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Molecular mechanism of mitogen action: Processing of receptor induced by epidermal growth factor

(radiolabeled hormone receptors/down regulation of hormone receptors/receptor internalization/DNA synthesis/mitogenic hormone)

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ABSTRACT An affinity labeling technique used previously for identification of a membrane receptor for epidermal growth factor (EGF) was exploited to investigate the physiological fate of receptor after binding of EGF. Incubation of affinity-labeled cells at 37° resulted in a time-dependent loss of radioactivity from the EGF–receptor covalent complex (M_r 190,000). Ninety percent of the radioactivity lost from the band of M_r 190,000 during a 1-hr incubation at 37° appeared in three bands of M_r 62,000, 47,000, and 37,000. The crosslinked EGF–receptor complex (M_r 190,000) on intact cells was accessible to the action of trypsin at 4° and cofractionated with the plasmalemmal fraction. The proteolytic processing products of receptor were inaccessible to trypsin and banded with the lysosomal fraction upon subcellular fractionation. The rate of internalization and proteolytic processing of radiolabeled receptor was the same as the rate of reduction of binding activity induced by EGF. A study of the relationship between EGF-induced receptor internalization and processing, and stimulation of DNA synthesis, showed that both these processes were half-maximally stimulated at approximately 0.1 nM EGF, a concentration at which only 10% of the receptor sites are occupied. These data indicate that at concentrations of EGF subsaturating for binding but optimal for biological activity, there is a slow, continuous process of receptor internalization and degradation which could be limiting for EGF-induced mitogenesis.

Epidermal growth factor (EGF), a single-chain polypeptide of M_r 6045 (1), belongs to a select group of polypeptides which are extremely potent mitogenic hormones for target cultured cells (2, 3). EGF is present in mouse serum (4) and exerts its growth stimulatory effects at the low concentrations (0.01–0.1 nM) normally present there (5–8). Besides being a powerful mitogen, EGF may also be an important physiological effector involved in the control of differentiation of cells (9, 10).

Binding of EGF to responsive cells is mediated through high-affinity surface receptor interactions (6, 11); the membrane receptor for EGF on mouse 3T3 cells is a protein of M_r approximately 185,000 (12). A study of the characteristics of interaction of EGF with human fibroblasts showed that the concentration of peptide required to achieve maximal binding was approximately 5-fold higher than that required for maximal thymidine incorporation (6, 8, 11). Similar deviations from a close quantitative correlation between binding and biological activity have been observed with other hormones (13–15). In order to gain insight into the transduction mechanism relating hormone binding to its bioactivity, the cellular events occurring after the binding of hormone to receptor must be followed. We recently described a procedure for *in situ* radiolabeling of the EGF receptor on murine 3T3 cells using a photoreactive derivative of EGF (12, 16). In this communication we report studies in which these radiolabeled cells were used to study the physiological fate of receptor after EGF binding.

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MATERIALS AND METHODS

Cell Culture. Monolayer cultures of Swiss mouse 3T3 cells were grown and maintained as described (12).

Synthesis of Heterobifunctional Crosslinking Reagents. Methyl-3-[(*p*-azidophenyl)dithio]propionimidate (PAPDIP) was synthesized by a procedure that did not vary significantly from that described by Kiehm and Ji for synthesis of ethyl(4-azidophenyl)-1,4-dithiobutyrimidate (17). 4,4′-Dithiobisphenylazide and 3-mercaptopropionitrile were used as the starting materials. *N*-(4-Azido-2-nitrophenyl)ethylenediamine (NAPEDE) was synthesized from 4-fluoro-3-nitrophenylazide and ethylenediamine as the starting materials by a procedure described by Darfler and Marionetti for the synthesis of *N*-(5-azido-2-nitrophenyl)ethylenediamine (18).

Preparation of Photoreactive Derivatives of EGF. Murine EGF was purified by the procedure of Savage and Cohen (19). ^{125}I-Labeled EGF (^{125}I-EGF) and PAPDIP–^{125}I-EGF, a derivative of radioiodinated EGF containing a photoreactive group at its NH_2 terminus, were prepared as described (12). The maximal molar ratio achieved for PAPDIP bound to EGF was approximately 0.3, and this ratio was not increased by a second treatment with PAPDIP. A noncleavable, heterobifunctional crosslinker, NAPEDE, was used to prepare a reduction-resistant derivative of radioiodinated EGF, containing photoreactive groups linked to carboxyl groups in the EGF molecule. A solution of ^{125}I-EGF (50 μg, 2×10^5 cpm/ng) in 1 ml of 10 mM Tris·HCl, pH 7.0/0.2 M NaCl, was adjusted to pH 4.0 with dilute HCl. The reaction was initiated by addition of 50 μl each of 50 mM aqueous solutions of NAPEDE and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl. After a 30-min incubation at 23°, 5 μl of glacial acetic acid was added, and the mixture was then applied to a 0.4 × 20 cm Sephadex G-10 column and eluted with 20 mM potassium phosphate, pH 7.5/0.2 M NaCl to obtain NAPEDE–^{125}I-EGF at the void volume.

Binding and Photolysis of PAPDIP–^{125}I-EGF and NAPEDE–^{125}I-EGF. The procedure for binding and crosslinking of PAPDIP–^{125}I-EGF to confluent cell monolayers in 35-cm dishes has been described (12), and was used without modification for NAPEDE–^{125}I-EGF.

Polyacrylamide Gel Electrophoresis and Autoradiography. The conditions are those described earlier (12).

DNA Synthesis. Cells grown to confluence in 35-mm culture dishes were incubated with EGF in 2 ml of Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Twenty-four hours later, [^{3}H]thymidine (New England Nuclear) was added at a final concentration of 1 μCi/ml and 0.65 μM. After a 1-hr

Abbreviations: EGF, epidermal growth factor; ^{125}I-EGF, ^{125}I-labeled EGF; PAPDIP, methyl-3-(*p*-azidophenyl)dithiopropionimidate; NAPEDE, *N*-(4-azido-2-nitrophenyl)ethylenediamine; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid.

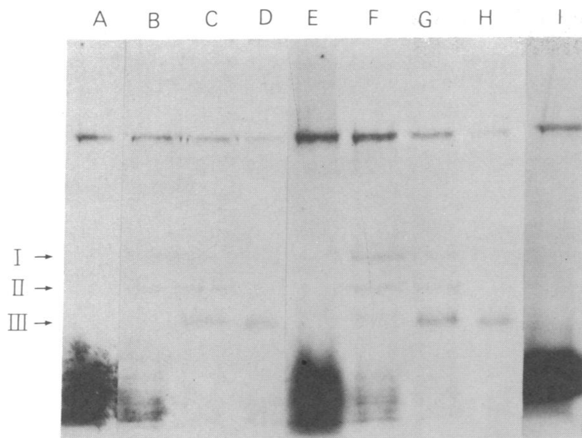

FIG. 1. Visualization of radiolabeled receptor and its degradation products by autoradiography of 5–20% polyacrylamide gradient gels. (Lanes A–D) Degradation of the receptor radiolabeled with PAPDIP–^{125}I-EGF. Confluent monolayers of 3T3 cells in 35-mm plastic dishes were incubated with 60 ng of PAPDIP–^{125}I-EGF (2×10^5 cpm/ng) in 1 ml of Earle's balanced salt solution containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, and 0.02% bovine serum albumin at 23° for 10 min in the dark. The monolayers were then washed with 3 ml of balanced salt solution containing 5 mM Hepes, pH 7.4, six times in the dark. After photolysis for 5 min at 4° as described (12), 1 ml of balanced salt solution/Hepes was added to each plate and the cells were incubated at 37° for (A) 0, (B) 30, (C) 60, and (D) 120 min. After the supernatant fraction was removed, 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. (Lanes E–H) Degradation of the receptor radiolabeled with NAPEDE–^{125}I-EGF. Cells were radiolabeled with NAPEDE–^{125}I-EGF (2×10^5 cpm/ng) as described above for labeling with PAPDIP–^{125}I-EGF. They were incubated at 37° for (E) 0, (F) 30, (G) 60, and (H) 120 min and then solubilized and subjected to gel electrophoresis under nonreducing conditions. (Lane I) 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 in the presence of 5% 2-mercaptoethanol.

incubation at 37°, trichloroacetic acid-insoluble radioactivity was determined (6).

Subcellular Fractionation. Monolayers of 3T3 cells were grown in 100-mm culture dishes, scraped from the plates, and homogenized (20). The homogenate was centrifuged at $500 \times g$ for 15 min, and the supernatant fraction was removed and centrifuged at $20{,}000 \times g$ for 60 min. The resulting pellet was suspended in 1 ml of 0.25 M sucrose/1 mM triethanolamine·HCl, pH 7.4, and then subjected to discontinuous sucrose gradient equilibrium centrifugation as described by Schimmel *et al.* (21). The fractions, I (8.3–20% sucrose), II (20–27%), III (27–32%), IV (32–40%), and V (40–55%), were assayed for phosphodiesterase I and N-acetyl-β-D-glucosaminidase activities (22).

RESULTS

Degradation of Radiolabeled EGF Receptor at 37°. Murine 3T3 cells were radiolabeled with either PAPDIP–^{125}I-EGF or NAPEDE–^{125}I-EGF. Photolysis of NAPEDE–^{125}I-EGF bound to cells resulted in the formation of a single species of crosslinked complex (receptor–NAPEDE–^{125}I-EGF) (Fig. 1, lane E) having an electrophoretic mobility essentially identical to that of the covalent receptor–PAPDIP–^{125}I-EGF complex (Fig. 1, lane A). This complex of M_r 190,000 did not dissociate into subunits when sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed under reducing conditions (Fig. 1, lane I), indicating that the receptor consists of a single, large

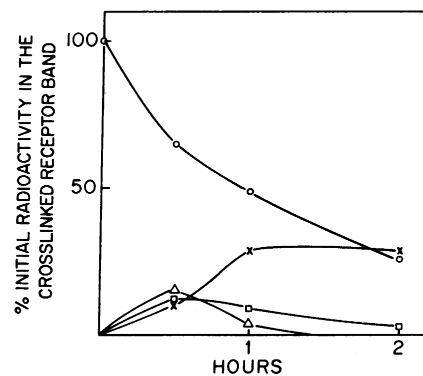

FIG. 2. Rate of degradation of radiolabeled receptor at 37°. The relative amounts of radioactivity in the radiolabeled receptor band (○) and in bands I (△), II (□), and III (×) were estimated by densitometric analysis of the autoradiograms, described in Fig. 1, lanes A–D.

polypeptide chain. When cells containing bound EGF and EGF–receptor covalent complex (either receptor–PAPDIP–^{125}I-EGF or receptor–NAPEDE–^{125}I-EGF) were incubated at 37°, there was loss of total cell-bound radioactivity with time and also a decrease in radioactivity in the covalent complex band, accompanied by appearance of new radioactive bands of M_r 62,000, 47,000, and 37,000 (Fig. 1, lanes A–H). The rate of loss of radioactivity from the EGF–receptor crosslinked complex band was the same irrespective of whether PAPDIP–^{125}I-EGF or NAPEDE–^{125}I-EGF was used for radiolabeling the receptor (Figs. 1, lanes A–H, and 2). Formation of the low molecular weight bands was not affected by the presence of native unlabeled EGF, unlabeled tyrosine, or cycloheximide in the medium during incubations at 37°. Radioactivity lost from the band of M_r 190,000 during the first hour of incubation at 37° was recovered almost quantitatively from the lower molecular weight bands (Fig. 2), suggesting a precursor–product relationship between these proteins.

Fate of Noncovalent EGF–Receptor Complex. In this experiment we took advantage of the fact that the proteolytic processing products of receptor retain their EGF-binding activities. Cells containing noncovalently bound PAPDIP–^{125}I-EGF were incubated at 37° and then subjected to photolysis (Fig. 3, lanes J and K). The pattern of protein labeling was the same as that observed with cells that were photolyzed prior to incubation at 37°, indicating that the noncovalent EGF–receptor complex has a physiological fate identical to that of the covalently linked complex. When the photolysis step was omitted, the radioactive bands of receptor and its degradation products were not observed (Fig. 3, lanes J′ and K′).

Cellular Localization of EGF–Receptor Complex and Its Degradation Products. The EGF–receptor covalent complex band was degraded by trypsin treatment of intact cells at 4°, indicating that this complex was located at the cell surface (Fig. 4, lanes L–O), but the lower molecular weight bands were insensitive to the action of trypsin on intact cells (Fig. 4, lanes L–O). The study was extended by subcellular fractionation of cells containing the radiolabeled receptor and its degradation products. The degradation products, but not the intact radiolabeled receptor, cofractionated with the lysosomes (Fig. 5).

To examine the possibility that the products may arise by lysosomal hydrolysis of the EGF–receptor complex, we tested the effects of chloroquine, a lysosomotropic reagent (23). Chloroquine almost completely inhibited the appearance of low molecular weight bands (Fig. 4, lanes P and Q). Inhibition of lysosomal degradation of receptor by chloroquine resulted in the appearance of a new radioactive band of approximately

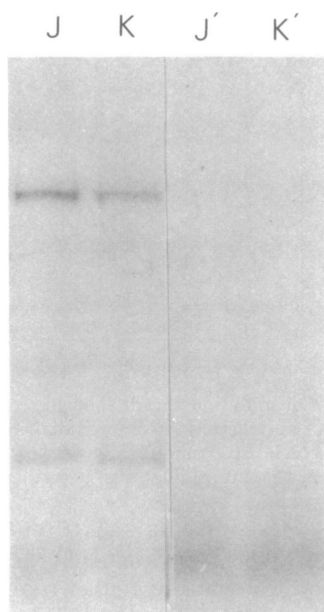

FIG. 3. Degradation of receptor not covalently linked to EGF. (Lanes J and K) Radiolabeling experiments with PAPDIP–^{125}I-EGF were performed under conditions identical to those described in the legend for Fig. 1, lanes C and D, except that the cells were first incubated at 37° for (J) 60 or (K) 120 min and then photolyzed. (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.

130,000 M_r (Fig. 4, lanes P and Q). Limited tryptic digestion of radiolabeled cells at 4° also produced this band (Fig. 4, lanes L–O), suggesting the presence of a trypsin-like protease that has receptor-degrading activity.

Relationship between Radiolabeled Receptor Degradation and EGF-Induced "Down Regulation" of Receptor Activity. EGF binding activity in 3T3 cells can be down regulated by incubating the cells with EGF (Fig. 6). A maximum of 80% binding activity was lost after incubation with high concentrations of EGF. However, an EGF concentration that produced <10% receptor site occupancy produced a relatively large (>30%) reduction in binding activity. The PAPDIP–EGF preparation was as effective as native EGF in causing down regulation (Table 1). Half-maximal reduction of binding activity occurred at approximately 0.1 nM EGF in both cases.

The relationship between EGF-induced receptor down regulation and radiolabeled receptor degradation was investigated by incubating cells containing covalently complexed PAPDIP–^{125}I-EGF or noncovalently bound EGF with a saturating concentration of native, unlabeled EGF at 37°. The time-dependent reduction in EGF-binding activity closely paralleled the degradation of radiolabeled receptors, indicating that the same mechanism is responsible for both phenomena (Fig. 7).

Stimulation of [^{3}H]Thymidine Incorporation. Native EGF and unphotolyzed PAPDIP–EGF were equally effective in stimulating [^{3}H]thymidine incorporation into DNA. The concentration required for half-maximal stimulation was approximately 0.1 nM. Polynucleotide synthesis was stimulated maximally at concentrations of EGF ranging from 0.5 to 1.5 nM; higher doses of EGF were less stimulatory (Table 2). In agreement with the results of others (8), at least a 7-hr period of continuous exposure to EGF was necessary for maximal induction of DNA synthesis. A similar requirement has recently

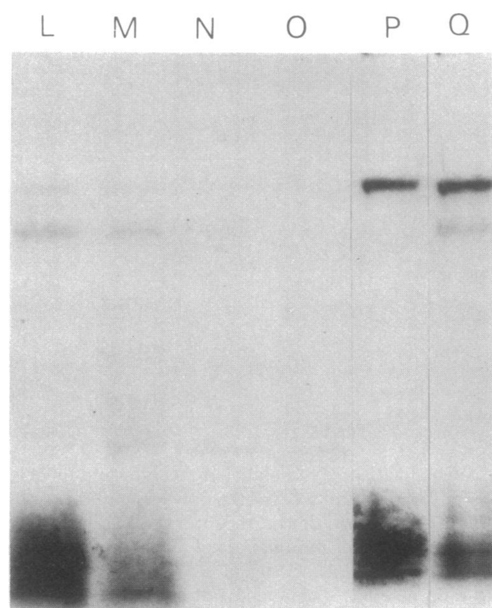

FIG. 4. Susceptibility of the radiolabeled receptor and its degradation products to the action of an extracellularly added protease, and the effects of an inhibitor of lysosomal protease activity on receptor degradation. (Lanes L–O) Trypsin treatment of intact cells containing radiolabeled receptor and its degradation products. Monolayers radiolabeled with PAPDIP–^{125}I-EGF and incubated at 37° for (L) 0, (M) 30, (N) 60, and (O) 120 min, as described for Fig. 1, lanes A–D, were treated for 30 min at 4° with 25 μg of trypsin in 1 ml of balanced salt solution/Hepes. Cell-bound radioactivity was analyzed by gel electrophoresis under nonreducing conditions. (Lanes P and Q) Effect of chloroquine on receptor degradation. Cells were radiolabeled with PAPDIP–^{125}I-EGF and incubated at 37° for (P) 30 and (Q) 120 min as described for Fig. 1, lanes B and D, except that 0.1 mM chloroquine was present during binding, photolysis, and incubation at 37°.

been elegantly demonstrated for the growth factor present in platelet-poor plasma (24).

DISCUSSION

The regulation of receptor-binding activity by hormones, i.e., down regulation, is a common phenomenon (25–27), but it has

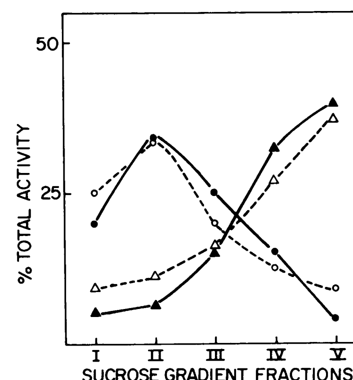

FIG. 5. Subcellular distribution of radiolabeled receptor and its degradation products. Cell monolayers in 100-mm culture dishes were radiolabeled with PAPDIP–^{125}I-EGF (12), incubated at 37° for 45 min with 5 ml of balanced salt solution/Hepes, and subjected to subcellular fractionation. The isolated 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.

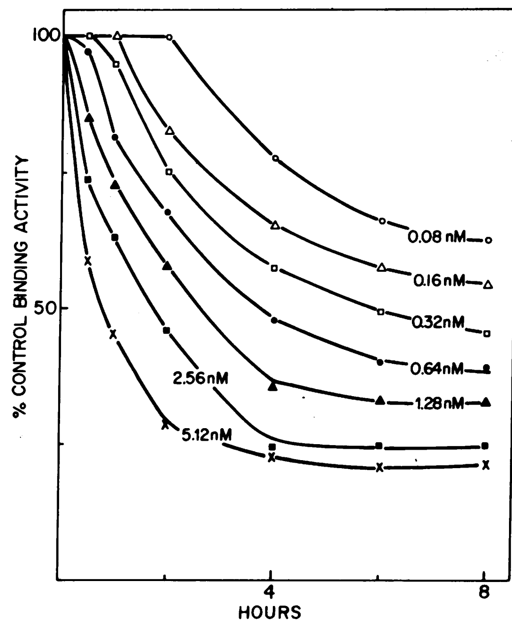

FIG. 6. Down regulation of EGF receptor by EGF. Monolayers of 3T3 cells in 35-mm culture dishes were incubated at 37° for the indicated time periods, with EGF in modified Eagle's medium containing 0.02% bovine serum albumin. Each monolayer was then washed hourly at 37° for 4 hr with 3 ml of modified Eagle's medium/bovine serum albumin for each wash. The washed cells were then incubated with 50 ng of ^{125}I-EGF (2×10^5 cpm/ng) in 1 ml of binding medium at 23° for 60 min and tested for EGF-binding activity (12).

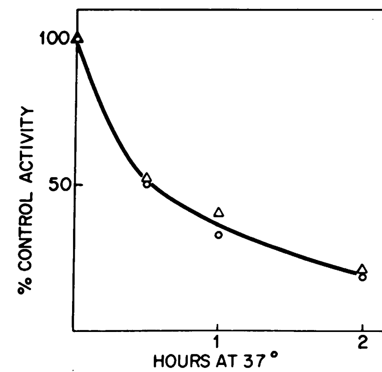

FIG. 7. Relationship between radiolabeled receptor degradation and down regulation. 3T3 cells grown to confluence in 35-mm culture dishes were incubated for 60 min at 23° in the dark with 30 ng of either PAPDIP-^{125}I-EGF or native unlabeled EGF in 1 ml of balanced salt solution/Hepes/bovine serum albumin. After incubation, each monolayer was washed six times with 3-ml portions of balanced salt solution/Hepes and then photolyzed (12). The cells were then incubated at 37° for the indicated periods of time with 1 ml of balanced salt solution/Hepes/bovine serum albumin 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 (O) 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 six times with 3-ml portions of balanced salt solution/Hepes, incubated with 60 ng of ^{125}I-EGF (3.5×10^5 cpm/ng) in 1 ml of binding medium at 23° for 80 min, and tested for EGF-binding activity (△) as described (12).

not been demonstrated whether down regulation proceeds by receptor degradation or some other process. While testing for correlations between cellular processing of radiolabeled receptor and the phenomenon of down regulation, we considered the possibility that a photoreactive derivative of hormone may induce events different from those induced by native hormone. A covalent complex of hormone and receptor might also elicit unphysiological cellular responses. The degradation of the radiolabeled EGF-receptor covalent complex reported here is not likely to result from such artifacts, since it meets the following criteria: (*i*) Receptor that is not internalized as a covalent complex undergoes degradation via the same pathway as the covalently linked complex (Fig. 3). (*ii*) High concentrations of native unlabeled EGF, which give rise to greater than 80% receptor site occupancy, markedly enhance the rate of degradation of radiolabeled receptor (compare data in Figs. 2 and 7). (*iii*) The kinetics of radiolabeled receptor degradation were identical to the kinetics of loss of EGF-binding activity from the cell surface (Fig. 7). Furthermore, EGF and PAPDIP-EGF have identical receptor-binding activities, abilities to down regulate receptors, and abilities to stimulate DNA synthesis. These data show that the system described here provides a valid model for studying the fate of receptor after EGF binding.

The concomitant loss of EGF-binding activity and EGF-receptor covalent complex from the cell surface is closely associated with the appearance of defined degradation products of receptor protein in the lysosomal fraction. This is explained most simply by a mechanism in which binding of EGF to receptor is followed by endocytosis of receptor and formation of

Table 1. Down regulation of receptor activity by EGF and PAPDIP-EGF

Hormone	Hormone-induced loss of specific binding activity, %
EGF	
0.06 nM	34
0.17 nM	47
0.5 nM	55
5.0 nM	79
PAPDIP-EGF	
0.06 nM	30
0.17 nM	43
0.5 nM	58
5.0 nM	78

Murine 3T3 cells grown in 35-mm plates were incubated at 37° for 6 hr with the indicated concentrations of either EGF or PAPDIP-EGF. They were then washed for 4 hr and tested for binding activity, as described in the legend for Fig. 6.

Table 2. Stimulation of [^{3}H]thymidine incorporation into DNA by EGF and PAPDIP-EGF

Hormone	[^{3}H]Thymidine incorporation, cpm
EGF	
0.03 nM	4,600
0.1 nM	14,700
0.3 nM	19,900
0.9 nM	24,700
9.0 nM	17,500
PAPDIP-EGF	
0.03 nM	4,800
0.1 nM	13,700
0.3 nM	20,900
0.9 nM	24,600
9.0 nM	18,100

[^{3}H]Thymidine incorporation was measured after incubation of 3T3 cells with EGF or PAPDIP-EGF, and was corrected for the incorporation observed (2860 cpm) in the absence of EGF.

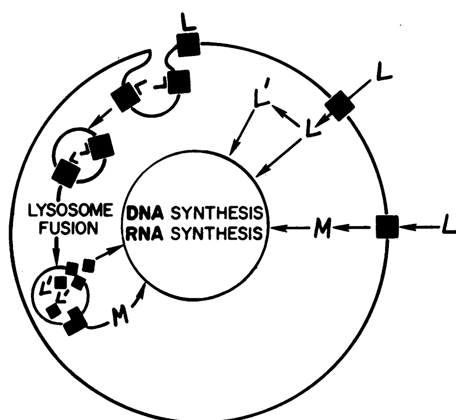

FIG. 8. Hypothetical mechanisms for production of the "second messenger" in EGF action. L and L′ denote the unmodified and modified ligand, respectively, i.e., EGF; M denotes a catalytically produced second messenger. Solid squares represent the cell surface receptor. See *Discussion* for further details.

a secondary lysosome where receptor is processed by proteolysis (Fig. 8). A similar conclusion was derived by inference from studies showing that cells degrade noncovalently bound EGF by a process that has many of the kinetic properties described here for degradation of the radiolabeled receptor–EGF covalent complex (11). An endocytic pathway for receptor removal after association with hormone implies that the equilibrium of the binding reaction will shift towards the formation of additional hormone–receptor complexes. A low steady-state receptor occupancy does ultimately lead to a large reduction in cell surface activity of the EGF receptor (Fig. 6). However, at low concentrations of EGF, the loss of EGF-binding activity occurred only after a substantial lag period. This suggests that reorganization of membrane components may be required for endocytosis. Reorganization of receptors after EGF binding could explain the observation of rapid masking of specifically bound EGF from antibody against EGF (11).

At least four distinct and testable transduction mechanisms can be proposed to explain how the EGF–receptor interaction can lead to production of a second messenger which drives the cell through G_1 and commits it to enter the S phase of the cell cycle (Fig. 8). These are: (*i*) stimulation of second messenger production by receptor-mediated catalysis at the cell surface or in pinosomes prior to receptor degradation; (*ii*) receptor-mediated transport of EGF, which then serves as the second messenger either with or without further modification; (*iii*) proteolytic processing of EGF in lysosomes, leading to the creation of a second messenger [the type of mechanism that best explains the properties of the low density lipoprotein receptor system (28)]; and (*iv*) proteolytic processing of receptor or some other endocytosed membrane protein to yield a species which is the second messenger or has the capabilities of producing one. The concentrations of EGF required for half-maximal receptor internalization and induction of DNA synthesis are identical. This suggests that the former process causes the latter, favoring the third and fourth hypotheses. Since controlled proteolysis can act from outside the cell in the absence of a mitogenic hormone to stimulate the initiation of DNA synthesis and mitogensis (29), we prefer the fourth hypothesis.

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