Characterization by Electrophoresis of Epidermal Growth Factor Stimulated Phosphorylation Using A-431 Membranes†

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ABSTRACT: Addition of epidermal growth factor (EGF) in vitro to membranes prepared from the human epidermoid carcinoma cell line A-431 stimulated ^{32}P incorporation from [γ-^{32}P]ATP into specific membrane components as detected by sodium dodecyl sulfate gel electrophoresis and autoradiography. Although EGF increased the phosphorylation of a number of membrane proteins, two components which appear to be glycoproteins with molecular weights of 170 000 and 150 000 were primarily affected. All of the membrane components phosphorylated in the presence or absence of EGF were digested by Pronase or trypsin except a low molecular weight band which presumably contains labeled phospholipids. Dibutyryl-cAMP, cAMP, or cGMP did not stimulate ^{32}P incorporation into any component of the A-431 membranes in the presence or absence of EGF. The A-431 membrane preparations were demonstrated to be capable of phosphorylating exogenous proteins such as histones by an EGF-sensitive reaction. The enhanced phosphorylation of the individual membrane components was specific for EGF; other peptide hormones and concanavalin A were ineffective. An EGF-stimulated ^{32}P incorporation also was detected in endogenous components of membrane preparations from normal human term placenta and cultured human foreskin fibroblasts. These findings suggest that one of the biochemical consequences of the formation of EGF receptor complexes is increased phosphorylation of several membrane proteins by a cyclic nucleotide independent, membrane-bound, protein kinase.

Epidermal growth factor is a mitogenic polypeptide found in many species including humans which enhances cell proliferation in vivo and in cell culture [see reviews by Carpenter & Cohen (1978, 1979)]. Critical to understanding the mechanism of action of this and other polypeptide hormones is the development and analysis, in biochemical terms, of subcellular systems that are responsive in vitro to addition of these extracellular signals. We have recently described a preparation of membranes from A-431 tumor cells that not only has a high binding capacity for [^{125}I]EGF[1] but also has a protein phosphorylation system that is stimulated in vitro by the addition of EGF (Carpenter et al., 1978, 1979). In this system EGF increased approximately threefold the net ^{32}P incorporation from [γ-^{32}P]ATP into the A-431 membranes. The EGF-sensitive membrane-associated phosphorylation system appeared to be capable of using both endogenous substrates and exogenous substrates such as histones, but not casein, in a cyclic nucleotide independent manner. The objectives of the investigation described herein are (1) to determine whether EGF or other reactants such as cyclic nucleotides have a selective effect on the phosphorylation of individual membrane components, (2) to characterize the phosphorylated membrane components, and (3) to demonstrate the direct phosphorylation of exogenous proteins by the A-431 membranes.

Materials and Methods

Materials. Mouse EGF was isolated by procedures previously described (Savage & Cohen, 1972). cAMP, dibutyryl-cAMP, cGMP, nucleotide triphosphates, and histones (Type II-A) were from Sigma. Ribonuclease was obtained from Worthington Biochemicals. Growth hormone (GH), follicle-stimulating hormone (FSH), thyrotropin, prolactin, and luteinizing hormone (LH) were obtained from NIH. [γ-^{32}P]ATP and [γ-^{32}P]GTP with specific activities of 20 and 8 Ci/mmol, respectively, were from New England Nuclear. The A-431 human epidermoid carcinoma cells were provided by Dr. J. DeLarco (NIH).

Growth of A-431 Cells and Preparation of Membrane Fractions. The A-431 cells were grown in 100-mm Falcon dishes containing Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (Flow Laboratories) and gentamycin (Microbiological Associates). Membranes were prepared by the procedures described by Thom et al. (1977) and characterized as described previously (Carpenter et al., 1978, 1979). Protein was quantitated by the procedure of Bradford (1976) using γ-globulin as a standard.

Standard Membrane Phosphorylation Reaction. The reaction mixtures (40 μL) used to investigate the endogenous phosphorylation contained the following unless otherwise indicated: A-431 membranes (30 μg); Hepes buffer (20 mM, pH 7.4); $MnCl_2$ (2 mM); 0.01% bovine serum albumin; [γ-^{32}P]ATP (15 μM, 8×10^5 cpm); EGF (35 ng, 120 nM). The reaction was initiated by the addition of labeled ATP. All incubations were carried out at 0 °C for the indicated times. The reaction was stopped by the addition of 40 μL of Na$DodSO_4$ sample buffer (Laemmli, 1970) and heating at 100 °C for 5 min.

Gel Electrophoresis and Autoradiography. The A-431 membrane components were separated by Na$DodSO_4$ gel electrophoresis (7.5% acrylamide unless otherwise noted) by the method of Laemmli (1970). Molecular weight standards used for calibration of the gels were the following: human erythrocyte spectrin, 240 000 and 220 000; β-galactosidase, 130 000; BSA, 68 000; carbonic anhydrase, 29 000; hemoglobin,

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[1] Abbreviations used: EGF, epidermal growth factor; cAMP (cGMP), adenosine (guanosine) cyclic 3′,5′-monophosphate; BSA, bovine serum albumin; Na$DodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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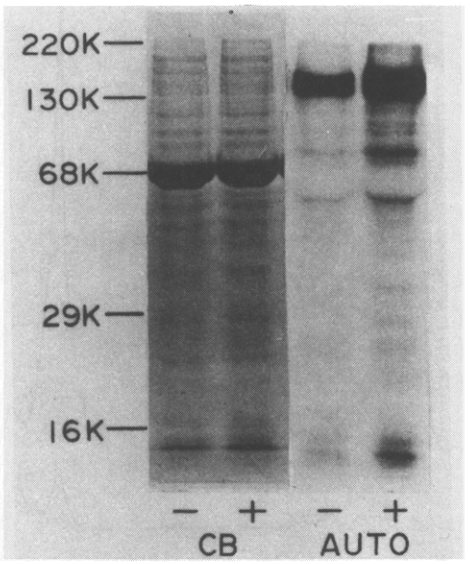

FIGURE 1: Electrophoresis, Coomassie Blue staining, and autoradiography of A-431 membranes incubated with [γ-^{32}P]ATP in the absence (−) or presence (+) of EGF. The standard phosphorylation assays of A-431 membranes (30 μg, 0 °C, 5 min) were performed, and the samples were subjected to $NaDodSO_4$ gel electrophoresis, Coomassie Blue staining (CB), and autoradiography (AUTO, 72-h exposure) as described under Materials and Methods. EGF was present at a final concentration of 120 nM.

16 000. The gels were fixed and stained with periodic acid–Schiff base (PAS), Coomassie Blue, or Stains-all as previously described (King & Morrison, 1976). The slab gels were dried under vacuum, and autoradiography (1–10 days) was performed with Kodak RP Royal X-O Mat film.

Quantitation of ^{32}P Incorporation into A-431 Membranes. The ^{32}P incorporation into membrane components separated on $NaDodSO_4$ gels was analyzed by two procedures. Autoradiographs of the gels were scanned on a Corning Model 740 densitometer, and portions of the densitometric tracings were cut out by hand and weighed. A more direct quantitation of ^{32}P incorporation was performed by preparing 2-mm slices of the slab gel by using a Mickle slicer (Eppendorf) and counting the radioactivity in a gas flow counter.

Results

$NaDodSO_4$ Gel Electrophoresis of Phosphorylated and Nonphosphorylated Proteins. The separation of A-431 membrane components into molecular weight classes by $NaDodSO_4$ gel electrophoresis is shown in Figure 1. Coomassie Blue staining (CB, Figure 1) indicates the presence of many protein components in this crude membrane preparation. The migration of protein components was not altered by incubation with EGF prior to electrophoresis. The heavily stained band which migrates at 68 000 in both the control preparation and the one incubated with EGF is BSA used in the reaction mixtures to prevent adsorption of EGF to plastic or glass. Also presented in Figure 1 are autoradiographs (AUTO) of A-431 membranes phosphorylated in the presence or absence of EGF and separated by $NaDodSO_4$ electrophoresis. The data show that (1) EGF increased the phosphorylation of many membrane components, particularly those having molecular weights of approximately 170 000, 150 000, and 80 000, (2) proteins phosphorylated in the presence of EGF are also phosphorylated, but to a lesser extent, in the absence of the growth factor, and (3) there is no apparent correlation between the intensity of Coomassie Blue staining and the amount of phosphorylation.

Quantitation of ^{32}P Incorporation. Two procedures have been used to quantitate the amount of ^{32}P present in membrane components separated on $NaDodSO_4$ gels. Densitometric tracing of autoradiographs from the gels is a reliable method to compare, on a relative basis, the level of ^{32}P incorporation into membrane components. By cutting out and weighing portions of the tracings corresponding to individual bands, the increased ^{32}P incorporation in the presence of EGF is approximately threefold for each of the 170 000, 150 000, and 80 000 molecular weight components (data not shown). The phosphorylation of other components with molecular weights of 60 000 and 22 500 was frequently increased in the presence of EGF but could not be consistently demonstrated. More direct and accurate quantitation of ^{32}P incorporation was obtained by slicing the gels and counting the radioactivity present in each slice. The results of this procedure (Figure 2) show two- to threefold increases of ^{32}P into the same EGF-sensitive bands. This method, however, produced less resolution of individual membrane components than was obtained by autoradiography and scanning.

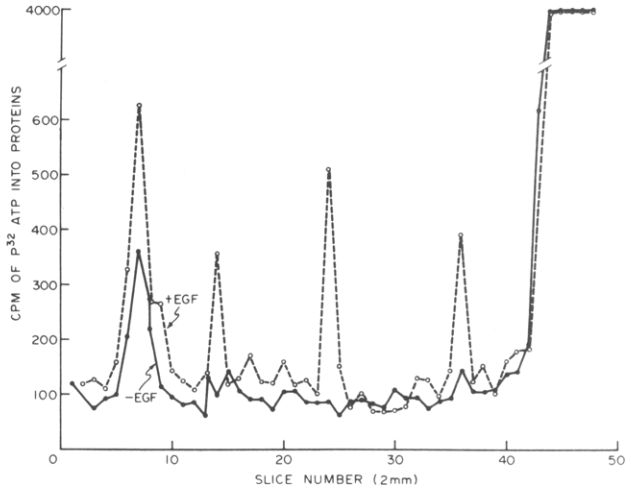

FIGURE 2: Direct quantitation of ^{32}P incorporated into A-431 membrane components in the absence (−) or presence (+) of EGF. The standard phosphorylation assays of A-431 membranes (30 μg, 0 °C, 5 min) were performed. The samples were subjected to $NaDodSO_4$ gel electrophoresis; the gels were cut into 2-mm slices, and the slices were counted in a gas flow counter as described under Materials and Methods. EGF was added to a final concentration of 120 nM.

Specificity of the EGF Stimulation of Membrane Phosphorylation. In our previous work we found that enhanced incorporation of total ^{32}P into A-431 cell membranes could not be produced by other peptide hormones or agents (Carpenter et al., 1979). Using the more sensitive technique of electrophoresis and autoradiography to examine the phosphorylation of separated membrane components, we have tested the ability of other hormones [fibroblast growth factor (FGF), 73 ng/mL; 100 ng/mL FSH, LH, prolactin, insulin, growth hormone, glucagon, or thyrotropin] and concanavalin A (M_r 30 000, 150 ng/mL) to affect the phosphorylation of individual membrane components. In these experiments (data not shown) no agent other than EGF significantly stimulated the endogenous phosphorylation.

Effect of EGF and Other Reaction Components on Endogenous Phosphorylation. The effects of increasing concentrations of EGF on the phosphorylation of separated membrane components are shown in Figure 3. These results indicated that the phosphorylation of many membrane components, especially those at the 170 000, 150 000, 80 000, and 22 500 molecular weight regions, was dependent upon the EGF concentration. Other minor phosphorylated membrane components were noted (M_r 60 000, 55 000, 40 000, or 30 000) in some experiments, but they could not be consistently repro-

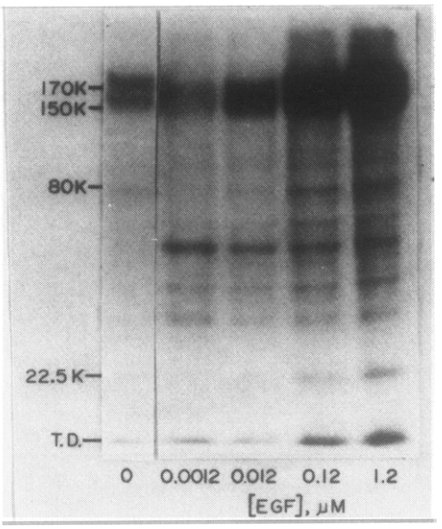

FIGURE 3: Effect of EGF concentration on endogenous phosphorylation of A-431 membranes. The standard phosphorylation assays of A-431 membranes (30 μg, 0 °C, 5 min) were performed, and the samples were subjected to $NaDodSO_4$ gel electrophoresis, Coomassie Blue staining, and autoradiography (48-h exposure) as described under Materials and Methods. EGF was added to the final concentrations indicated. T.D. = tracking dye front.

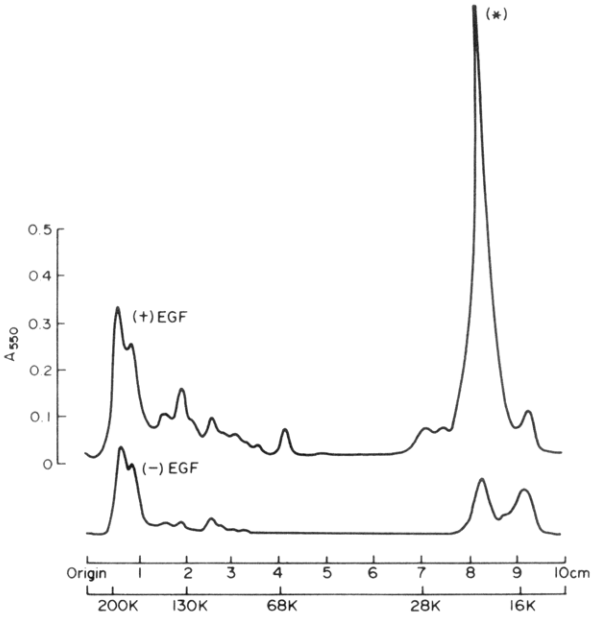

FIGURE 4: Phosphorylation of histones by A-431 membranes in the absence (−) or presence (+) of EGF. The standard phosphorylation assays of A-431 membranes (30 μg, 0 °C, 3 min) were performed in the presence of histone fraction II-A (25 μg/40 μL of reaction mixture). Samples were prepared for electrophoresis, Coomassie Blue staining, autoradiography (48-h exposure), and densitometry as described under Materials and Methods. The correspondence of the position of the histone on the Coomassie Blue stained gel with the position of a major phosphorylated peak on the autoradiograph is indicated by (*). The absorbance scale is the same for both densitometric tracings.

duced. Nearly maximal increases in the phosphorylation of the EGF-stimulated components was observed at an EGF concentration of ~120 nM.

Maximal stimulation of the phosphorylation of each of the EGF-sensitive components occurred in the presence of 1 mM Mn^{2+}. Mg^{2+} at much higher concentrations (20–50 mM) could effectively substitute for Mn^{2+}, and it produced the same pattern of phosphorylation of individual membrane components in both the basal and EGF-stimulated reactions (data not shown). Qualitatively similar autoradiographic patterns of basal and EGF-stimulated protein phosphorylation were seen at all membrane concentrations tested (10–200 μg of protein, data not shown) using either labeled ATP or GTP (data not shown).

Phosphorylation of Exogenous Substrates. Our previous data (Carpenter et al., 1978, 1979) suggested that EGF increased the membrane-catalyzed phosphorylation of certain exogenous proteins such as histones and ribonuclease. Those studies, however, measured total ^{32}P incorporation and did not demonstrate directly whether the exogenous proteins were substrates for the phosphorylation reactions or whether they interacted with membranes in a manner that increased the phosphorylation of endogenous membrane proteins. We, therefore, subjected the phosphorylated mixture of endogenous and exogenous proteins to $NaDodSO_4$ gel electrophoresis, Coomassie Blue staining, and autoradiography. The results (Figure 4) show that exogenous histones are phosphorylated under these conditions and that their phosphorylation is enhanced by EGF, approximately fivefold. Ribonuclease was also shown to be phosphorylated by the A-431 membranes (data not shown).

Effect of cAMP and cGMP on Membrane Phosphorylation. Cyclic nucleotides are known to activate many protein kinases; however, our previous data (Carpenter et al., 1979) showed no stimulatory effect of cyclic nucleotides on total ^{32}P incorporation into A-431 membranes in the presence or absence of EGF. We have considered the possibility that if the phosphorylation of a few individual membrane components were affected by the presence of cyclic nucleotides, this effect might be masked by measuring total ^{32}P incorporation. The addition of 10^{-6} or 10^{-8} M cAMP, dibutyryl-cAMP, or cGMP (data not shown) did not increase the basal or EGF-stimulated phosphorylation of any membrane protein. A small inhibitory effect (~40%) on phosphorylation was noted at higher concentrations of dibutyryl-cAMP (10^{-4} M) but not of cGMP (data not shown).

Nature of the Phosphorylated Components. The phosphorylated membrane components, detected on $NaDodSO_4$ gels, appear to be sensitive to proteolytic enzymes. A-431 membranes were phosphorylated in the presence or absence of EGF for 2.5 min at 0 °C and then incubated briefly with proteases (2 min at 25 °C). Figure 5 shows that trypsin (or Pronase) treatment removed almost all of the phosphorylated components detectable by gel electrophoresis except those at the tracking dye front, which presumably are phospholipids. Examination of the corresponding Coomassie Blue stained gels showed that protease treatment removed much of the stained protein components.

The possible glycoprotein nature of the phosphorylated components was examined by staining membrane components in the gels by the PAS method (King & Morrison, 1976). PAS-stained material was seen in positions corresponding to the 170 000, 150 000, and low molecular weight regions of the $NaDodSO_4$ gels (data not shown). Samples were electrophoresed in $NaDodSO_4$ gels with differing acrylamide concentrations to confirm that this comigration of labeled ^{32}P and PAS-stained components was not coincidental. The PAS-stained components in 5, 7.5, and 10% acrylamide gels always corresponded to the position of the 170 000, 150 000, and low molecular weight phosphorylated components. In the 5% acrylamide gels the 170 000 and 150 000 molecular weight components not only migrated farther into the gels, as expected, but also were comparatively much more diffuse, as detected both by autoradiography and PAS staining. The autoradiographic patterns of other components such as the

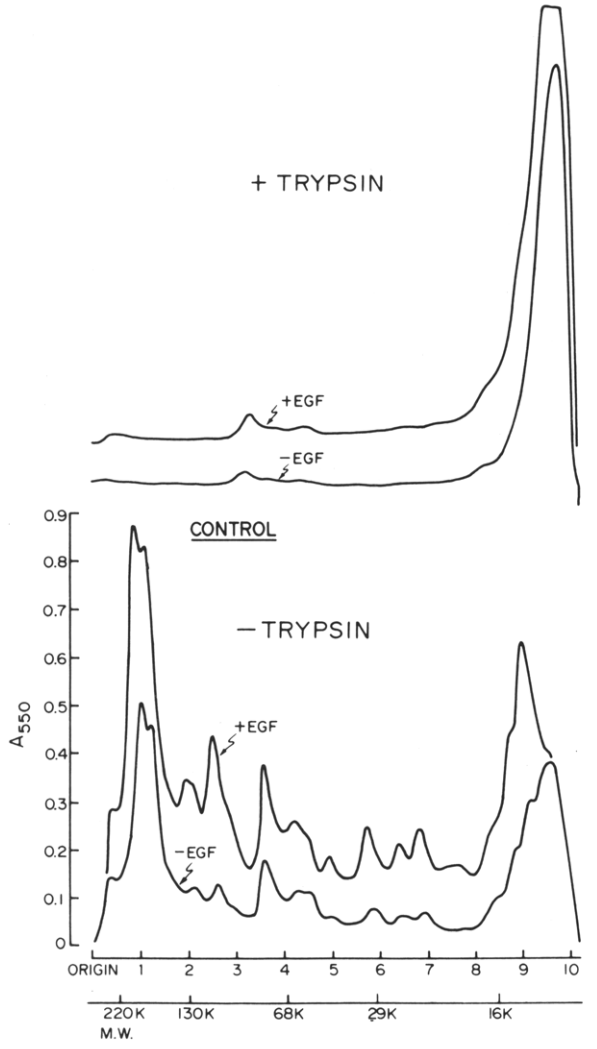

FIGURE 5: Effect of trypsin on A-431 membranes phosphorylated in the absence (–) or presence (+) of EGF. The standard phosphorylation assays of A-431 membranes (30 μg, 0 °C, 2.5 min) were performed in the presence or absence of EGF (120 nM). TPCK-treated trypsin (10 μg/40 μL of mixture) was added to the reaction mixtures and incubated at 25 °C for 2 min with the phosphorylated samples. Laemmli sample buffer (40 μL) was added, the mixture boiled for 5 min, and the sample subjected to electrophoresis, Coomassie Blue staining, autoradiography (48-h exposure), and densitometry as described under Materials and Methods. The absorbance scale is the same for all tracings but is shown only for the untreated control sample.

80 000 or 22 500 molecular weight bands remained as compact, well-defined bands (data not shown).

Membrane Extraction Studies. For determination of whether the EGF-stimulated protein kinase activity and the endogenous substrates were firmly associated with the A-431 membranes, the membrane preparations were washed with a variety of solvents prior to performing the phosphorylation assay. Figure 6 shows that neither the basal nor EGF-stimulated reactions were greatly affected by washing the membranes with 1 M urea, 1 M KCl, or 1 M KSCN. These results suggest that both the endogenous substrates and the EGF-stimulated protein kinase are firmly associated with the A-431 membranes.

Phosphorylation in Other Cell Types. We have examined the effect of EGF on ^{32}P incorporation into membrane preparations derived from cultured human fibroblasts and from term human placenta. Autoradiographs of these membranes, phosphorylated in the presence or absence of EGF, are shown in Figure 7. The results show an enhancement by EGF of ^{32}P incorporation into components of the placental membranes

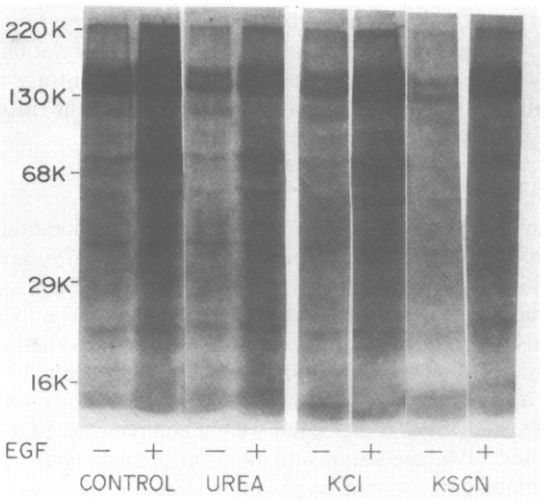

FIGURE 6: Effects of washing and extraction methods on endogenous phosphorylation of A-431 membranes in the absence (–) or presence (+) of EGF. Membrane samples (80 μL, 240 μg of protein) were centrifuged at 50000*g* for 10 min. The supernatant was removed, and the pellets were resuspended in 200 μL of 20 mM Hepes buffer, pH 7.4, containing the following additions: none (control); 1 M KCl and 0.5 mM EDTA; 1 M KSCN and 0.5 mM EDTA; 1 M urea. Each of the mixtures was allowed to stand on ice for 10 min and recentrifuged. The pellets were resuspended in 20 mM Hepes buffer, pH 7.4, and aliquots (40 μg of protein) were assayed by the standard phosphorylation, electrophoresis, and autoradiographic (48-h exposure) procedures described under Materials and Methods.

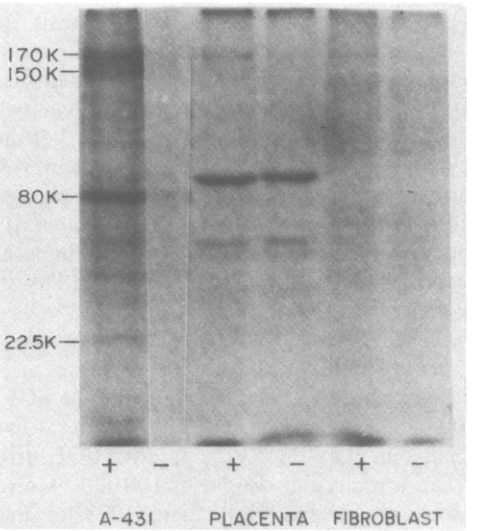

FIGURE 7: Endogenous phosphorylation of membrane components from A-431 cells, human placenta, and human foreskin fibroblasts in the absence (–) or presence (+) of EGF. The standard phosphorylation assays (0 °C, 15 min) were performed on A-431 membranes (30 μg), term human placental membrane fractions (34 μg), or human foreskin fibroblast membranes (100 μg) in the absence or presence of EGF (120 nM). Samples were prepared for electrophoresis and autoradiography (144-h exposure) as described under Materials and Methods.

whose migration in the gel coincides with the 170 000 molecular weight component of the A-431 membranes. Similarly, an EGF-stimulated band in the membranes from human fibroblasts can be detected in the 170 000 molecular weight region of the gel. In neither instance, however, is the EGF effect as intense as in A-431 membranes. The decreased responsiveness of the fibroblast membranes is due, most likely, to the 20-fold lower concentration of EGF receptors in human fibroblasts compared to A-431 cells. The placental membranes, which have been reported to have a binding capacity for [^{125}I]EGF similar to that of A-431 membranes (O'Keefe

et al., 1974), were prepared by a completely different method which may account for the lower level of phosphorylation. Of course, other factors, such as the relative concentrations or activities of protein kinases and phosphatases in the different membranes, also may be important.

Discussion

The observations that EGF stimulates ^{32}P incorporation into isolated membranes (Carpenter et al., 1978, 1979) are extended in this report by gel electrophoresis and autoradiographic methods. It was possible by these procedures to show that EGF stimulated ^{32}P incorporation into individual components of A-431 cell membranes. Although the effects of EGF on membrane phosphorylation most clearly involved the 170000 and 150000 molecular weight components, EGF also increased ^{32}P incorporation into many other components. Since exogenous proteins can be phosphorylated in this system, it is not known whether all of the endogenous phosphorylated components detected are intrinsic membrane proteins. Except for the low molecular weight material, all of the major and minor phosphorylated components appeared to be proteins based on their susceptibility to proteases. This was expected as the major phosphorylated compound detected in A-431 membranes after limited acid hydrolysis was phosphothreonine (Carpenter et al., 1979).

The two components (M_r 170000 and 150000) which are the most heavily phosphorylated in the presence of EGF appear to be glycoproteins based upon the correspondence of PAS staining and radioactivity after electrophoresis in gels of varying acrylamide concentrations. Also, the migration of these components in 5% acrylamide gels was diffuse, which perhaps is indicative of carbohydrate heterogeneity as previously demonstrated for the glycosylated band 3 of the human erythrocyte membrane (Steck, 1974). Carpenter & Cohen (1977) proposed that the EGF receptor was a glycoprotein or glycolipid based on the ability of lectins to block [^{125}I]EGF binding in human fibroblasts. The glycoprotein nature of the EGF receptor also was suggested by Pratt & Pastan (1978) based on their studies of mutant BALB/c 3T3 cells defective in glycoprotein synthesis. Das et al. (1977) have used covalent cross-linking to identify EGF receptor complexes in 3T3 cells. They have estimated the molecular weight of the EGF receptor as 190000 in 3T3 cells (Das et al., 1977). In rat liver membranes (Sahyoun et al., 1978) the putative EGF–urogastrone receptor had a molecular weight of 100000. However, the same laboratory (Hock & Hollenberg, 1978) estimated that the EGF receptor in human placenta was identifiable as a doublet of 180000 and 160000 molecular weight in placenta. It is possible, therefore, that the 170000 and 150000 molecular weight glycosylated components we have observed in the A-431 membranes may represent the EGF receptor which is phosphorylated as an early result of the binding of EGF. On the basis of differing susceptibility to heat denaturation (Carpenter et al., 1979), the EGF receptor itself does not appear to be a protein kinase.

To date our studies of the EGF-stimulated phosphorylation reactions in the A-431 membranes have suggested that the binding of EGF "activates" a membrane-bound, cyclic nucleotide independent protein kinase which is capable of phosphorylating endogenous substrates. All of these interactions (binding, "activation", and phosphorylation) occur rapidly at 0 °C, suggesting that the EGF receptor, the protein kinase, and the endogenous phosphate acceptors are in close proximity. However, direct evidence to describe these topological relationships is not available. It is of interest in this regard that the active site of the membrane-bound, EGF-stimulated, protein kinase is accessible to both endogenous *and* exogenous substrates.

The data in this report also demonstrate that the EGF-sensitive phosphorylation reaction is not unique to the neoplastic A-431 cells; EGF stimulated ^{32}P incorporation in membranes from normal human fibroblasts and from term human placenta. Enhanced membrane protein phosphorylation, therefore, may be a general consequence of EGF binding to membrane receptors.

Acknowledgments

We acknowledge the technical assistance of Linda Coats, Katherine Chen, Marty Reich, and Elaine West.

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