A Nonmitogenic Analogue of Epidermal Growth Factor Induces Early Responses Mediated by Epidermal Growth Factor

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ABSTRACT Cyanogen bromide-cleaved epidermal growth factor (CNBr-EGF) binds to EGF receptors with reduced affinity compared to the native hormone but fails to induce DNA synthesis. However, at similar receptor occupancy, CNBr-EGF is as potent as EGF in activating early cell responses to the hormone. The phosphorylation of membrane proteins, the stimulation of Na^+-K^+-ATPase as reflected by the ouabain-sensitive uptake of ^{86}Rb of fibroblasts, changes in the organization of microfilaments and in cell-morphology, and the activation of the enzyme ornithine-decarboxylase are all induced by CNBr-EGF as well as EGF.

Our results are consistent with the notion that EGF-induced phosphorylation could act as a "second messenger" for the activation of various EGF-induced responses such as activation of Na^+-K^+-ATPase, changes in the cytoskeleton and cell morphology, and the activation of the enzyme ornithine decarboxylase. However, the stimulation of phosphorylation of membrane proteins and other early responses are either not required or necessary but insufficient for the induction of DNA synthesis.

Suboptimal concentrations of EGF together with CNBr-EGF stimulate DNA synthesis in human fibroblasts. Other growth factors such as insulin, fibroblast growth factor, and prostaglandin $F_{2\alpha}$, which potentiate the mitogenic response of EGF, do not effect the response to CNBr-EGF. This suggests that the restoration of the mitogenic properties of CNBr-EGF by suboptimal doses of EGF occurs at the level of EGF receptors or during their processing.

Epidermal growth factor (EGF), a single chain polypeptide hormone isolated from the mouse submaxillary gland (28), is a potent mitogen for various epidermal and epithelial cells both in vivo and in vitro (7). The initial interaction of EGF with target cells occurs at specific receptors which are homogeneously distributed over the cell surface. Subsequent to binding, the mobile (31) hormone-receptor complexes rapidly aggregate into clusters and collect over coated regions of the plasma membrane (12, 13, 15, 23, 31, 32). The EGF-receptor complexes are then endocytosed by an energy-dependent process and eventually degraded in lysosomes (12, 15). At the same time, EGF induces an array of metabolic alterations in cells. These include early effects such as stimulation of nutrient uptake (7, 14) and ion fluxes (26), enhanced phosphorylation of endogenous membrane proteins (5, 6, 19), changes in cell morphology (8), and cytoskeletal organization (34). Delayed effects of EGF include the activation of cytoplasmic enzymes and the stimulation of protein and DNA synthesis (7). The question remains open of when and how EGF generates bio-

chemical signals during the topographical rearrangement and processing of hormone-receptor complexes. Furthermore, it is not clear yet whether all EGF-induced effects are related to each other by a single mediator and whether the observed hormone responses converge into a common pathway leading to DNA synthesis.

The importance of EGF-receptor aggregation for the induction of the EGF mitogenic response is indicated by the use of an EGF derivative, obtained by cyanogen bromide cleavage (CNBr) of the hormone (37). Cyanogen bromide-cleaved EGF (CNBr-EGF) binds with reduced affinity to EGF receptors but fails to induce receptor clustering or DNA synthesis. The cross-linking of this derivative with bivalent anti-EGF antibodies restores both cluster formation and stimulation of DNA synthesis (33, 37).

In this report we characterize the biological activity of CNBr-EGF on cultured cells. Surprisingly, at equal receptor occupancy, the nonmitogenic CNBr-EGF appears to behave as a full agonist of EGF in all the biological assays studied except

DNA synthesis. Our results present CNBr-EGF as a versatile tool for investigating the mode of action of EGF and are discussed in the light of a possible mechanistic uncoupling of EGF early and delayed responses.

MATERIALS AND METHODS

Materials

EGF was purified from the submaxillary glands of adult male mice by the method of Savage and Cohen (28). ^{125}I-EGF was prepared by the chloramine T method (17) to a specific activity of 80,000–120,000 cpm/ng. CNBr-EGF was prepared as reported and characterized by amino acid analysis (36, 37). ^{86}Rubidium chloride (^{86}RbCl) and L-[1-^{14}C]ornithine (54.1 mCi/mmol) were obtained from New England Nuclear (Boston, MA). [γ-^{32}P]-ATP (16 Ci/mmol) and [^{3}H-methyl]thymidine (47 Ci/mmol) were purchased from the Radiochemical Centre, Amersham Corp. (Arlington Heights, IL). 7-Nitrobenz-2-oxa-1,3-diazole phallacidin (NBD-phallacidin) was a generous gift from Dr. L. Barak. Ouabain and porcine insulin were purchased from Sigma Chemical Co. (St. Louis, MO). Prostaglandin F$_{2α}$ (PGF$_{2α}$) was a gift from Dr. J. Pike (Upjohn College). Bovine pituitary fibroblast growth factor (FGF) was a kind gift from Dr. I. Vlodavsky.

Cells

Human epidermoid carcinoma cells (A-431 cell line) were kindly provided by Dr. G. Todaro. Human foreskin fibroblasts (HFF) from primary cultures were a gift from Drs. D. Rothman and M. Gabbay. Swiss mouse 3T3 fibroblasts were obtained from Dr. A. Ben-Zeev. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS).

Binding Studies

Binding of ^{125}I-EGF was performed on confluent monolayers of living cells in 24 well Costar trays (Costar, Data Packaging, Cambridge, MA) as described elsewhere (43).

Microscopy

Cells were plated at low density in 35-mm plastic culture dishes. 1 and 2 h after the addition of either 50 ng/ml EGF or 500 ng/ml CNBr-EGF, in DMEM containing 0.1% bovine serum albumin (BSA), the cells were observed with a Zeiss inverted microscope IM 35. For fluorescent labeling of actin, cells were fixed with 3% paraformaldehyde and treated with 0.1% Triton X-100 at room temperature. Permeabilized cells were then incubated with 2 ng/ml NBD-phallacidin for 20 min. Photographs were taken on Kodak Tri-X film.

Membrane Phosphorylation Reaction

A membrane preparation of A-431 cells was obtained by the procedure of Thom et al. (39). Phosphorylation reactions were conducted as reported previously (5, 6, 19, 35). Briefly, 30 μg of membrane protein were preincubated at 0°C for 15 min with increasing concentrations of either EGF or CNBr-EGF in 20 mM HEPES buffer (pH 7.4) containing 50 mM MgCl and 0.01% BSA in a final volume of 50 μl before addition of [γ-^{32}P]-ATP (50 nM) for 10 min at 0°C. Aliquots were pipetted on Whatman No. 3 filter papers (Whatman, Inc., Chemical Separation Div., Clifton, N. J.) which were immediately dropped into cold 10% trichloroacetic acid (TCA)–0.1 M pyrophosphate, extensively washed with this solution, and extracted with ethanol and ether. The radioactivity of the filters was then counted in a liquid scintillation counter.

^{86}Rb$^+$ Uptake Measurements

HFF were grown to confluence in 35-mm dishes (Nunc, Kampstrup, Denmark) and starved in serum-free medium two days before the assay. After 10 min of preincubation at 37°C with either EGF or CNBr-EGF, cells were incubated at 37°C for 10 min with 0.5 μCi of ^{86}RbCl in a final volume of 250 μl. Cells were then rapidly washed with 10 ml of ice-cold phosphate-buffered saline, and 0.5 ml of ice-cold 5% TCA was added to each well. The TCA-soluble radioactivity was determined directly in a liquid scintillation counter. Control systems were incubated for 1 min with 1 mM ouabain before the addition of ^{86}RbCl. Zero time uptake was measured and subtracted from the average of triplicates.

Induction of Ornithine Decarboxylase Activity

Mouse fibroblasts (Swiss 3T3) were grown to confluence in 35-mm dishes and

then maintained for 2 d in medium containing 0.5% FCS before the assay. EGF or CNBr-EGF were added to the cells for various times at 37°C. Cells were washed with ice-cold buffer solution A (50 mM Tris-HCl, 1 mM EDTA, pH 7.4), collected with a rubber policeman in 0.2 ml of buffer solution A and frozen and thawed three times. After centrifugation (10 min at 4°C, 12,000 g), ornithine decarboxylase activity was determined by incubating 180 μl of the supernatant solution with 20 μl of assay buffer (0.5 mM pyridoxalphosphate, 12.5 mM dithiothreitol, and 0.2 μCi [1-^{14}C]ornithine in buffer solution A). The reaction was conducted in plastic tubes covered with a rubber stopper supporting a polyethylene center well (Kontes Co., Vineland, NJ) filled with Whatman No. 3 filter paper soaked in NCS Tissue Solubilizer (Amersham/Searle Corp., Ill.). After incubation at 37°C for 1 h, 0.5 ml of 50% TCA was added and incubation at 37°C was allowed to proceed for 30 min. Center wells were then removed and their radioactive content was determined.

Stimulation of Thymidine Incorporation

Cells for assay were grown to confluence in 24 well Costar trays (Costar, Data Packaging) and then maintained for 2 d in medium containing 0.5% FCS. Hormones were added to the cells 18 h before a 4-h pulse of [^{3}H-methyl]-thymidine (0.5 μCi). Cells were washed three times with phosphate-buffered saline, and the TCA nonextractable radioactivity was determined in a liquid scintillation counter.

RESULTS

EGF has a single methionyl residue at position 21 (7), and cleavage with cyanogen bromide results in the formation of two polypeptide chains connected by the three disulfide bonds of the protein molecule. Amino acid analysis of an acid hydrolysate of CNBr-EGF revealed the full conversion of methionine into homoserine lactone. CNBr-EGF was shown to have a tertiary structure similar to that of EGF, but it is less stable than the native hormone to denaturation by 4 M of guanidinum chloride (16). However, CNBr-EGF is a stable protein and intact EGF is a very stable protein (16). CNBr-EGF binds to EGF-receptors on A-431 cells and membranes prepared from these cells and also to EGF receptors on HFF and 3T3 fibroblasts with an affinity 1/10 that of EGF (36, 37) (Table I) and fails to induce visible clustering of EGF receptors (33, 37). The nonmitogenic activity of CNBr-EGF is not related to its reduced stability, since either anti-EGF antibodies (37) or suboptimal concentration of native EGF restore its full mitogenic activity.

TABLE I

Comparison of Binding and Biological Properties of EGF and CNBr-EGF

	EGF	CNBr-EGF
	ng/ml	
50% Displacement of 10 ng/ml [^{125}I]EGF binding to A-431 cells*	10	100
Stimulation of ouabain-sensitive ^{86}Rb influx (200% above control) in HFF	20	150
Enhancement of endogenous membrane protein phosphorylation (250% above control) (A-431 membrane preparation)	150	1,200
Induction of ornithine decarboxylase in 3T3 cells (350% above control)	10	100
Induction of morphological changes in A-431 cells	50	500
Reorganization of the microfilament system in A-431 cells	50	500
Stimulation of DNA synthesis in 3T3 and HFF cells‡	10	—

* Similar results for 50% displacement of [^{125}I]EGF were obtained for HFF and 3T3 cells.

‡ Maximal stimulation of DNA synthesis (1,000%–2,000% above control) in 3T3 and HFF cells is achieved at 10 ng/ml EGF. CNBr-EGF does not stimulate DNA synthesis at doses as high as 100 ng/ml when added to 3T3 and HFF cells.
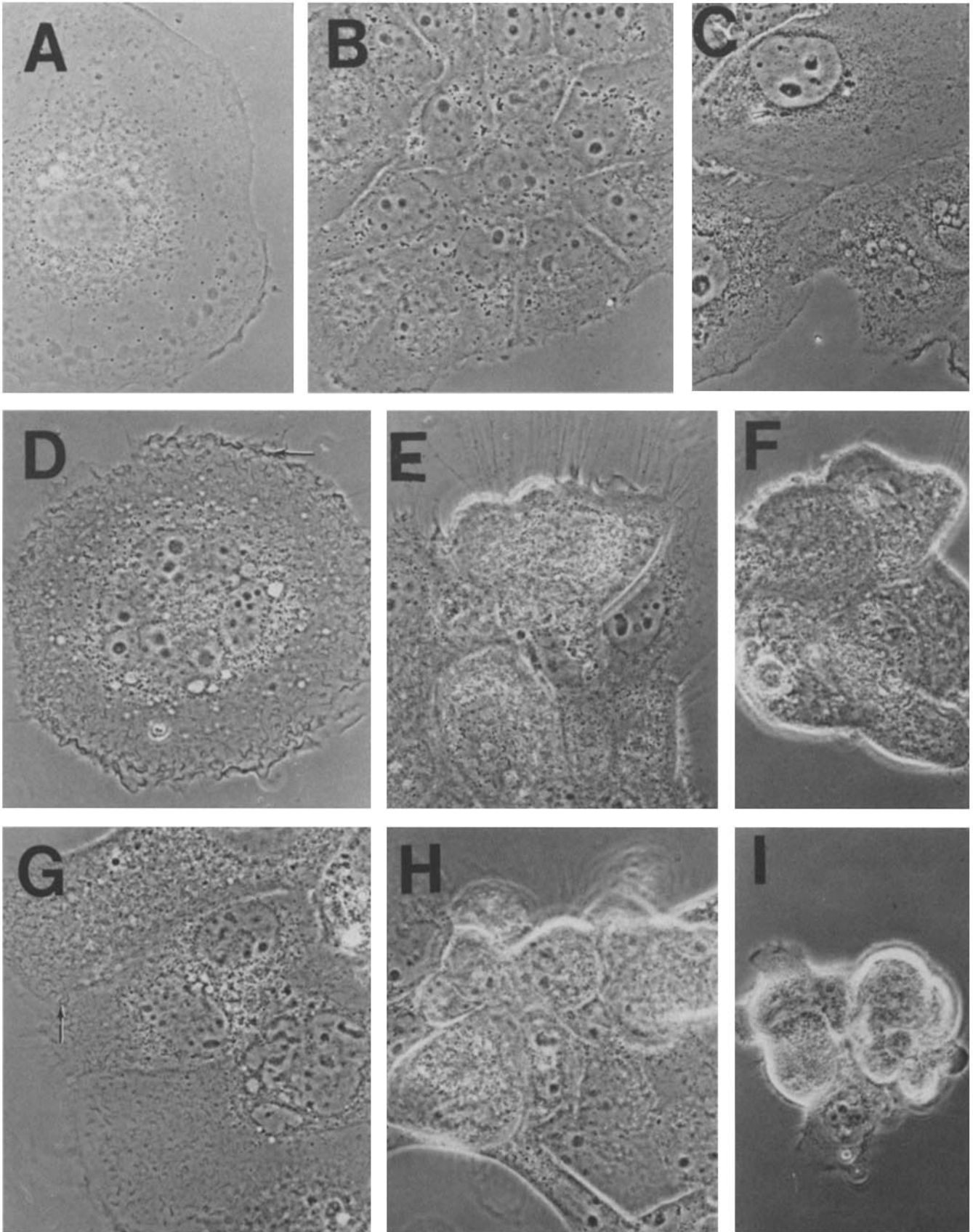
FIGURE 1 Morphological changes induced by EGF or CNBr-EGF in A-431 cells. Phase-contrast micrographs of A-431 cells grown to sparse density in 35-mm plastic dishes and incubated for 1 h at 37°C with medium (A, B, and C), 50 ng/ml EGF (D, E, and F), and 500 ng/ml CNBr-EGF (G, H, and I). A-431 cells appear as flat, closely associated cells (B and C) with a smooth plasma membrane (A). In the presence of either EGF or CNBr-EGF, the cells round up and pile up to form multilayered colonies (E–G) while exhibiting membrane activity (see arrows in D and G). × 732.

EGF mediates physiological responses in target cells which occur at different times after the addition of the hormone. We undertook a comparison of the effects of EGF and CNBr-EGF on these responses.

Membrane Phosphorylation

EGF rapidly activates a cyclic nucleotide independent protein kinase specific for tyrosine residues (41). This protein kinase has been copurified with the EGF membrane receptor (9). Addition of EGF and [γ-^{32}P]ATP to a membrane preparation from A-431 cells enhances the phosphorylation of endogenous membrane proteins. Addition of CNBr-EGF yields the same effect (Table I), at a concentration of the derivative about tenfold that of the native hormone. It has been shown previously (36) that EGF and CNBr-EGF stimulate the phosphorylation of the same membrane components.

^{86}Rb Uptake

EGF stimulates transiently the Na$^+$-K$^+$-ATPase of target cells shortly after binding to cell membrane receptors (26). The effect of EGF and CNBr-EGF on ouabain-sensitive ^{86}Rb$^+$ uptake which reflects the activity of Na$^+$-K$^+$-ATPase was studied in HFF. As can be seen from Table I, both EGF and CNBr-EGF enhanced the ouabain-sensitive ^{86}Rb$^+$ influx about twofold over basal activity, provided the same receptor occupancy is reached. Basal influx was 1,230 ± 300 cpm; EGF (10 ng/ml) induced influx of 2,160 ± 360 cpm, and CNBr-EGF (100 ng/ml) induced influx of 2,120 ± 200 cpm into HFF cells. Similar effects of EGF and CNBr-EGF on ^{86}Rb influx were observed on A-431 cells but measurements on these cells were less accurate due to nonselective pinocytosis induced by the hormones (14).

Morphological and Cytoskeletal Changes

EGF transiently alters the morphology (8) and the cytoskeletal organization (34) of A-431 cells. Similar effects can be observed on other EGF-sensitive cell types but they are most striking on A-431 cells which bear an unusually high number of EGF receptors (11, 42). A-431 cells grow in culture as flat, closely associated cells (Fig. 1 A–C). After incubation at 37°C for 2 h with either 50 ng/ml EGF (Fig. 1 D–E) or 500 ng/ml CNBr-EGF (Fig. 1 G–H), the cells show extensive membrane activity (see arrows in Fig. 1 D and G), round up (Fig. 1 E and H), and form multilayered colonies (Fig. 1 F and I). The effects of EGF or CNBr-EGF on the organization of actin filaments in A-431 cells are depicted in Fig. 2. Actin was visualized after paraformaldehyde fixation and Triton X-100 permeabilization of the cells by labeling with NBD-phallacidin (2). In A-431 cells, actin is organized in fiber bundles which often span the entire length of the cell and are oriented in different directions; some of the actin bundles appear in beltlike structures (34) (Fig. 2 A). After incubation with either 50 ng/ml EGF (Fig. 2 B) or 500 ng/ml CNBr-EGF (Fig. 2 C) at 37°C for 1 h, most of the well-organized actin bundles are no longer visible; rather, an intense diffuse labeling is seen in the cytoplasm. The double-labeling experiment with NBD-phallacidin and anti-actin antibodies indicated that the two reagents decorate similar patterns (microfilaments) in cultured cells (40). For both EGF and CNBr-EGF, the morphological and microfilament alterations are transient; after more than 8 h of incubation at 37°C, cells resumed their original morphology and cytoskeletal organization (34).

Induction of Ornithine Decarboxylase

The increase in activity of the cytoplasmic enzyme ornithine decarboxylase (ODC), which catalyzes the first step in the
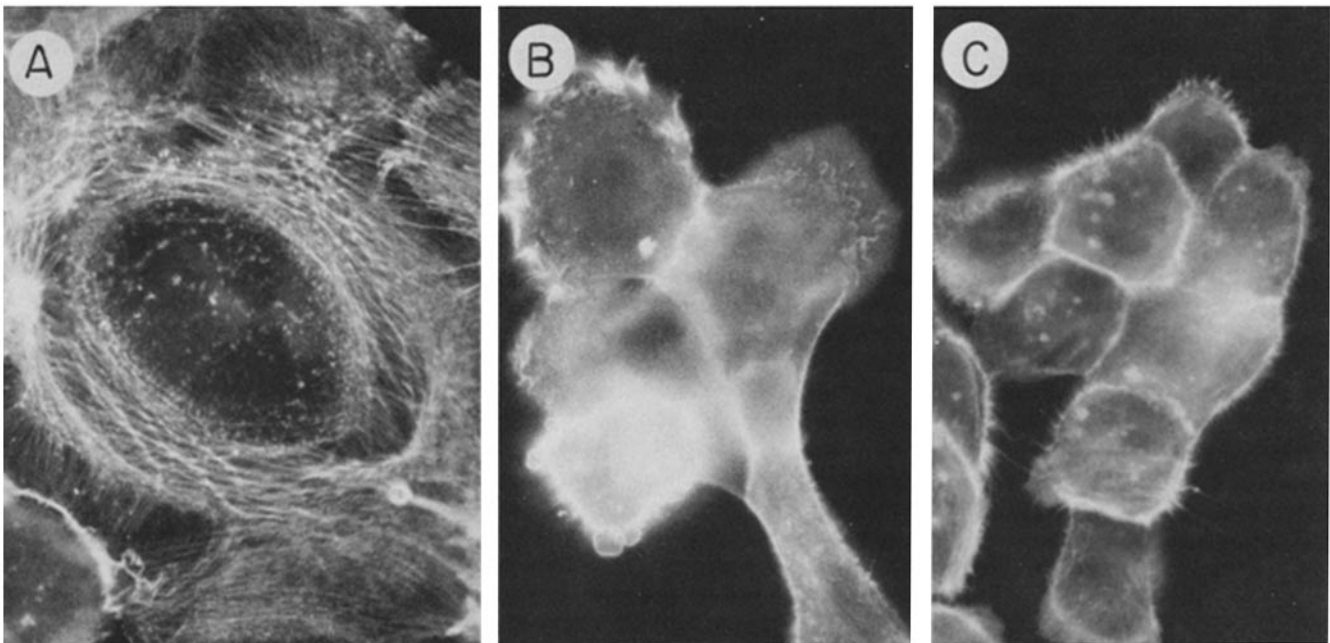

FIGURE 2   Reorganization of actin filaments induced by EGF or CNBr-EGF in A-431 cells. A-431 cells growing on glass cover slips were incubated for 1 h at 37°C with medium (A), 50 ng/ml EGF (B), and 500 ng/ml CNBr-EGF (C). The cells were fixed with 0.1% formaldehyde, permeabilized with 0.1% Triton X-100, and labeled with 2 ng/ml of NBD-phallacidin, a fluorescently labeled mushroom toxin which specifically labels F-actin inside the cells. The fluorescence micrographs show the well-organized pattern of the actin-containing microfilament system in untreated (A) A-431 cells. Both EGF (B) and CNBr-EGF (C) induce the reorganization of the F-actin-containing filaments in these cells. × 732.

synthesis of polyamines, is an important component of the proliferative response of cells to a mitogenic stimulus. Maximal stimulation of ODC activity in 3T3 fibroblasts is observed after 4 h of incubation with EGF (Fig. 3). CNBr-EGF appears to be a full agonist of EGF in the induction of this delayed effect of EGF, provided a tenfold higher concentration of CNBr-EGF is used to compensate for its reduced affinity for EGF-receptors (Figs. 3 and 4).

DNA Synthesis

The mitogenic activity of EGF is measured in terms of the stimulation of thymidine incorporation into target cells induced by the hormone. Maximal stimulation of thymidine incorporation occurs 16–18 h after binding of EGF. As previously reported for 3T3 fibroblasts (37) and human fibroblasts (Fig. 5), CNBr-EGF is a hundredfold less potent than EGF in inducing DNA synthesis. CNBr-EGF is also inactive in the in vivo assay for EGF, namely precocious opening of eyelids of newborn mice (16).

As CNBr-EGF competes for binding of EGF to its membrane receptors and behaves as an agonist of the EGF-mediated short-term effects, we examined the possibility that it acts as an antagonist of EGF for the induction of DNA synthesis. Surprisingly, low concentrations of EGF together with CNBr-EGF clearly work synergistically on the enhancement of thymidine incorporation into HFF (Fig. 5); similar results were obtained when EGF and CNBr-EGF were added to 3T3 fibroblasts (data not shown). It should be noted that at all concentrations of EGF and CNBr-EGF incubated together the

stimulation effect does not exceed the maximal effect of EGF. As synergistic effects between hormones are well documented (18, 27), we investigated whether other hormones and growth-factors which modulate the mitogenic effect of EGF would also restore the potency of CNBr-EGF for induction of DNA synthesis. Results for insulin, FGF, and $PGF_{2\alpha}$ are shown in Fig. 6. Insulin and $PGF_{2\alpha}$ alone are practically nonmitogenic for HFF, whereas FGF at 50 ng/ml enhances thymidine incorporation about twofold above control. All three hormones

FIGURE 5 Induction of DNA synthesis in quiescent fibroblasts by the addition of EGF together with CNBr-EGF. Enhancement of [^{3}H-methyl]thymidine incorporation in quiescent human foreskin fibroblasts incubated with increasing concentrations of CNBr-EGF alone (A, O—O), or together with 0.25 ng/ml EGF (A, ●—●), 0.5 ng/ml EGF (A, △—△), 1 ng/ml EGF (▲—▲), and 2 ng/ml EGF (▽—▽): the effect of induction with increasing concentrations of EGF alone (□—□) is shown in B. Cells were incubated at 37°C for 18 h with the hormone(s) before a 4-h pulse with [^{3}H-methyl]thymidine and determination of the trichloroacetic acid–nonextractable radioactivity.
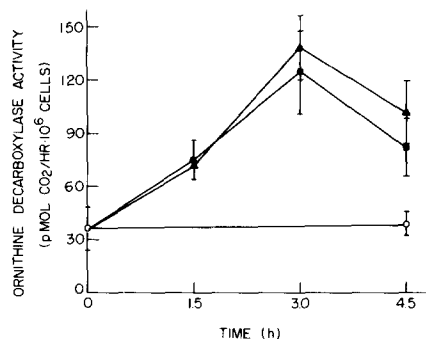

FIGURE 3 Kinetics of the stimulation of ODC activity by EGF or CNBr-EGF in mouse 3T3 fibroblasts. 3T3 cells were grown to confluence in 35-mm plastic dishes and further grown in medium containing 0.5% fetal calf serum before the assay. Cells were incubated for various periods of time with either 10 ng/ml EGF (●———●), 100 ng/ml CNBr-EGF (▲———▲), or buffer (O———O). Measurement of ODC activity was performed as described under Materials and Methods. Results are the average of three measurements.
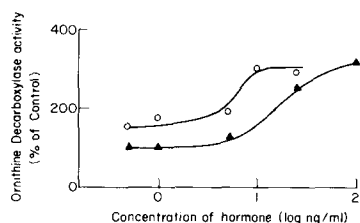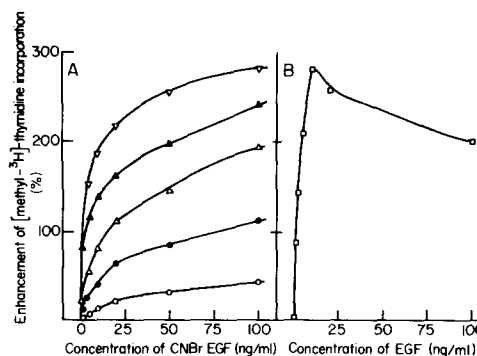

FIGURE 4 Stimulation of ODC activity by either EGF or CNBr-EGF in mouse 3T3 fibroblasts. Cells were incubated with increasing concentrations of either EGF (O—O) or CNBr-EGF (▲—▲). Measurements of ODC activity are described in Materials and Methods. Results are the average of three measurements.
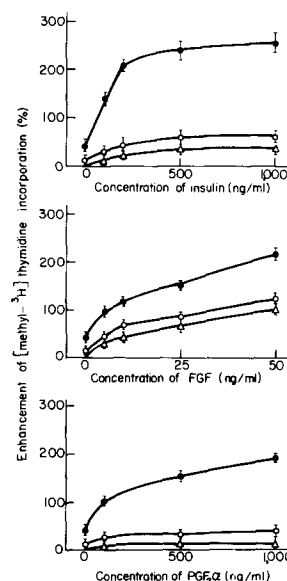

FIGURE 6 Synergistic effects of insulin, FGF, or $PGF_{2\alpha}$ with either EGF or CNBr-EGF on the initiation of DNA synthesis in quiescent fibroblasts. Enhancement of [^{3}H-methyl]thymidine incorporation in quiescent human foreskin fibroblasts incubated with increasing concentrations of insulin (top), FGF (middle), and $PGF_{2\alpha}$ (bottom) alone (△—△) or together with either 10 ng/ml CNBr-EGF (O—O) or 1 ng/ml EGF (●—●). Cells were incubated at 37°C for 18 h with the hormone(s) before a 4-h pulse with [^{3}H-methyl]thymidine and determination of the trichloroacetic acid–nonextractable radioactivity.

act clearly in synergy with EGF but only in an additive fashion with CNBr-EGF over the entire concentration range examined.

DISCUSSION

EGF binding to cells initiates a series of changes in cell metabolism which begins within minutes of hormone binding but which persists for many hours afterward. The last change in the series is an increase in DNA synthesis and ultimately mitosis.

The binding of EGF to cell surface receptors is followed by internalization of the hormone-receptor complexes. This raises at least three different mechanisms for the transduction of its biological activity.

(a) EGF acts at the level of the cell surface to generate directly, or indirectly via a "second messenger" (e.g., phosphorylation, or intracellular concentration of ions and metabolites), a signal that triggers both rapid and delayed responses. Alternatively, one or more of the rapid responses may produce the signal for the long-term effects. In this model, internalization does not participate in the signaling process; it is instead related to the desensitization of the cell to the growth factor.

(b) EGF generates a signal at the level of the plasma membrane for the rapid responses, and the internalized hormone or its fragments produce an independent signal by interaction with putative intracellular sites for the long-term effects.

(c) EGF binding to the cell surface receptors creates the appropriate perturbation in the receptor molecule which by itself is the active species.

One approach to resolve between these possibilities is to use drugs which inhibit specific stages in the processing of EGF-receptor complexes and to examine their effects on the biological response mediated by the hormone (3, 20, 24, 29, 43). Another approach to resolve between these possibilities is to establish analogues of EGF which bind to EGF receptors and then analyze their biological properties. We have recently reported on the generation of monoclonal antibodies directed against EGF-receptors (35). These antibodies mimic both the early and delayed responses mediated by EGF, suggesting that the receptor rather than the hormone is the important moiety for the transduction of the biological signal.

Here we show, using an EGF analogue CNBr-EGF, that stimulation of DNA synthesis need not occur even if the early part of the series, including changes in metabolism up to 4 h after hormone binding, does occur. It appears, then, that events of activation of protein phosphorylation, ion transport, and induction of enzyme activities either are not required, or else are necessary but insufficient, to trigger DNA synthesis (21). Of course, this interpretation is based on the premise that CNBr-EGF and native-EGF bind to the same class of receptors which, when properly stimulated, initiate the full series of metabolic changes leading to mitosis. The possibility that CNBr-EGF binds to a subset of receptors, whose stimulation triggers only part of the pathways to mitosis, is unlikely since Scatchard analysis of EGF binding to cells indicates the presence of only one class of binding sites (4). Moreover, CNBr-EGF blocks the binding of EGF to its receptors and hence must bind to the same sites as the native hormone (35, 37).

EGF-induced phosphorylation of membrane proteins does not lead to activation of DNA synthesis. However, EGF- or CNBr-EGF-induced phosphorylation could lead to the activation of Na$^+$-K$^+$-ATPase (26) and also to structural changes in the cell's microfilament system. Such a mechanism has been suggested for the src gene product, which in many aspects

resembles the EGF-receptor-associated kinase and which has been shown to affect the activity of Na$^+$-K$^+$-ATPase and the organization of the cytoskeleton (1, 10, 22, 25, 30, 41). Our results make it unlikely that the initial portion of the cascade of protein phosphorylation is a sufficient trigger for mitosis. If the src gene product and EGF-receptor-associated kinase indeed operate via a common pathway (41), then other steps must be required after or in parallel with protein phosphorylation to trigger DNA synthesis.

Although CNBr-EGF is not mitogenic by itself, suboptimal amounts of EGF restore its mitogenic activity. A synergism between hormones was reported for fibroblast growth factor which induces DNA synthesis in 3T3 cells only in the presence of insulin (27). Synergistic effects between hormones are usually interpreted as the convergence of several pathways into a common step initiating DNA synthesis, by increasing the probability for the cell to enter the S state of its cycle (38). In the case of CNBr-EGF, only EGF possesses a synergistic potential while insulin, PGF$_{2\alpha}$ and FGF are unable to restore the mitogenic activity of the inactive CNBr-EGF. Apparently, the critical event for DNA synthesis in which CNBr-EGF is defective seems to be related to EGF receptor and its processing. It is noteworthy that cross-linking of the cell-bound CNBr-EGF by anti-EGF-antibodies restores mitogenic activity and receptor clustering, thus suggesting that receptor clustering plays a role in the initiation of DNA synthesis (33, 37).

The separation of early and late EGF functions tells against models of EGF action in which early changes in cell metabolism, for example, phosphorylation of membrane proteins, provide internal stimuli for the late events of DNA synthesis. Hence our results favor a model of EGF action which involves several biochemical signals. These signals may arise during the various stages of the processing of EGF-membrane receptor complexes (clustering, internalization, and degradation). It may be that a combination of stimuli from surface and internalized hormone-receptor complexes leads to DNA synthesis. Such a combination of stimuli would explain the synergistic effects on DNA synthesis of native EGF and CNBr-EGF.

REFERENCES

1. Ash, J. F., P. K. Vogt, and S. J. Singer. 1976. Reversion from transformed to normal phenotype by inhibition of protein synthesis in rat kidney cells infected with a temperature sensitive mutant of Rous sarcoma virus. Proc. Natl. Acad. Sci. U. S. A. 73:3603-3607.
2. Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole phallacidin. Proc. Natl. Acad. Sci. U. S. A. 77:980-984.
3. Brown, K. D., M. Friedkin, and E. Rozengurt. 1980. Colchicine inhibits epidermal growth factor degradation in 3T3 cells. Proc. Natl. Acad. Sci. U. S. A. 77:480-484.
4. Carpenter, G., K. J. Lembach, M. M. Morrison, amd S. Cohen. 1975. Characterization of the binding of ^{125}I-labeled epidermal growth factor to human fibroblasts. J. Biol. Chem. 250:4297-4304.
5. Carpenter, G., L. King, and S. Cohen. 1978. Epidermal growth factor stimulates phosphorylation in membrane preparation in vitro. Nature (Lond.). 276:409-410.
6. Carpenter, G., L. King, and S. Cohen. 1979. Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. J. Biol. Chem. 254:4884-4891.
7. Carpenter, G., and S. Cohen. 1979. Epidermal growth factor. Annu. Rev. Biochem. 48:193-216.
8. Chinkers, M., J. A. McKanna, and S. Cohen. 1979. Rapid induction of morphological changes in human carcinoma cells A-431 by epidermal growth factor. J. Cell. Biol. 83:260-265.

9. Cohen, S., G. Carpenter, and L. King, Jr. 1979. Epidermal growth factor-receptor-protein kinase interactions. *J. Biol. Chem.* 255:4834–4842.

10. Collett, M. S., A. F. Purchio, and R. L. Erikson. 1980. Avian sarcoma virus transforming protein pp60^src shows protein kinase activity specific for tyrosine. *Nature (Lond.).* 285:167–169.

11. Fabricant, R. N., J. E. DeLarco, and G. J. Todaro. 1977. Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* 74:565–569.

12. Gorden, P., J. L. Carpentier, S. Cohen, and L. Orci. 1978. Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 75:5025–5029.

13. Haigler, H., J. F. Ash, S. J. Singer, and S. Cohen. 1978. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. U. S. A.* 75:3317–3321.

14. Haigler, H. T., T. J. McKanna, and S. Cohen. 1979. Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J. Cell Biol.* 83:82–90.

15. Haigler, H. T., J. A. McKanna, and S. Cohen. 1979. Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J. Cell Biol.* 81:382–395.

16. Holladay, L. A., C. R. Savage, Jr., S. Cohen, and D. Puett. 1976. Conformation and unfolding thermodynamics of epidermal growth factor and derivatives. *Biochemistry.* 15:2624–2633.

17. Hunter, M. W., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.).* 194:495–496.

18. Jimenez de Asua, L. 1980. An ordered sequence of temporal steps regulates the rate of initiation of DNA synthesis in cultured mouse cells. *In* Control Mechanisms in Animal Cells. L. Jimenez de Asua, R. Levi-Montalcini, R. Shields, and S. Iacobelli, editors. Raven Press, New York. pp. 173–198.

19. King, L., G. Carpenter, and S. Cohen. 1980. Characterization by electrophoresis of epidermal growth factor stimulated phosphorylation using A-431 membranes. *Biochemistry.* 19:1524–1528.

20. King, A. C., L. Hernaez-Davis, and P. Cuatrecasas. 1981. Lysosomotropic amines inhibit mitogenesis induced by growth factors. *Proc. Natl. Acad. Sci. U. S. A.* 78:717–721.

21. Lever, J. E., K. M. V. Richmond, A. M. Otto, and L. Jimenez de Asua. 1980. Dissociation of uridine and ^86Rb^+ uptake from stimulation of DNA synthesis in Swiss 3T3 cells. *Cell Biology International Report.* 4:907–916.

22. Marx, J. L. 1981. Tumor viruses and the kinase connection. *Science (Wash. D. C.).* 211:1336–1338.

23. Maxfield, F. R., J. Schlessinger, Y. Schechter, M. C. Willingham, and I. Pastan. 1978. Collection of insulin, EGF and α_2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. *Cell.* 14:805–810.

24. Maxfield, F. R., P. J. A. Davies, L. Klempner, M. C. Willingham, and I. Pastan. 1979. Epidermal growth factor stimulation of DNA synthesis is potentiated by compounds that inhibit its clustering in coated pits. *Proc. Natl. Acad. Sci. U. S. A.* 76:5731–5735.

25. Pollack, R., M. Osborn, and K. Weber. 1975. Patterns of organization of actin and myosin in normal and transformed cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:994–998.

26. Rozengurt, E., and L. A. Heppel. 1975. Serum rapidly stimulates ouabain sensitive ^86Rb^+ influx in quiescent 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:4492–4495.

27. Rozengurt, E. 1980. Stimulation of DNA synthesis in quiescent culture cells: exogenous agents, internal signals, and early events. *Curr. Top. Cell. Regul.* 17:59–88.

28. Savage, C. R. Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative. *J. Biol. Chem.* 247:7609–7611.

29. Savion, N., I. Vlodavsky, and D. Gospodarowicz. 1980. Role of degradation process in the mitogenic effect of epidermal growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 77:1466–1470.

30. Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart. 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. *Cell.* 20:807–816.

31. Schlessinger, J., Y. Schechter, P. Cuatrecasas, M. C. Willingham, and I. Pastan. 1978. Quantitative determination of the lateral diffusion coefficients of the hormone-receptor complexes of insulin and epidermal growth factor on the plasma membrane of cultured fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 75:5353–5357.

32. Schlessinger, J., Y. Schechter, M. C. Willingham, and I. Pastan. 1978. Direct visualization of binding, aggregation and internalization of insulin and epidermal growth factor on living fibroblastic cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:2659–2663.

33. Schlessinger, J. 1980. On the mechanism and role of hormone induced clustering of membrane receptors. *Trends Biochem. Sci.* 5:210–214.

34. Schlessinger, J., and B. Geiger. 1981. Epidermal growth factor induces redistribution of actin and α-actinin in human epidermal carcinoma cells. *Exp. Cell Res.* 134:273–279.

35. Schreiber, A. B., I. Lax, Y. Yarden, Z. Eshhar, and J. Schlessinger. 1981. Monoclonal antibodies against the receptor for epidermal growth factor induced early and delayed effects of epidermal growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 78:7535–7539.

36. Schreiber, A. B., Y. Yarden, and J. Schlessinger. 1981. A nonmitogenic analogue of epidermal growth factor enhances the phosphorylation of membrane proteins. *Biochem. Biophys. Res. Commun.* 101:517–523.

37. Shechter, Y., L. Hernaez, J. Schlessinger, and P. Cuatrecasas. 1979. Local aggregation of hormone-receptor complexes is required for activation by epidermal growth factor. *Nature (Lond.).* 278:835–838.

38. Smith, J. A., and L. Martin. 1973. Do cells cycle? *Proc. Natl. Acad. Sci. U. S. A.* 70:1263–1267.

39. Thom, D., A. S. Powell, C. W. Lloyd, and D. A. Rees. 1977. Rapid isolation of plasma membranes in high yield from cultured fibroblasts. *Biochem. J.* 168:187–194.

40. Tramontano, D., A. Avivi, F. S. Ambesi-Impiombato, L. Barak, B. Geiger, and J. Schlessinger. 1982. Thyrotropin induces changes in the morphology and the organization of microfilament structures in cultured thyroid cells. *Exp. Cell Res.* In press.

41. Ushiro, H., and S. Cohen. 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membrane. *J. Biol. Chem.* 255:8363–8365.

42. Wrann, M. M., and C. F. Fox. 1979. Identification of epidermal growth factor receptors in a hyperproducing human epidermoid carcinoma cell line. *J. Biol. Chem.* 254:8083–8086.

43. Yarden, Y., M. Gabbay, and J. Schlessinger. 1981. Primary amines do not prevent the endocytosis of epidermal growth factor into 3T3 fibroblasts. *Biochem. Biophys. Acta.* 674:188–203.