Vol. 101, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1981 Pages 517-523

A NON-MITOGENIC ANALOGUE OF EPIDERMAL GROWTH FACTOR ENHANCES THE PHOSPHORYLATION OF ENDOGENOUS MEMBRANE PROTEINS

Alain B. Schreiber, Yosef Yarden and Joseph Schlessinger

Department of Chemical Immunology
The Weizmann Institute of Science, Rehovot 76100, Israel

Received April 30, 1981

Summary: The cyanogen bromide cleaved analogue of Epidermal Growth Factor (EGF) binds to EGF receptors with reduced affinity but fails to induce DNA synthesis. However, at similar receptor occupancy, cyanogen bromide cleaved EGF is as potent as EGF in enhancing the phosphorylation of endogenous membrane proteins mediated by the EGF specific, cyclic nucleotide independent protein kinase. Two possibilities arise concerning the biological role of the EGF induced membrane protein phosphorylation: 1) it is not related to the triggering of DNA synthesis by EGF and does not serve as "second messenger" for the growth factor. 2) it is a necessary but not sufficient biochemical signal for the activation of DNA synthesis by EGF.

INTRODUCTION

Epidermal Growth Factor (EGF) is a potent mitogen for various cells *in vitro* and *in vivo* (1). After binding to specific cell membrane receptors EGF induces an array of early and delayed physiological processes. Early responses include the stimulation of nutrient transport (1), the stimulation of Na^+-K^+-ATPase (2) and the induction of alterations in cell morphology (3) and in cytoskeletal organization (4). Delayed effects of EGF include the activation of cytoplasmic enzymes and the stimulation of DNA synthesis (1). Recently Cohen and coworkers have shown that EGF enhances the activity of a membrane associated, cyclic nucleotide independent protein kinase (5-7) specific for tyrosine residues (8). Since various growth factors were shown to increase the phosphorylation of cellular proteins in intact cells it was proposed that the EGF associated protein phosphorylation may serve as a "second messenger" for the induction of the mitogenic response of the hormone (5,8-10).

EGF has a single methionyl residue at position 21. Cleavage with cyanogen bromide results in the formation of two polypeptide chains connected by the

0006-291X/81/140517-07$01.00/0

the three disulphide bonds of the molecule (11). The cyanogen bromide cleaved EGF (CNBr-EGF) retains approximately 10% of the binding activity of EGF but is virtually devoid of mitogenic activity *in vitro* (11) and *in vivo* (12). In contrast to EGF, CNBr-EGF does not induce the patching of EGF-membrane receptor complexes. The addition of bivalent anti-EGF antibodies restores both patch formation and mitogenic activity of this analogue (11).

In this communication we report that, at similar receptor occupancy, the non-mitogenic CNBr-EGF is as potent as EGF in stimulating the phosphorylation of endogenous membrane proteins. Hence CNBr-EGF may serve as a molecular tool to study the involvement of membrane protein phosphorylation in the regulation of the mitogenic activity of EGF.

MATERIALS AND METHODS

Materials. EGF was purified from the submaxillary glands of adult male mice by the method of Savage and Cohen (13). ^{125}I-EGF was prepared by the chloramine T method (14) to a specific activity of 80,000 - 120,000 cpm/ng. CNBr-EGF was prepared and characterized for purity as previously reported (12). [γ-^{32}P]-ATP (16 Ci/mmol) and [methyl-^{3}H]-thymidine (47 Ci/mmol) were purchased from the Radiochemical Centre, Amersham.

Cells. Human epidermoid carcinoma cells (A-431 cell line) were kindly provided by Dr. G. Todaro. Human foreskin fibroblasts (HFF) from primary cultures were obtained from Dr. M. Gabbay. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Membranes from A-431 cells were prepared and characterized as reported (6).

Binding assays. a) to living cells: A-431 cells or HFF were plated in 24 wells Costar trays and assayed at confluence in DMEM containing 0.1% bovine serum albumin (BSA) buffered at pH 7.4 with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes). After incubation at 25°C for thirty minutes with varying amounts of CNBr-EGF or native EGF, ^{125}I-EGF was added (final concentration of 10 ng/ml) and cells were further incubated at 25°C for thirty minutes. Cells were washed four times with buffer, lysed with 0.1 N NaOH and the cell-associated radioactivity was counted.

b) to membranes: 10 µg A-431 membrane protein were incubated with varying amounts of CNBr-EGF or native EGF prior to addition of ^{125}I-EGF as for cells. The reaction mixture was filtered rapidly on Millipore GSWP filters, washed extensively with buffer and the radioactive content of the filters was counted. Nonspecific binding was assessed both for cells and membranes by measuring the radioactivity in the presence of 2 µg/ml EGF and it did not exceed five percent of the total binding. The variability in all the binding studies was less than 5% of the corresponding values.

Stimulation of DNA synthesis. Cells were grown to confluence in 24-well Coster trays and then maintained in medium containing 0.5% fetal calf serum for two days prior to the assay. EGF or CNBr-EGF were added to the cells for eighteen

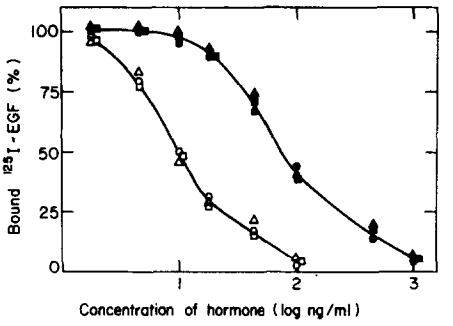

Fig. 1. Competition of binding of ^{125}I-EGF by either EGF or CNBr-EGF. Inhibition of binding of 10 ng/ml ^{125}I-EGF to monolayers of HFF (Δ-Δ), A-431 cells (o-o) and to a membrane preparation from A-431 cells (□ -□) by increasing concentrations of native EGF (open symbols) or CNBr-EGF (closed symbols). Results are the average of three independent experiments.

hours prior to a four hours pulse with 0.5 μCi [methyl-^{3}H] thymidine. Cells were washed three times and the acid precipitable radioactivity was determined in a liquid scintillation counter.

Phosphorylation reaction. 30 μg A-431 membrane protein were preincubated at 0°C for fifteen minutes with varying concentrations of EGF or CNBr-EGF in 20 mM Hepes buffer (pH 7.4) containing 50 mM $MgCl_2$ and 0.01% BSA in a final volume of 50 μl. The phosphorylation reaction was initiated by the addition of 10 μl [γ-^{32}P]-ATP (50 μM) and the incubation at 0°C was continued for ten minutes. The reaction was stopped by pipetting 50 μl onto Whatmann no. 3 filter squares which were dropped into cold 10% trichloroacetic - 0.01 M pyrophosphate. Filter papers were washed extensively with this solution, extracted with ethanol and ether, dried and their radioactive content measured. Alternatively phosphorylation reactions were terminated by the addition of sample buffer for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (15) and boiling for five minutes. Membrane components were separated by SDS-PAGE on 6-15% gradient gels by the method of Laemmli (15). Gels were vacuum dried and autoradiography was performed with preflashed Kodak RP Royal X-omat films and intensifying screens.

RESULTS AND DISCUSSION

The binding properties of CNBr-EGF to monolayers of HFF or A-431 cells and to A-431 plasma membranes are presented in Fig. 1. CNBr-EGF competes for the binding of ^{125}I-EGF on these three preparations at concentrations approximately one order of magnitude higher than the native hormone. CNBr-EGF has less than 0.2% of the potency of native EGF in stimulating thymidine incorporation into quiescent HFF (Fig. 2). Hence CNBr-EGF exhibits at least a fiftyfold disparity between its binding capacity towards EGF receptors and its ability to stimulate

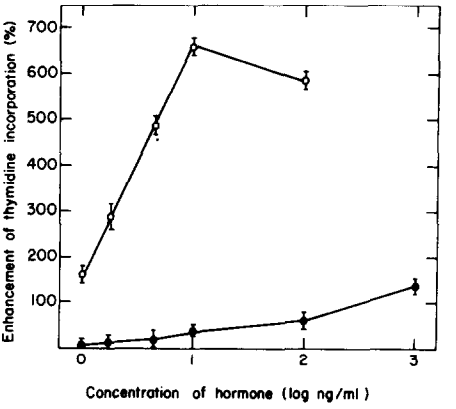

Fig. 2. Induction of thymidine incorporation in quiescent fibroblasts by either EGF or CNBr-EGF. Enhancement of [methyl-3H] thymidine incorporation in quiescent HFF by EGF (o-o) and CNBr-EGF (•-•). Cells were incubated at 37°C for 18 hours with the growth factor prior to a 4 hours pulse with [methyl-^{3}H]-thymidine and determination of acid precipitable radioactivity.

DNA synthesis. Similar binding and mitogenic properties of CNBr-EGF were reported for mouse 3T3 fibroblasts (11). Moreover CNBr-EGF was reported to be non-mitogenic in vivo (12).

Cohen and coworkers have shown that plasma membranes isolated from various cell sources including human epidermoid carcinoma cells (A-431 cell line), HFF and human placental cells contain a cyclic nucleotide independent protein kinase which is specifically activated by EGF (5,6). The EGF sensitive protein kinase phosphorylates both exogenous substrates and several endogenous membrane proteins. The major phosphorylated membrane proteins are two glycoproteins of molecular weight 170,000 and 150,000 (7). Membranes prepared from A-431 cells contain a high number of EGF receptors and therefore serve as the most sensitive system to analyze the properties of the EGF specific protein kinase. In a striking analogy to oncogenic RNA virus-associated protein kinases essential for the malignant transformation (3,10,16,17),the EGF sensitive kinase phosphorylates tyrosine residues (8). Moreover both EGF and transformation with oncogenic RNA viruses induce similar effects on cell morphology (3,18) and on cytoskeletal organization (4,19). This together with the observation that trans-

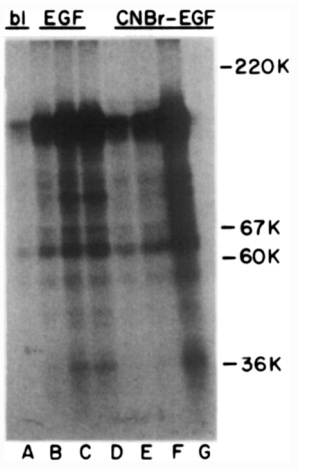

Fig. 3. Enhancement of membrane protein phosphorylation by either EGF or CNBr-EGF. Autoradiography (24 hours exposure) of SDS-PAGE (6-15% gradient slab gel) analysis of A-431 membranes incubated with [γ-^{32}P]-ATP for 10 minutes at 0°C and preincubated for 15 minutes at 0°C with buffer (lane A, blank), EGF (60 ng/ml, lane B; 120 ng/ml, lane C; 300 ng/ml, lane D) or CNBr-EGF (300 ng/ml, lane E; 600 ng/ml, lane F; 1200 ng/ml, lane G). Molecular weight markers are ferritin half unit (220K), BSA (67K), catalase subunit (60K) and lactate dehydrogenase subunit (36K).

formation by murine sarcoma virus or Abelson leukemia virus decreases the number of available EGF receptors on infected cells (20,21) may indicate that EGF and transformation specific effects operate via related or even common pathways. Consequently and because of the pleiotypic substrate specificity of protein kinases it was suggested that the EGF induced protein phosphorylation acts as a "second messenger" for the mitogenic response of the hormone (5,8,22).

In order to examine this possibility we have tested the ability of the non-mitogenic CNBr-EGF to stimulate the EGF sensitive protein kinase. Fig. 3 shows the effect of increasing concentrations of EGF or CNBr-EGF on the phosphorylation of endogenous proteins in membranes prepared from A-431 cells. Similar patterns were obtained with EGF and CNBr-EGF with two major phosphorylated proteins of 170,000 and 150,000 dalton. The quantitative enhancement of protein phosphorylation induced by EGF or CNBr-EGF is shown in Fig. 4. For a similar degree of phosphorylation a tenfold higher concentration of CNBr-EGF

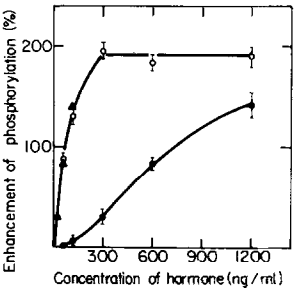

Fig. 4. Enhancement of protein phosphorylation in A-431 membrane preparation. Membranes were preincubated for 15 minutes at 0°C with increasing concentrations of either EGF (o-o) or CNBr-EGF (●-●) prior to the addition of [γ-^{32}P)-ATP and quantitation of [^{32}P]-phosphoproteins as described under Materials and Methods. Calculated values of the stimulation of phosphorylation by CNBr-EGF at same receptor occupancy as native EGF after correction for the decreased affinity of CNBr-EGF towards EGF receptors (▲-▲).

over EGF must be used in order to correct for the reduced affinity of CNBR-EGF towards EGF receptors. This means that at the same receptor occupancy the non-mitogenic CNBr-EGF, which also fails to induce receptor patching, is a full agonist of EGF for inducing membrane protein phosphorylation.

Two interesting possibilities arise concerning the biological role of the EGF induced protein phosphorylation:

1) The EGF associated phosphorylation does not play any role in the process of EGF induced receptor patching and does not serve as a "second messenger" for the mitogenic effect of the hormone.

2) More likely, the EGF induced protein phosphorylation is a necessary but not sufficient signal for the induction of DNA synthesis. This would indicate that the activation of DNA synthhsis by EGF is not the consequence of a single step involving receptor occupancy followed by the induction of protein phosphorylation (a "single hit" mechanism). Rather, several biochemical signals are generated during the various stages of the processing of EGF-membrane receptor complexes (clustering, internalization and degradation)(23). CNBr-EGF is defective in one or more of these signals which are triggered subsequent to the induction

of membrane protein phosphorylation and which are required for the mitogenic effect of EGF.

ACKNOWLEDGEMENTS

This study was supported by grants from NIH (CA-25820) and from U.S.-Israel Binational Science Foundation. A.B.S. is a recipient of a long term EMBO fellowship. We acknowledge fruitful discussions with Drs. G. Hillman and Y. Shechter.

REFERENCES

1. Carpenter, G. and Cohen, S. (1979) Ann Rev. Biochem. 48, 193-216.
2. Rozengurt, E. and Heppel, L. (1975) Proc. Natl. Acad. Sci. USA 72, 4492-4495.
3. Chinkers, M., McKanna, T.J.A. and Cohen, S. (1979) J. Cell Biol. 83, 260-265.
4. Schlessinger, J. and Geiger, B. (1981) Exp. Cell Res. (in press).
5. Carpenter, G., King, L. Jr. and Cohen, S. (1978) Nature 276, 409-410.
6. Carpenter, G., King, L. Jr. and Cohen, S. (1979) J. Biol. Chem. 254, 4884-4891.
7. King, L. Jr., Carpenter, G. and Cohen, S. (1980) Biochemistry 19, 1524-1528.
8. Ushiro, H. and Cohen, S. (1980) J. Biol. Chem. 255, 8363-8365.
9. Chinkers, M. and Cohen, S. (1981) Nature 290, 516-519.
10. Kudlow, E.J., Buss, E.J. and Gill, G.N. (1981) Nature 290, 519-521.
11. Shechter, Y., Hernaez, L., Schlessinger, J. and Cuatrecases, P. (1979) Nature 278, 835-838.
12. Holladay, L.A., Savage, C.R. Jr., Cohen, S. and Puett, D. (1976) Biochemistry 15, 2624-2633.
13. Savage, C.R. Jr. and Cohen, S. (1972) J. Biol. Chem. 247, 7609-7611.
14. Hunter, M.W. and Greenwood, F.C. (1962) Nature 194, 495-496.
15. Laemmli, U.K. (1970) Nature 227, 680-682.
16. Sefton, B.M., Hunter, T., Beemon, K. and Eckhart, W. (1980) Cell 20, 807-816
17. Collett, M.S., Purchio, A.F. and Erikson, R.L. (1980) Nature 285, 167-169.
18. Ash, J.F., Vogt, P.K. and Singer, S.J. (1976) Proc. Natl. Acad. Sci. USA 73, 3603-3607.
19. David-Pfeuty, T. and Singer, S.J. (1980) Proc. Natl. Acad. Sci. USA 77, 6687-6691.
20. Todaro, G., De Larco, J.E. and Cohen, S. (1976) Nature 264, 26-30.
21. Blomberg, J., Reynolds, J.H. Jr, Van de Ven, W.J.M. and Stephenson, J.R. (1980) Nature 286, 504-507.
22. Marx, J.L. (1981) Science 211, 1336-1338.
23. Schlessinger, J. (1980) Trends in Biochem. Sci. 5, 210-214.