or any other of its phenotypic responses remains obscure.

Our initial interest in EGF stemmed both from these intriguing biological properties and from the sensitivity of the binding assay for $^{125}$I-EGF peptides [10, 11]. When radioactively labeled murine EGF is incubated with cultured murine cells at concentrations 10- to 50-fold times the $K_d$, over 90% of the cell-associated radioactivity is specifically bound to what Scatchard analysis reveals to be a single species of receptor in both human [10] and murine [12] fibroblasts. Our involvement in structural chemical crosslinking studies [13–16] had led T. Miyakawa of this laboratory to synthesize a heterobifunctional, photoactive crosslinking reagent. The highly specific properties of the EGF binding assay and the directly assessable biological effects of EGF on cells made the EGF system the one of choice for initial application of this heterobifunctional crosslinking reagent to receptor identification [12]. In this paper, we briefly review the methodology employed for affinity labeling of the EGF receptor and describe the biological fate of the affinity labeled receptor in cultured murine 3T3 cells. The binding capacity of cultured cells for many hormones, eg, insulin [17], human growth hormone [18], and epidermal growth factor [8], decreases when the cells are incubated with the hormone. The term "down regulation" has been coined to describe this general phenomenon, which could occur by any of a number of mechanisms. Our studies show that the receptor is internalized in response to EGF by cultured cells and degraded in the lysosomes [19]. Furthermore, the EGF-induced steady state level of receptor internalization and DNA synthesis are half maximal at the same low EGF level, a concentration at which the EGF receptor is approximately ten percent occupied.

## MATERIALS AND METHODS

Swiss mouse 3T3 cells derived from clone 42 and obtained from George Todaro were used exclusively in these studies. These were routinely grown in Dulbecco modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and used on or before the 8th passage from our frozen stock. Cells were maintained in this medium with FCS adjusted to 5% for at least 3 cell doublings prior to assays for EGF binding, EGF-induced DNA synthesis, down regulation and affinity labeling [12, 19]. The procedures for isolation of EGF from mouse submaxillary glands [5], radioiodination of EGF [12], assay for EGF binding, and EGF-induced down regulation of DNA synthesis [19] have been described.

## RESULTS AND DISCUSSION

**Identification of the EGF receptor.** A few years ago, Tokichi Miyakawa had synthesized a heterobifunctional probe, which we had initially intended to use in structural crosslinking studies to determine nearest neighbor protein interactions in membranes. At that time, we had been utilizing "reversible" crosslinking reagents, such as bis-dithiopropionimidate (DTBP) and two-dimensional gel electrophoresis with reducing reagent

treatment prior to electrophoresis in the second dimension, to identify both the crosslinked oligomers and their monomeric progenitors in membranes of paramyxoviruses and cultured murine cells [13–16]. Reagents like DTBP have a number of limitations, the foremost of which is the limitation in reaction specificity imposed by the site-specific nature of the reactive group [16, 20]. For this reason, we synthesized a reagent that contained a site specific group at one terminus, but an aryl azido group at the other; this was assigned the acronym PAPDIP. Aryl azido groups are generally quite unreactive when maintained in light-free conditions, but when illuninated with the appropriate wave length of light, they are converted to highly reactive nitrenes. Though nitrenes do exhibit selective reactivity under appropriate conditions, they have a broad repertoire of reactivity, eg, with hydrocarbons by extraction by hydrogen. We felt that this property would considerably extend the range of crosslinked products. PAPDIP also contains a readily cleavable disulfide bridge, since it was initially synthesized for use in structural crosslinking, rather than as a reagent for affinity labeling of receptors.

Once this reagent was at hand, its possible application to affinity labeling of receptors became too appealing an opportunity to ignore. A colleague at UCLA, Harvey Herschman, and his postdoctoral associate, Aaron Aharanov, were at this time engaged in studies on modulation of EGF binding in murine 3T3 cells. This set the stage for a collaboration that led in short order to the specific labeling of the EGF receptor in murine 3T3 cells [12]. The EGF system was a most fortunate choice. The specific binding assay for EGF is excellent. Under the normal conditions of the binding assay, over 90% of the cell-associated EGF is bound to receptor. Also, the amino terminus of EGF can be reacted with a broad range of amino site-specific reagents with no loss of binding activity; nor is there a change in the dissociation constant. Not all hormone receptor systems share these two essential properties. Insulin, for example, binds with excellent specificity to its receptor but is readily inactivated by a variety of site-specific reagents; fibroblast growth factor (FGF) is not so readily inactivated by amino site-specific reagents, but it binds relatively non-specifically to cells. Additionally, EGF can be labeled at high specific activity with carrier-free radioiodine with no change in its biological activity, and EGF receptors are present on the surface at a density of approximately 100,000 binding sites per cell. This amount of receptor does not encourage attempts at direct isolation; approximately 1 mg of receptor protein is present on cells grown to confluence on 10,000 dishes of 10-cm diameter. However, 100,000 sites per cell provides a number that is more than adequate for affinity labeling studies.

Figure 1 describes the experimental procedures that have been used to affinity label the EGF receptor. Affinity labeling can be achieved not only with PAPDIP-EGF, but also with NAPEDE-EGF, formed with a reagent that reacts site-specifically with carboxyl groups on EGF. In both cases, the aryl azido EGF derivatives are first interacted with cells or isolated membranes to permit binding of EGF to its receptor. With cells, this is usually done at low temperature to prevent the metabolism of the probe and, as it also turns out, the receptor. Once binding equilibrium is achieved, the sample is subjected to photolysis resulting in the covalent addition of a portion of the specifically bound EGF to a single cell surface protein. In practice, the photoaffinity labeled receptor is visualized by first solubilizing the cellular proteins in detergent solution and then subjecting the proteins to gel electrophoresis followed by autoradiography.

**Evidence that the affinity labeled, crosslinked, complex is an EGF receptor complex.** The kinetic parameters that are determinants in the formation of the crosslinked complex of EGF with a high molecular weight surface protein all indicate that complex formation

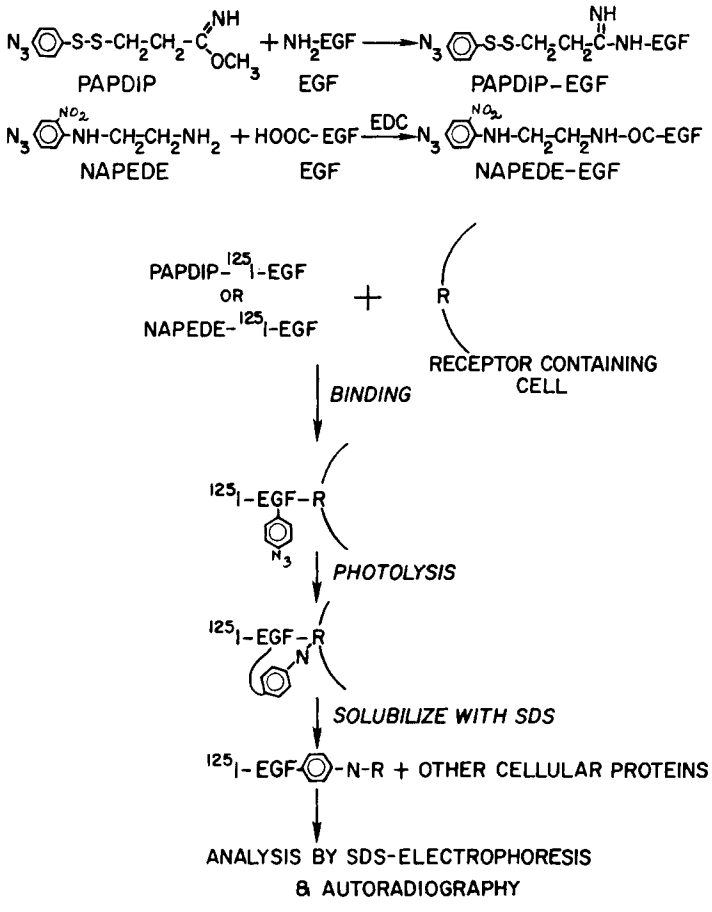



Fig. 1. Procedures for affinity labeling of EGF receptor. Two photoaffinity reagents have been used to covalently affix EGF to its receptor, a cell surface protein of approximately 170,000 daltons. A reagent, assigned the acronym PAPDIP, reacts site specifically with the terminal amino group of EGF. NAPEDE attaches site specifically to one or more of the carboxyl residues on EGF in a reaction driven by a water soluble carbodiimide (EDC). Once PAPDIP or NAPEDE is affixed to $^{125}$I-EGF, the radiolabeled photoaffinity probe is incubated with cells, usually at low temperature to prevent metabolism of receptor during this initial binding period. The sample is then subjected to photolysis to convert the aryl azido group to the highly reactive nitrene.

**TABLE I. Evidence Which Indicates That the Crosslinked Complex is an EGF-Receptor Complex**

1. It is specific for a single membrane protein.
2. No crosslinking is observed with a non-binding, non-responding variant clone of 3T3.
3. The amount of crosslinked complex formation and specific binding decrease in parallel when unlabeled EGF is added as a competitor.
4. Under a variety of other conditions, a direct proportionality exists between binding activity and crosslinked complex formation.

From the experiments of Das et al [12].

and specific binding enjoy a direct proportionality (Table I). While these data show that formation of the crosslinked complex requires specific binding, they do not exclude the possibility, however slight, that the crosslinked complex consists of EGF plus some protein that is not the EGF receptor. Experiments summarized later in this article show that the metabolism of the crosslinked complex and EGF receptor are tightly coupled; these indicate that the affinity labeled macromolecule is the EGF receptor, but do not exclude the possibility that EGF becomes crosslinked to a protein other than the EGF binding portion of an oligomeric EGF receptor. Recently, we found that a small fraction of underivatized EGF becomes so tightly associated with receptor that it resists dissociation in boiling sodium dodecyl sulfate solution [21]. Since this product has a molecular weight identical to that of the crosslinked complex, we now feel reasonably certain that this high molecular weight membrane protein component of the crosslinked complex is the true EGF receptor.

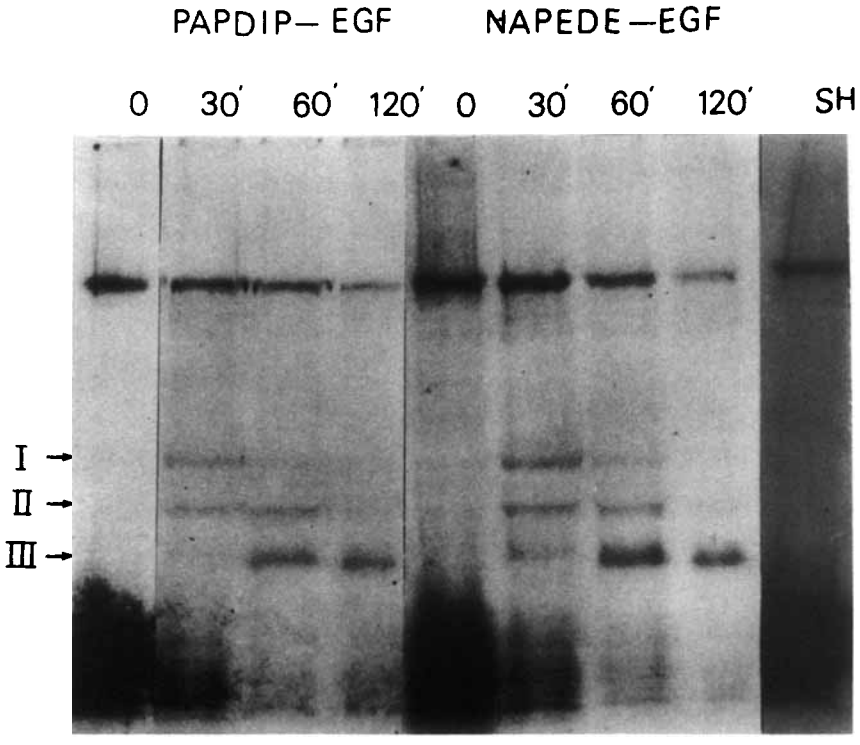



Fig. 2. Visualization of radiolabeled receptor and its degradation products by autoradiography of 5–20% polyacrylamide gradient gels. (PAPDIP-EGF). Degradation of the receptor radiolabeled with PAPDIP-$^{125}$I-EGF. Confluent monolayers of 3T3 cells in 35-mm plastic dishes were incubated with 60 ng of PAPDIP-$^{125}$I-EGF ($2 \times 10^5$ cpm/ng) at 23° for 10 min in the dark. The monolayers were then washed six times in the dark. After photolysis for 5 min at 4°, the cells were incubated at 37° for 0, 30, 60, 120 min. The cells were solubilized with 70 μl of 0.1 M Tris-HCl, pH 6.8/3% sodium dodecyl sulfate/0.6% N-ethylmaleimide. Electrophoresis was done under nonreducing conditions. (NAPEDE-EGF). Degradation of the receptor radiolabeled with NAPEDE-$^{125}$I-EGF ($2 \times 10^5$ cpm/ng) was followed as described above for PAPDIP-$^{125}$I-EGF. Cells were incubated at 37° for 0, 30, 60, and 120 min and then solubilized and subjected to gel electrophoresis under nonreducing conditions. (-SH). Molecular weight of the receptor in reducing sodium dodecyl sulfate gels. Cells were radiolabeled with NAPEDE-$^{125}$I-EGF as described for lane E, and electrophoresis was done after treatment with 5% 2-mercaptoethanol. From Das and Fox [12].

**Processing of the EGF receptor.** When labeled EGF is incubated with human cells at 37°, it first binds to receptor and is then rapidly degraded and released as iodotyrosine [8]. Since this behavior is paralleled by a loss of EGF binding activity when unlabeled EGF is incubated with cells, Carpenter and Cohen concluded that EGF and, by inference, the EGF receptor are internalized and subsequently degraded by cells. Our ability to affinity label the EGF receptor provided us with a means to test directly for the behavior of receptor upon EGF binding. Figure 2 describes experiments in which photoaffinity EGF probes were first bound to cells at low temperature to prevent receptor and EGF metabolism, and then photolyzed at low temperature prior to a temperature upshift to 37°C. After the temperature upshift, the radioactivity initially present in the EGF-receptor complex appears in three lower molecular weight processing products, termed Bands I, II, and III, in order of decreasing molecular weight. The rate and pattern of appearance of these products is highly cold sensitive. Lowering the temperature of incubation from 37°, to 31°, to 27°, or 23° results in a delay in the appearance of all three bands and in the failure of formation of Bands II and III at the lowest temperatures (Fig. 3). The processing pattern proceeds independently of the probe employed (PAPDIP-EGF vs. NAPEDE-EGF). Treatment of the EGF-receptor complex formed upon binding and photolysis of NAPEDE-EGF with 2-mercaptoethanol results in no apparent change in the electrophoretic mobility of the complex, indicating that the receptor is a single, high molecular weight polypeptide (Fig. 2).

**Receptor down regulation as assessed by loss of EGF binding activity from cells is equivalent to internalization and degradation of the EGF receptor.** Table II summarizes experiments that show that the internalization and proteolytic processing of the covalent

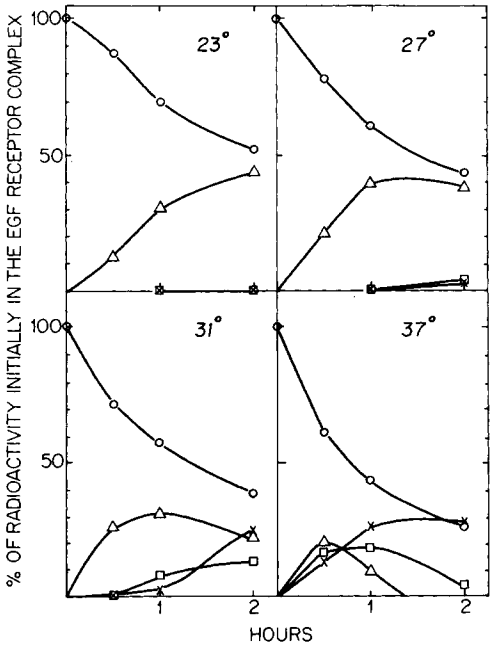



Fig. 3. Rate of degradation of radiolabeled receptor at 23°, 27°, 31°, and 37°. The relative amounts of radioactivity in the radiolabeled receptor band (○) and in bands I (△), II (□), and III (×) were estimated by densitometric analysis of the autoradiograms. The experimental procedures were those used for PAPDIP-EGF in Figure 2.

**TABLE II. Relationship Between Degradation of Radiolabeled Receptor and Loss of Hormone Binding Activity**

1. Receptor not covalently linked to EGF undergoes degradation and the fragments (I + II + III) bind and react with internalized PAPDIP-EGF.
2. High concentrations of native unlabeled EGF (causing greater than 80% receptor site occupancy) markedly enhance the rate of degradation of radiolabeled receptor.
3. The kinetics of degradation of radiolabeled receptor are identical to the kinetics of loss of receptor from the cell surface as measured by reduction of EGF binding activity.
4. EGF and PAPDIP-EGF are identical in their receptor binding activities, abilities to "down regulate" receptors, and abilities to stimulate DNA synthesis.

From the experiments of Das and Fox [19].

**TABLE III. Down Regulation of Receptor Activity and Stimulation of [$^3$H]-Thymidine Incorporation Into DNA by EGF and PAPDIP-EGF**

A) Hormone-induced loss of receptor activity (% total specific binding activity lost)

| Hormone concentration (nM) | 0.005 | 0.167 | 0.5 | 5 |
|---|---|---|---|---|
| PAPDIP-EGF | 34 | 47 | 55 | 79 |
| EGF | 30 | 43 | 58 | 78 |

B) [$^3$H]-thymidine incorporated into DNA (cpm)

| Hormone concentration (nM) | 0.033 | 0.1 | 0.3 | 0.9 | 9 |
|---|---|---|---|---|---|
| PAPDIP-EGF | 4,600 | 14,700 | 19,900 | 24,700 | 17,500 |
| EGF | 4,840 | 17,500 | 20,900 | 24,600 | 18,100 |

From Das and Fox [19].

EGF-receptor complex mimics the behavior of the normally formed EGF-receptor complex. First, both EGF and PAPDIP-EGF have identical biological properties. Receptor internalization and mitogenic stimulation occurring in response to EGF or PAPDIP-EGF are indistinguishable (Table III). Figure 4 describes an experiment in which the photolysis was not conducted until after receptor internalization and processing to lower molecular weight products had occurred. This experiment shows that the processing products which form in response to EGF are identical to those that form when the non-covalent EGF-receptor covalent complex is internalized (Fig. 2). This experiment also shows that the lower molecular weight processing products retain EGF binding capacity. Figure 5 describes an experiment in which the rate of internalization of the EGF-receptor covalent complex was compared with the rate of loss of EGF binding capacity by cells; these two phenomena are essentially indistinguishable. Figure 6 describes the result of an experiment in which the covalent complex was first formed by photolysis and the excess PAPDIP-EGF removed. The rate of EGF-receptor covalent complex degradation was then determined in the presence and absence of added EGF. This experiment shows that unlabeled EGF stimulated the internalization of the covalent complex, a further indication that the internalization and processing behavior of the complex is that of the normally formed EGF-receptor complex.

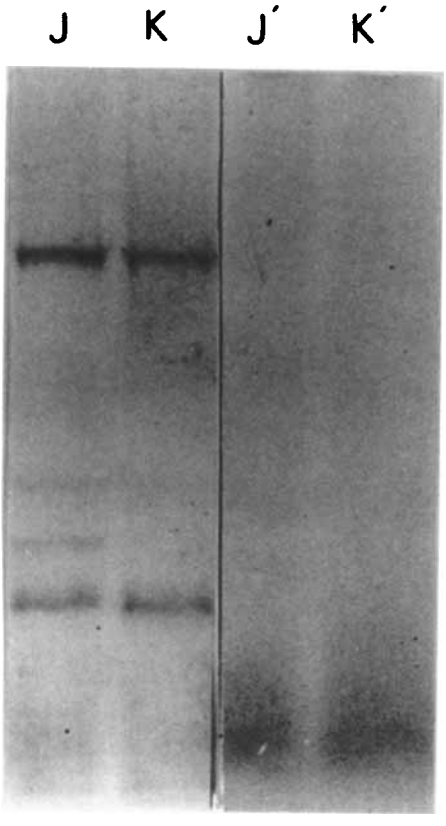



Fig. 4. Degradation of receptor not covalently linked to EGF. (Lanes J and K). Radiolabeling experiments with PAPDIP-$^{125}$I-EGF were performed under conditions identical to those described in the legend for Figure 2, except that the cells were first incubated in the dark at 37° for (J) 60 or (K) 120 min and then photolyzed at low temperature and processed immediately for gel electrophoretic analysis. (Lanes J′ and K′). Binding of PAPDIP-$^{125}$I-EGF to cells and incubation at 37° for (J′) 60 and (K′) 120 min were performed under the same conditions as those used for J and K, except that the photolysis step was omitted. From Das and Fox [19].

**Receptor processing occurs in the lysosomes.** The degradation of cell associated EGF is inhibited by chloroquine, a lysosomotropic drug that inhibits the proteolytic action of lysosomes [8]. Figure 7 describes the isopycnic banding profiles of lysosomal and plasmalemmal enzyme markers and shows that the EGF-receptor complex fractionates with the plasmalemmal fraction, and that Bands I, II, and III, the proteolytic processing products, with the lysosomes. The lysosomal processing of the EGF-receptor covalent complex is inhibited by chloroquine (Fig. 8), another indication of the role of lysosomal proteases in the processing of the EGF receptor. The metabolic fate of the EGF receptor upon EGF addition is summarized schematically in Figure 9. Upon addition of EGF (1), the receptor is internalized (2), probably in a pinocytic step (3). The internalized vesicles then either fuse with lysosomes or mature to form primary lysosomes (4) in which the receptor molecules are degraded to the products designated Bands I, II, III, and R′ (5). We have detected the formation of a fourth processing product, here designated R′. R′accounts

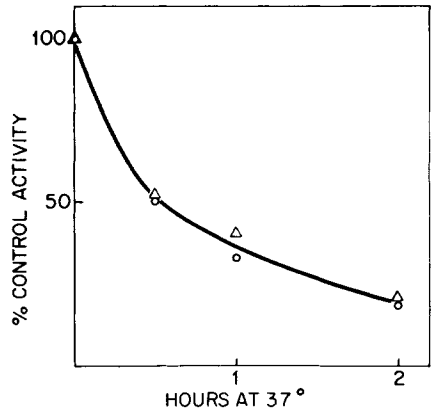



Fig. 5. Relationship between radiolabeled receptor degradation and EGF-induced loss of EGF binding activity. 3T3 cells grown to confluence were incubated for 60 min at 23° in the dark with 30 ng of either PAPDIP-$^{125}$I-EGF or native unlabeled EGF. Each monolayer was then washed 6 times and photolyzed, and the cells were incubated at 37° for the indicated periods of time with 1 ml of a solution containing 30 ng of native unlabeled EGF per ml. The medium was renewed after every 30 min of incubation. After incubation, the cells that had been exposed to PAPDIP-$^{125}$I-EGF were solubilized and subjected to electrophoresis and autoradiography. Radioactivity present in the radiolabeled receptor band (○) was determined by densitometric analysis of the band of $M_r$ 190,000. The cells that had been incubated at 23° with native unlabeled EGF were washed 6 times, incubated with 60 ng of $^{125}$I-EGF ($3.5 \times 10^5$ cpm/ng) at 23° for 80 min, and tested for EGF-binding activity (△). From Das and Fox [19].

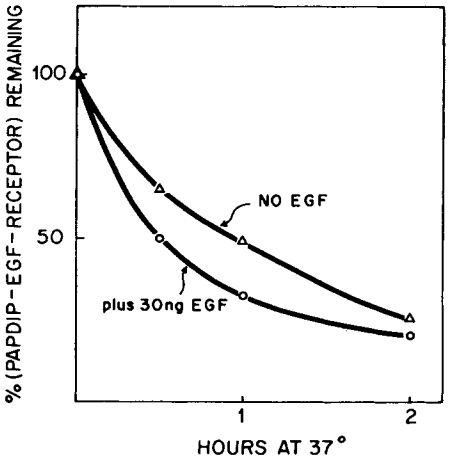



Fig. 6. Degradation of the radiolabeled EGF-receptor covalent complex is enhanced by the addition of unlabeled EGF. The PAPDIP-$^{125}$I-EGF covalent complex was formed, and the cells washed to remove PAPDIP-EGF from the incubation medium, as described in Figure 2. The cells were then incubated at 37° in 1 ml of balanced salt solution/HEPES/bovine serum albumin [19] with or without 30 ng of unlabeled EGF. At the times indicated, samples were processed for gel electrophoresis and radioautography as described by Das and Fox [19], the regions on the gels corresponding to the EGF-receptor covalent complex were cut from the gels, and the radioactivity determined by scintillation counting.

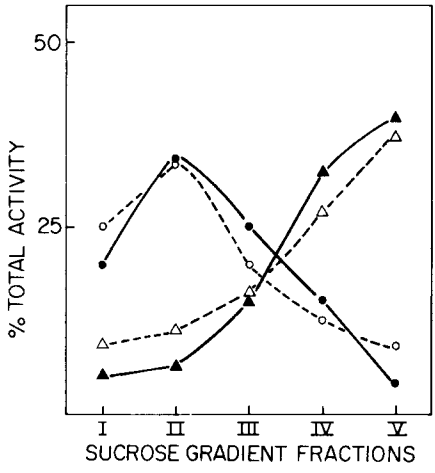
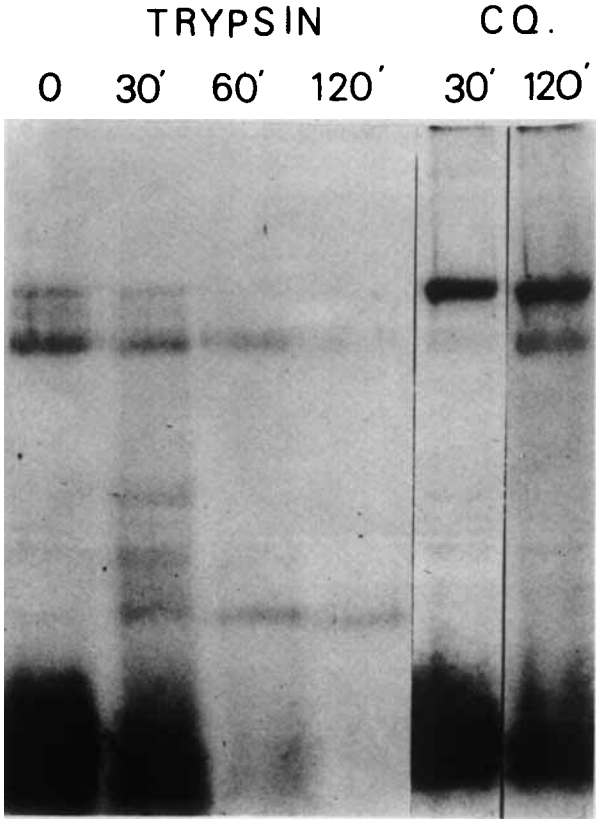



Fig. 7. Subcellular distribution of radiolabeled receptor and its degradation products. Cell monolayers were radiolabeled with PAPDIP-$^{125}$I-EGF at low temperature, incubated at 37° for 45 min and subjected to subcellular fractionation. The isolated fractions were tested for activity of phosphodiesterase I (○), a plasma membrane marker, and N-acetyl-β-D-glucosaminidase (△), a lysosomal marker, and were analyzed by electrophoresis and autoradiography. Radioactivity present in the radiolabeled receptor band ($M_r$ 190,000) (●) and in all three degradation products (bands I + II + III) (▲) was estimated by densitometric analysis of the autoradiograms. From Das and Fox [19].

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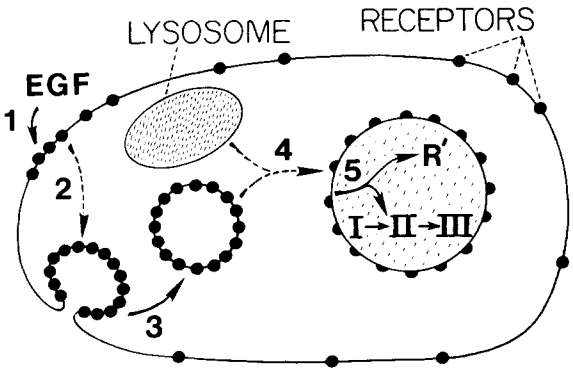


Fig. 9. Fate of the EGF receptor following EGF binding. After binding, EGF-induced internalization of the EGF and receptor-associated EGF are transported inside the cell, probably by an endocytic process (step 3). This event may require a prior patching of the EGF receptors (step 2). The internalized receptor covalently tagged with radioactive EGF bands with the lysosomal fraction upon centrifugation to equilibrium in sucrose density gradients. The intracellular radioactivity is then found not in the receptor, but almost entirely in four major proteolytic processing products (I, II, III, and R'). Products I, II, and III all form and turn over rapidly, whereas R', a polypeptide of approximately 80,000 daltons, accumulates slowly, but does not appear to turn over rapidly. The proteolysis of receptor yielding products I, II, and III is inhibited by chloroquine (Fig. 8), an inhibitor of lysosomal protease action.

for approximately 10% of the total processed receptor, has an apparent molecular weight of approximately 80,000, and does not appear to be further degraded to give rise to I, II, or III.

**Receptor internalization and induction of DNA synthesis have similar EGF requirements.** The evidence presented in the preceding sections shows that EGF-induced loss of EGF binding activity in cells proceeds by internalization and proteolytic digestion of EGF receptors. The rate and extent of receptor internalization, as indicated by the loss of EGF binding capacity, depends upon EGF concentration (Fig. 10). Increases in EGF concentration decrease the steady state level of receptor display; receptor internalization is a saturable process with respect to EGF concentration. An apparent lag in receptor internalization is evident at the lower EGF concentrations; this is overcome at high EGF concentration. This lag is due at least in part to the display of newly synthesized receptor molecules when the experiment is done as described (Fig. 10). It is less evident when cells are not incubated in medium for a duration of an hour or more after removal of unlabeled EGF prior to the binding assay [22].

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Fig. 8. Susceptibility of the radiolabeled receptor to extracellularly added protease and the effects of a lysosomal protease inhibitor on receptor degradation. (Trypsin). Trypsin treatment of intact cells containing radiolabeled receptor and its degradation products. Monolayers radiolabeled with PAPDIP-$^{125}$I-EGF and incubated at 37° for 0, 30, 60, and 120 min, as described for Figure 2, were treated for 30 min at 4° with 25 μg of trypsin. Cell-bound radioactivity was analyzed by gel electrophoresis under nonreducing conditions. (Chloroquine). Effect of chloroquine on receptor degradation. Cells were radiolabeled with PAPDIP-$^{125}$I-EGF and incubated at 37° for 30 and 120 min; 0.1 mM chloroquine was present during photolysis and incubation at 37°. From Das and Fox [19].

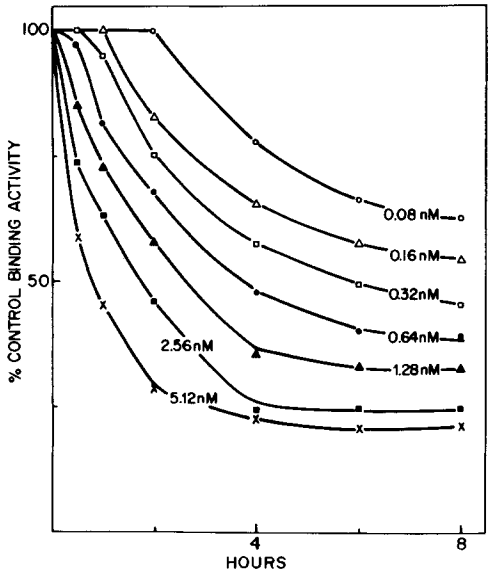


Fig. 10. Effects of EGF concentration on receptor internalization. Monolayers of 3T3 cells were incubated at 37° for 4 h. The washed cells were incubated with 50 ng of $^{125}$I-EGF (2 X $10^5$ cpm/ng) at 23° for 60 min and tested for EGF-binding activity. From Das and Fox [19].

Figure 11 describes three experiments done in parallel in which the concentration dependence of EGF binding, EGF-induced receptor internalization and EGF-induced stimulation of DNA synthesis were compared. EGF-induced receptor internalization and DNA synthesis are both half maximal at approximately 0.1 nM EGF, a concentration at which receptor occupancy is only 10%. When these data are plotted according to Lineweaver and Burke, the points representing EGF-induced receptor internalization and stimulation of DNA synthesis fall on the same curve. This data treatment yields information consistent with the possibility that EGF-induced receptor internalization and stimulation of DNA synthesis have the same limiting step, and moreover, with the possibility that receptor internalization is itself obligatory for cell cycle events which lead to stimulation of DNA synthesis.

The experiment described in Figure 12 shows that there is no simple proportionality between maximal rate or extent of decrease in EGF binding (receptor internalization) and stimulation of DNA synthesis. The cell cultures used for the experiment in Figure 12 were all treated with the stated concentrations of EGF at time zero. At various times during the next 24 h, the medium was removed from certain groups of the cell cultures, the cells were thoroughly washed four times with medium to remove the vestiges of dissociable EGF, and the cells were then incubated in fresh medium. Under these conditions, the bulk of the EGF associated with the cells after three h of incubation is nondissociable (data not shown). In all cases, 24 h had elapsed between the time of initial addition of EGF and the time of addition of $^3$H-thymidine to measure DNA synthesis during a 1-h pulse. When cells were incubated with EGF concentrations ranging from 0.053 to 4.3 nM for either 6 h or the full 24 h prior to assessment of the rate of $^3$H-thymidine uptake into DNA, the expected dependence on EGF concentration was observed at the lower EGF concentrations only,

ie, through 0.5 nM, 5-fold the concentration required for half-maximal stimulation of $^3$H-thymidine uptake into DNA. At concentrations of 1.4 nM and greater, stimulation of DNA synthesis was suboptimal. Inspection of Figure 11 reveals similar behavior; we have, in fact, consistently observed suboptimal stimulation of DNA synthesis at high EGF concentrations,

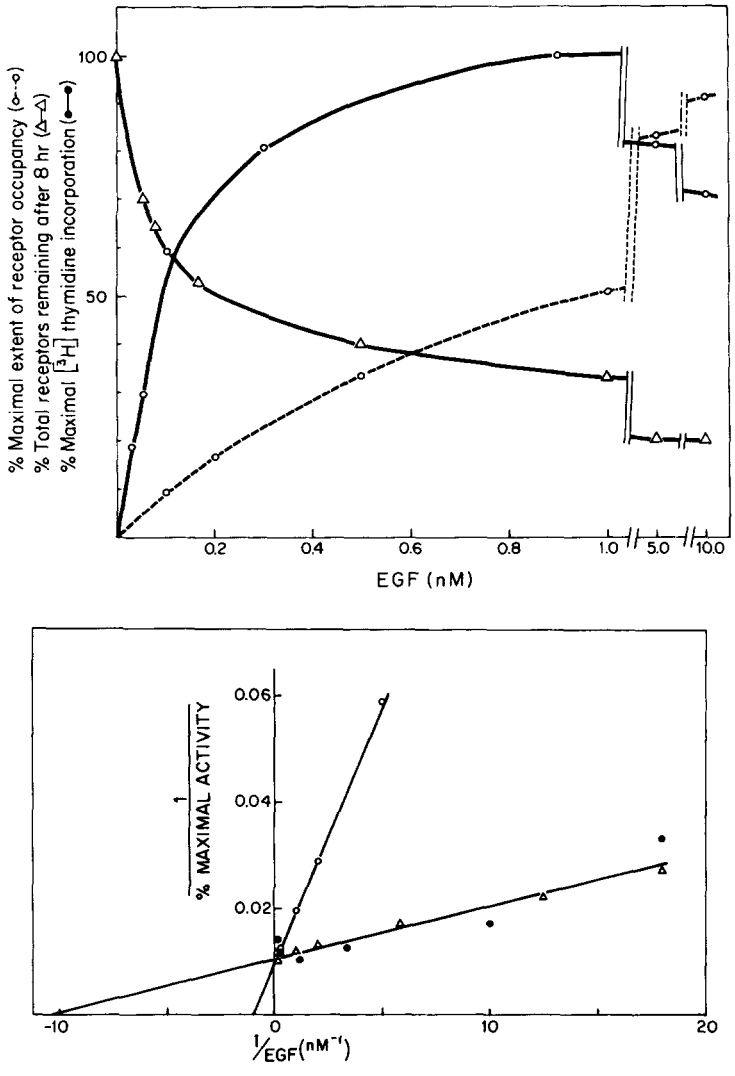


Fig. 11. Effects of EGF concentration on EGF specific binding, EGF-induced internalization of the EGF receptor, and increased rate of incorporation of $^3$H-thymidine into DNA. Top: Swiss mouse 3T3 cells were grown to saturation density in medium containing 5% calf serum. A portion of the cultures was used for determination of receptor occupancy as a function of EGF concentration (open circles, dashed line). Other portions were used to determine EGF induced receptor internalization 8 h after EGF addition (triangles) or stimulation of $^3$H-thymidine incorporation into DNA during a one-h pulse labeling 24 h after EGF addition (open circles, solid line). All points are the mean values from six independent determinations. Bottom: The data from Figure 11 (top) have been replotted by the method of Lineweaver and Burke. Receptor accupancy (○). Receptors remaining after incubation of cells with EGF (△). Thymidine incorporation into DNA (●).

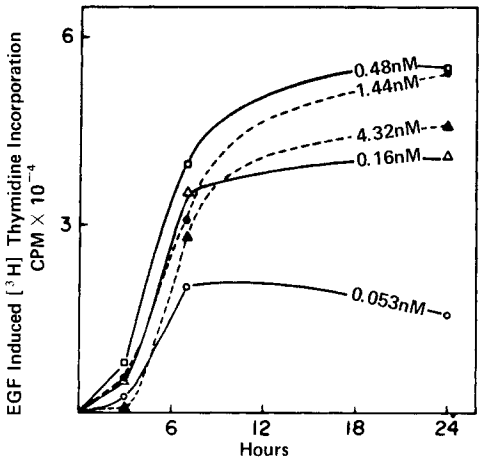


Fig. 12. Effects of EGF concentration and time of exposure of cells to EGF on EGF-induced stimulation of thymidine incorporation into DNA. Two ml of Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum and EGF at 0.053, 0.16, 0.48, 1.44, or 4.32 nM was added at time 0 to quiescent cultures of 3T3 cells in 35-mm dishes at 37°. At the times indicated, the medium was removed and the cells washed 4 times with 3-ml portions of Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum (DMEM-5% FCS). Two ml of DMEM-5% FCS was added to each monolayer, and incubation was continued at 37°. Twenty-four h after EGF addition (time 0), $^3$H-thymidine was added, and the cells were incubated at 37° for 1 h. Incorporation of $^3$H-thymidine into DNA is expressed as counts per minute per monolayer and is corrected for the DNA synthesis in control samples, where EGF was deleted. Other procedural details are those described by Das and Fox [19]. The maximal stimulation of DNA synthesis was approximately five-fold. All points are the mean values from six independent determinations.

and similar findings have been reported by Chen et al [23]. One of the most dramatic examples of suboptimal stimulation of DNA synthesis at high EGF concentrations was observed when EGF was removed after three h of incubation (Fig. 12). Under this condition, no stimulation was observed at the highest concentration used. Four clear conclusions can be drawn from these data. First, optimal stimulation requires approximately 6 h of continuous incubation of cells with EGF. Second, the intensity of the signal issued in response to EGF is not simply a product of EGF concentration multiplied by the time of incubation with EGF. Third, at low to moderate EGF concentration, stimulation of DNA synthesis correlates with receptor internalization. Fourth, rapid initial receptor internalization at high EGF concentration leads to a suboptimal DNA synthesis response. While these data pose a mechanistic enigma, this can be resolved by the postulate that the stimulation of DNA synthesis induced by EGF occurs in response to the steady state level of EGF receptor internalization occurring throughout the requisite 6-h period of incubation with EGF. This steady state level of internalization is the sum of internalization of receptor initially present before EGF addition plus the internalization of receptor synthesized and displayed during the period of incubation with EGF. This concept is indicated by the inhibition of DNA synthesis encountered at high EGF concentration. Under this condition, the bulk of the receptor is internalized early during the period of incubation with EGF, and the only receptors available for internalization during the later portions of the requisite 6-h incubation period are the newly synthesized ones. This concept is also supported by the data ob-

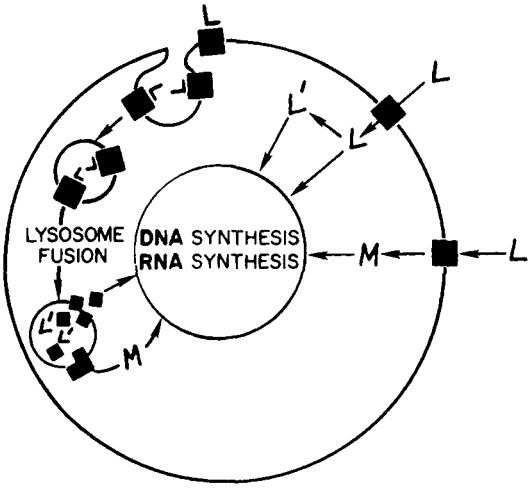


Fig. 13. Possible mechanisms for production of second messengers in EGF action. EGF could act to produce second messengers in at least three different ways. First, EGF could act on receptor, converting it or some associated cell surface membrane protein to a form which can produce second messengers by enzymic catalysis (the Receptor-Transducer Model). Second, EGF might be transported into the cytosol in a receptor-mediated reaction. The transported EGF, or a product derived from it, might then act directly as a second messenger (Receptor-Transducer-Internalization Model). These first two possibilities have been presented in greater detail by Steiner with reference to insulin action [24]. A third possible mechanism is the Endocytic Activation Model. Here, hormone action leads to internalization of both ligand and receptor. The receptor and/or other proteins internalized with receptor are then processed by lysosomal proteases leading to activation in one of two ways: 1) Products produced by proteolysis are converted from proenzymes to enzymes which in turn catalyze the production of second messengers. 2) The internalized and cleaved proteins are themselves the second messengers acting directly either in a lysosome-associated or lysosome dissociated form.

tained with samples incubated with EGF for a three-h pulse. At high EGF concentration, the receptor level is rapidly decreased to a low level (see Fig. 10). Once the medium is removed from the culture dish, the small amount of EGF remaining associated with the cells is not likely to be sufficient to produce further receptor internalization from the already depleted receptor pool. At lower EGF concentration, eg, 0.48 nM, the amount of EGF remaining bound to cells 3 h after EGF addition is similar to that bound at the higher concentrations, but the amount of receptor remaining and available for internalization is far greater. Thus, the chemical potential for receptor internalization at times after the wash step is greater at low to intermediate EGF concentration than at high EGF concentration.

Figure 13 summarizes the principal models for explaining the actions of hormones on cells. Two of these, the Receptor-Transducer Model and the Receptor-Transducer-Internalization Model, have been discussed thoroughly by Steiner [24]. Our data with the EGF system indicate a third model, Endocytic Activation, wherein the ligand functions to induce internalization of the receptor, rather than to activate second messenger production directly. This model predicts that the receptor, and possibly the hormone-receptor complex, is internalized and then activated by proteolytic processing to function as an enzyme in second messenger production or to liberate an active fragment which itself functions as the second messenger.

## ACKNOWLEDGMENTS

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