Journal of Cellular Biochemistry 19:205–222 (1982)
Evolution of Hormone-Receptor Systems 1–18

# Modulation of Epidermal Growth Factor-Dependent Protein Phosphorylation in Cell Membrane Preparations by Receptor Down Regulation

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We have reported previously [6] that epidermal growth factor (EGF)-induced down regulation of EGF receptors in normal rat kidney (NRK) cells results in a selective decrease in the in vitro EGF-dependent $^{32}$P-phosphorylation of two membrane phosphoproteins of Mr 170K and Mr 150K. In this report, we further characterized the modulation of $^{32}$P-phosphorylation of the 170K- and 150K-dalton proteins by down regulation with EGF in NRK cells.

While EGF binding to its receptors was a necessary condition to induce loss of EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins, it was not sufficient. Thus, reduction in the temperature of the incubation of cells with EGF from 37°C to 4°C abolished the loss of EGF-dependent phosphorylation of the 170K- and 150K-dalton membrane proteins. When EGF was removed from the medium the EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins was quickly replenished; by 3 hr one-half of the "down regulated" phosphorylation was restored. All EGF-dependent phosphorylating capacity of the 170K- and 150K-dalton protein bands returned by 6 hr after removal of the growth factor. The loss of EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins occurred at physiological EGF concentrations (0.25–25 ng/ml) that span the concentration range which is mitogenic for NRK cells. Exposure of confluent nondividing NRK cells to 1 ng/ml EGF, followed by incubation for 5 hr at 37°C, led to a 50% reduction in the EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins. Maximal reduction (~95%) in the EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins was noted with 10 ng/ml EGF for 5 hr. The EGF-induced loss of EGF-dependent phosphorylation was specific; several other growth factors did not produce phosphorylation loss of the 170K-

Abbreviations used: EGF, epidermal growth factor; NRK, normal rat kidney; DME, Dulbecco-Vogt modified Eagle's medium; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ATP, adenosine 5'-triphosphate; DME/F12+F, DME and Ham's F12 mixed 1:1 and supplemented with glutamine (4 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), insulin (10 μg/ml), and transferrin (5 μg/ml); TENG-N, 50 mM Tris-HCL, pH 7.4/5 mM EDTA/150 mM NaCl/0.25% gelatin/0.05% nonidet P-40; DBM, diazobenzyloxymethyl.

Received April 12, 1982; accepted May 24, 1982.

0730-2312/82/1903-0205$05.00 

and 150K-dalton proteins. Interestingly, NRK cells exposed to growth hormone or transferrin showed a time- and concentration-dependent increase in the EGF-dependent phosphorylation of numerous membrane components, including the 170K- and 150K-dalton proteins.

In addition, in this paper we report the use of immunoautoradiographic method for the detection and analysis of EGF receptors in A431 membranes. A comparison of the membrane receptor for EGF of untreated and down-regulated A431 cells by immunoautoradiography revealed that the EGF binding activity of the 150K-dalton membrane phosphoprotein, which most likely is a proteolytic degradation product of the "native" 170K-dalton EGF receptor protein [19], is decreased in cells subjected to down regulation with EGF. The loss of EGF binding activity by the 150K-dalton membrane protein during down regulation closely paralleled a decrease in the EGF-dependent $^{32}$P-phosphorylation of the 170K- and 150K-dalton protein bands. This loss of both EGF-receptor binding activity and EGF-dependent phosphorylating activity was influenced by cell density.

These results suggest that modulation of EGF receptor activity and membrane protein phosphorylation by EGF and heterologous growth factors may be an important mechanism for regualtion of cellular responsiveness to EGF.

**Key words: epidermal growth factor, immunoautoradiography, down regulation, membrane phosphoproteins, transferrin, growth hormone**

Epidermal growth factor (EGF) is a potent mitogen for many types of cells in culture [1]. EGF also is inhibitory to A431 and $GH_4C_1$ cell proliferation [2,3]. EGF initiates its action by binding to specific receptors on the surfaces of target cells [1]. Following this highly specific association with its receptor, EGF, probably together with its receptor, is internalized by cells and degraded by lysosomal proteases [4]. The internalization and degradation of EGF receptors is associated with a substantial reduction in the number of cell surface receptors for EGF [1,4]. This phenomenon, termed receptor down regulation, depends on EGF concentration, time, and temperature, and has been attributed to internalization and degradation of EGF receptors [1,4]. After removal of EGF, cells in which the EGF receptor has been down-regulated recover their full complement of receptors within several hr [1]. It is not known when in this sequence of events are biochemical signals generated for the numerous metabolic and morphological alterations that are required for mitogenesis.

Among the cell types used to investigate the mitogenic effect of EGF is the NRK cell line [5,6]. NRK cells have been shown to possess EGF receptors and to be responsive to the mitogenic stimuli produced by EGF [7]. Thus, the cultured NRK cells provide a useful system for 1) investigating EGF-mediated protein phosphorylation in cell membranes and its possible connection with cell proliferation [5,6]; 2) correlating changes in EGF-dependent membrane protein phosphorylation that occur when NRK cells change from normal to transformed phenotype [8]; and 3) investigating the possible modulatory effects of heterologous growth factors [9] on EGF-dependent membrane protein phosphorylation that may provide important information on the ways in which one growth factor biologically interacts with a second growth factor.

We have reported previously that in NRK membranes there are numerous components whose phosphorylation can be stimulated by EGF [5]. Among these phosphoproteins, two components of Mr 170K and 150K were primarily affected by EGF [5]. We have also noted that down regulation of EGF receptors in NRK cells by

EGF results in a specific decrease in phosphorylation of the 170K- and 150K-dalton membrane components to subsequent stimulation with EGF in vitro [6]. In this report, we present further characterization of the latter phenomenon with respect to temperature, reversibility of the EGF effect, physiological EGF concentration, and the modulating effects of heterologous growth factors. In addition, we report the use of a powerful immunoautoradiographic method for the detection and analysis of EGF receptors and a comparison by this immunoautoradiographic procedure of the membrane receptor for EGF of untreated and down-regulated A431 cells.

## MATERIALS AND METHODS

### Materials

Mouse EGF (receptor grade) and anti-mouse EGF rabbit antiserum were obtained from Collaborative Research (Waltham, Massachusetts). Bovine luteinizing hormone, rat follicle-stimulating hormone, bovine thyrotropin, rat growth hormone, and rat prolactin were provided by the National Institutes of Health. Human growth hormone (purity determined by radioimmunoassay: thyrotropin $<0.02\%$, luteinizing hormone $<0.05\%$, follicle-stimulating hormone $<0.01\%$, prolactin $<0.05\%$) was from Boehringer Mannheim Biochemicals (West Germany). Sources of other materials were as described previously [5,6].

### Preparation of Transferrin

Human transferrin (Behring Diagnostics, Sommerville, New Jersey) was converted to the 90% iron-saturated form and purified as previously indicated [10]. The purity of the preparation was determined by Coomassie blue staining, $^{125}$I-labeling of transferrin (Enzymobeads, Bio-Rad, Richmond, California), $NaDodSO_4$-polyacrylamide gel electrophoresis, and autoradiography. Only one major Coomasie blue stained or labeled transferrin band was observed (purity greater than 99%).

### Cell Culture and Membrane Isolation

NRK-B clone 18 were provided by Dr. R. Ting (Biotech Research, Inc. Rockville, Maryland). Human epidermal carcinoma A-431 cells were provided by Dr. G. Todaro (NCI). The cells were grown in 100-mm culture dishes containing DME supplemented with 10% (v/v) calf serum as previously described [5]. Membranes were prepared by the method of Thom et al [11] as described previously [5].

### Down Regulation of EGF Receptors

Cells were grown in 100-mm culture dishes containing DME supplemented with 10% calf serum. After 48 hr, confluent or subconfluent monolayers were washed twice with serum-free DME medium. Then, unless otherwise indicated, the cells were incubated with 5 ml of defined medium (DME/F12 + F) containing EGF at 37°C in the tissue culture incubator. EGF was added at 0.01-1200 ng/ml. After set incubation periods, the incubation media were removed and the monolayers were processed for membrane isolation as indicated above.

### Phosphorylation of Membrane Proteins and Gel Electrophoresis

The reaction mixtures contained the following: NRK membranes (62.5 $\mu$g of protein) or A-431 membranes (100 $\mu$g of protein); Hepes buffer (20 mM, pH 7.4); $MnCl_2$ (2mM); 0.125% bovine serum albumin; [$\gamma$ -$^{32}$P] ATP (0.5 $\mu$M, $1 \times 10^6$

cpm); EGF (36 ng, 120 nM) in a final volume of 50 $\mu$l. The phosphorylation assay was performed at 0°C as described previously [5].

$NaDodSO_4$ polyacrylamide gel electrophoresis was carried out in exponential gradient gels ranging from 5% to 9% acrylamide using the buffer system described by Laemmli [12].

### Electrophoretic Transfer of Membrane Proteins From $NaDodSO_4$/ Polyacrylamide Gels to DBM-Paper

$NaDodSO_4$/polyacrylamide gel electrophoresis was carried out as indicated above. Membrane samples containing 100 $\mu$g of protein were applied per lane. After electrophoresis, the proteins were transferred to DBM-paper [13]. Aminobenzyloxymethyl (ABM)-paper was prepared according to Alwine et al [13] or was obtained from Schleicher & Schuell (Keene, New Hampshire). ABM-paper was converted to DBM form as described by Alwine et al [13]. The apparatus utilized in the transfer procedure was from EC Apparatus (St Petersburg, Florida) or Bio Rad (Richmond, California). The transfer conditions were similar to those described by Symington et al [14].

### $^{125}$I-Labeled Protein A

$^{125}$I-labeled protein A was purchased from New England Nuclear (Boston, Massachusetts) or was prepared by lactoperoxidase procedure (Enzymobeads, Bio Rad). Protein A (Sigma, St Louis, Missouri) labeled with $^{125}$I by the lactoperoxidase procedure was separated from unreacted $^{125}$I by exclusion chromatography with a G-25 Sephadex column.

### Immunoautoradiography of DBM-Paper Containing Gel Protein Transfers

To detect EGF receptors by immunoautoradiography, the DBM-paper protein transfers were treated as follows: The DBM-paper onto which the proteins had been transferred was inactivated [15], rinsed in water and blotted on Whatman 3 MM paper [14]. Unless otherwise noted, the DBM-paper was incubated with EGF (100 ng/ml) in TENG-N [14], 50–100 $\mu$l/cm$^2$, in plastic boiling bags (Seal-N-Save Sears) for 1 hr at 37°C. The paper was then washed for 3 hr at 37°C with 800 ml of the same buffer (without EGF). The DBM-paper was next incubated in plastic bags for 16 hr at 37°C with anti-EGF antiserum diluted 1:16 in TENG-N and then washed as above. The final incubation was for 2 hr at 37°C with $^{125}$I-labeled protein A ($3 \times 10^5$ cpm/ml) in TENG-N in a plastic bag. After the DBM-paper was rinsed briefly with water, it was washed extensively in 250 ml of 50 mM Tris HCl, pH 7.4/5 mM EDTA/ 1 M NaCl/0.25% gelatin/0.4% Sarkosyl (Sigma) at 37°C for approximately 24 hr [14]. The DBM-paper was rinsed with water, blotted, air dried, and autoradiographed by using Kodak X-Omat film and a DuPont Cronex$^R$ Quanta III intensifying screen at −70°C.

The DBM-paper protein transfers were incubated with urea and 2-mercaptoethanol to remove $^{125}$I-labeled protein A and antibody as described elsewhere [15]. After this treatment, the erased DBM-paper transfer can be assayed again as described above.

### Other Procedures

Protein was determined in triplicate by the method of Lowry [16] using bovine serum albumin as standard. Radioactive bands were quantitated by scanning with an

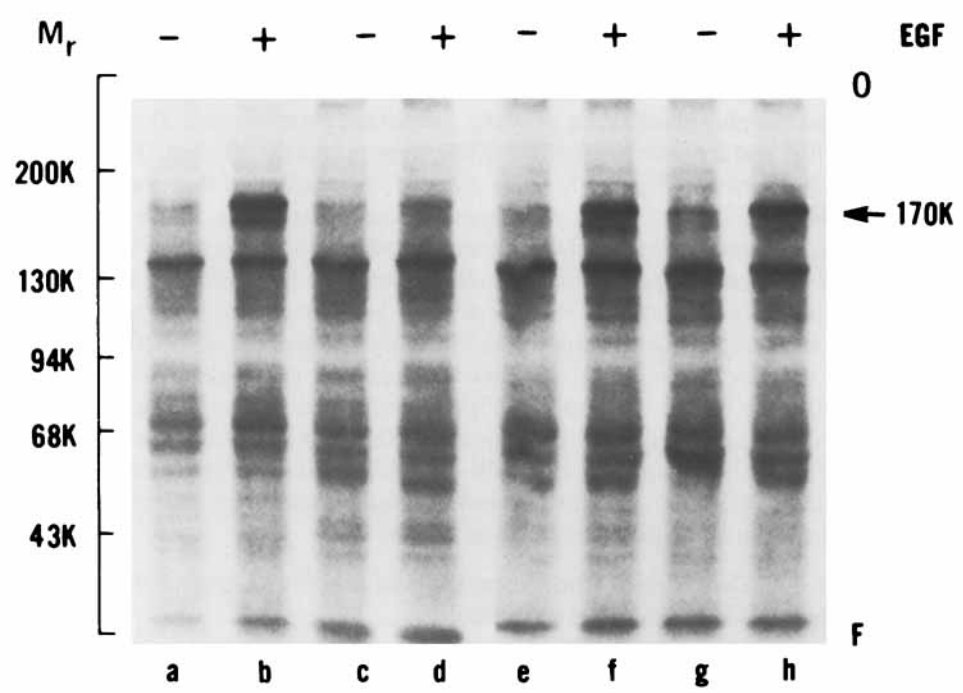



Fig. 1. Temperature-dependent effects of EGF-induced down regulation on the extent of in vitro phosphorylation of NRK membrane components incubated in the absence (−) or presence (+) of EGF. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated for 5 hr at 37°C or at 4°C with DME/F12 + F medium without or with EGF. After removal of EGF, the cells were collected and the membranes were isolated. The membranes were phosphorylated with [$\gamma$-$^{32}$P] ATP and the samples were subjected to NaDodSO$_4$ gel electrophoresis. The $^{32}$P-labeled membrane components were visualized by autoradiography (24-hr exposure). The samples were analyzed in exponential 5–9% acrylamide gels; 50 $\mu$g of protein was applied to each lane. 0, origin; F, front. In the phosphorylation assays, EGF was present at a final concentration of 120 nM. (a,b,c,d) Cells incubated at 37°C; (e,f,g,h) cells incubated at 4°C; (a,b,e,f) cells incubated in the absence of EGF; (c,d,g,h) cells incubated with medium containing EGF at 25 ng/ml.

ISCO densitometer. The amount of radioactivity in the bands of interest was determined by an Elographic Digitizer. The results reported in this paper were reproduced in at least four separate experiments each. Several different batches of anti-EGF antiserum were used and all of them produced virtually identical results.

## RESULTS

### NaDodSO$_4$Gel Electrophoresis of Phosphorylated Membrane Components of Untreated and Down-Regulated NRK Cells at 0°C and 37°C

Membranes from untreated cells and cells exposed to 25 ng/ml EGF at 37°C or at 0°C were phosphorylated in vitro in the presence and absence of EGF and analyzed by NaDodSO$_4$ gel electrophoresis and autoradiography to determine if incubation of cells with EGF at 0°C would result in a specific decrease in $^{32}$P phosphorylation of the 170K- and 150K-dalton membrane proteins, which has been previouly observed

in membranes from cells incubated with EGF at 37°C [6]. The data shown in Figure 1, lanes a and b, showed that in membranes from untreated cells incubated at 37°C 1) in the absence of EGF numerous membrane components are phosphorylated, 2) EGF increased the phosphorylation of many membrane proteins, and 3) one component of Mr 170K and a minor band of Mr 150K were primarily affected. The patterns of radioactivity shown in Figure 1, lanes c and d, demonstrated that incubation of cells with EGF at 37°C produced a decrease in the radioactivity of the 170K and 150K Mr bands. Figure 1, lanes e and f, demonstrated that the patterns of radioactivity of membrane components from untreated cells incubated at 0°C were similar to those of untreated cells incubated at 37°C (Figure 1, lanes a and b). The patterns of radioactivity shown in Figure 1, lanes g and h, demonstrated that incubation of cells with EGF at 0°C did not produce a significant reduction in the radioactivity of the 170K and 150K Mr bands. The results (Fig. 1) confirmed the original experiments [6] that down regulation of EGF receptors by EGF at 37°C results in a specific decrease in $^{32}P$ phosphorylation of the 170K- and 150K-dalton components to subsequent stimulation with EGF in vitro and further demonstrated that this phenomenon does not occur when the cells are exposed to EGF at 0°C.

## Recovery of EGF-Dependent Phophorylation of the 170K- and 150K-Dalton Proteins After Down Regulation with EGF

NRK cells in which the EGF-receptor has been down-regulated recover their EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins fully within approximately 6 hr following removal of EGF from the medium (Fig. 2). Incubation for 1 hr and 3 hr in fresh medium is required to restore approximately 30% and 65%, respectively, of the total EGF-dependent phosphorylating capacity of the 170K- and 150K-dalton protein bands. This time-dependent recovery of the EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins was accompanied by a parallel recovery of similar extent in the EGF-dependent phosphorylation of numerous other proteins (data not shown). The results also demonstrated that the basal phosphorylation of the 170–150K-dalton duplet and numerous other proteins increased gradually as a function of time after removal of EGF from the incubation medium (data not shown).

## Dose-Dependent Effects of EGF-Induced Down Regulation on Extent of In Vitro Phosphorylation of the 170K and 150K Mr Membrane Components

Our initial experiments [6] designed to investigate the relationship between receptor down regulation in cultured NRK cells and in vitro EGF-dependent phosphorylation of NRK membrane components were performed with partially purified EGF. In the dose-dependent experiments described here we have used highly purified preparations of EGF. As expected, major differences in the dose-response curves of in vitro EGF-dependent phosphorylation of the 170K and 150K Mr proteins in function of EGF concentration in culture medium became apparent when the results obtained with partially purified EGF [6] and highly purified EGF (FG. 3) were examined.

Figure 3 shows the effects of increasing concentrations of EGF in the incubation media on the subsequent phosphorylation of the 170K Mr membrane protein in the presence and absence of EGF. At the lowest concentration of EGF tested (1 ng/ml), 60% of the EGF-dependent phosphorylation of the 170K Mr protein was lost within 5 hr (Fig. 3C). Approximately 90% reduction in the EGF-dependent phosphorylation

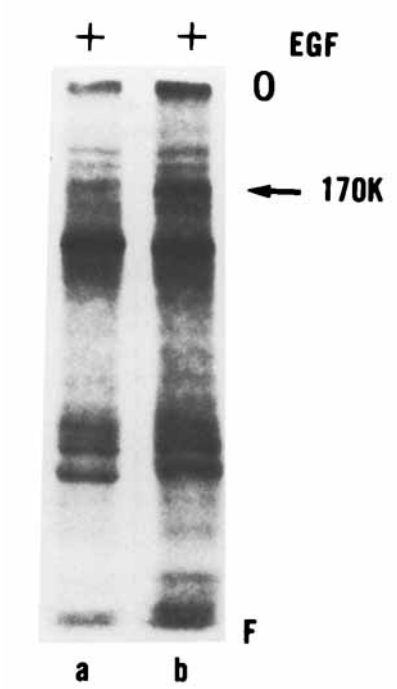
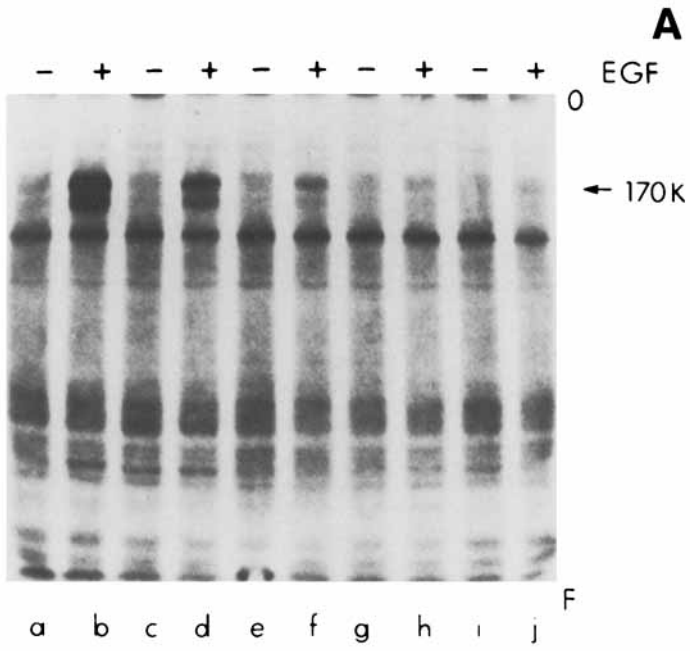



Fig. 2. Recovery of EGF-dependent phosphorylation of NRK membrane components after down regulation with EGF. Confluent NRK cells were down regulated with 50 ng/ml EGF as indicated in Materials and Methods. After down regulation, the medium was removed and the monolayers were washed and incubated at 37°C with DME/F12 + F medium without EGF. After 0 hr (a) or 6 hr (b) of incubation in the absence of EGF the cells were collected and the membranes were isolated. The membranes were phosphorylated in the presence (+) of 120 nM EGF and the samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (24-hr exposure). The samples were analyzed in exponential 5-9% acrylamide gels; 50 $\mu$g of protein was applied to each lane, O, origin; F, front.

of the 170K-dalton protein occurred in the presence of 10 ng/ml EGF (Fig. 3C). The reduction in $^{32}P$ incorporation in the 170K Mr protein was accompanied by a parallel decline in the radioactivity of a band of 150K-dalton (Fig. 3A, B, C).

The patterns of radioactivity in Figures 1 and 3 demonstrate that only two phosphoprotein bands in down-regulated cells have consistently reduced radioactivity while the radioactivity of other bands either remained constant or was reduced by down regulation with EGF but to a much lower extent. For example, Figure 3B shows that the radioactivity in one prominet band of 130K-daltons was unaffected by down regulation with concentrations of EGF up to 25 ng/ml. It is pertinent to note here that in some experiments (Fig. 3A), but not all (data not shown), a minor band of low Mr was significantly affected by down regulation with low concentrations (1 to 10 ng/ml) of EGF.

The results (Fig. 3) confirm the original experiments [6] that preincubation of NRK cells with EGF results in a selective reduction in the EGF-dependent $^{32}P$ phosphorylation of the 170K- and 150K-dalton protein bands and demonstrate that loss of EGF-dependent $^{32}P$ phosphorylation of the 170K- and 150K-dalton bands


A
– + – + – + – + – +
EGF
0
← 170K
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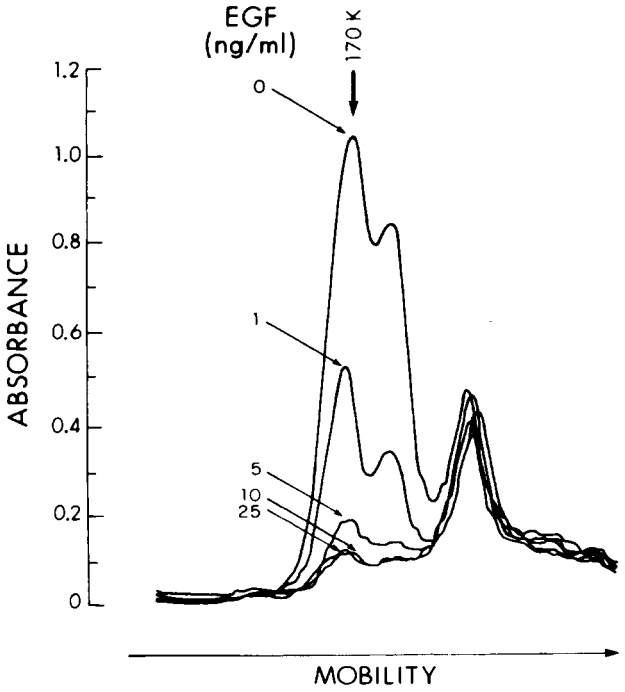
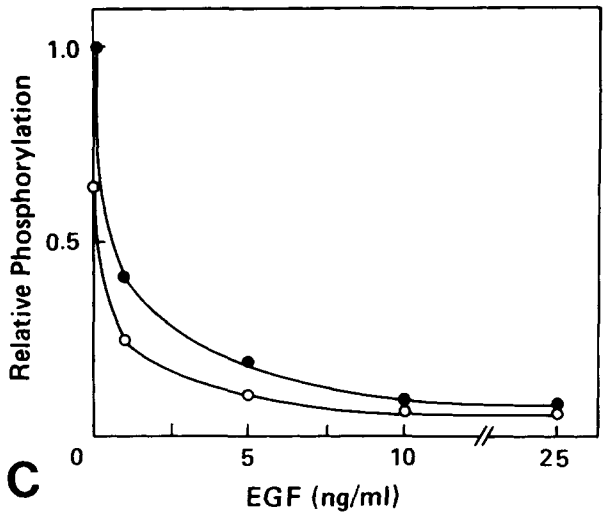

EGF
(ng/ml)
170K
0
1
5
10
25
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
MOBILITY




occurs at physiological EGF concentrations (1–10 ng/ml) that span the concentration range which is mitogenic for NRK cells [7].

## Modulation of EGF-Dependent NRK Membrane Protein Phospholrylation by Polypeptide Growth Factors

A wide variety of polypeptide growth factors were added to cultured NRK cells in place of EGF to test for their influence on the subsequent in vitro EGF-dependent phosphorylation of NRK membrane components. The data shown in Figure 4, lanes e and f, demonstrated that incubation of cells with growth hormone (25 ng/ml) for 5 hr produced a significant increase in the radioactivity of numerous protein bands, including the 170K and 150K Mr components. The growth hormone-induced increase in the EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins was noted with as little as 5 ng/ml growth hormone and was maximal at 50 ng/ml for 5 hr (data not shown). Similar results have been obtained with both relatively crude

Fig. 3. Effect of EGF concentrations on extent of in vitro phosphorylation of NRK membrane components incubated with [$\gamma$-$^{32}$P] ATP in the absence (−) or presence (+) of EGF. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated for 5 hr at 37°C with DME/F12 + F medium containing various concentration of EGF. After removal of EGF, the cells were collected and the membranes were isolated. Samples were prepared for phosphorylation, electrophoresis, Coomassie blue staining, autoradiography (24-hr exposure), and densitometry as described under Materials and Methods. Radioactive bands were quantitated with an electronic planimeter and expressed as peak area. (A) Photographs of representative autoradiographs are shown. (a,b) Cells incubated in the absence of EGF; (c,d; e,f; g,h; i,j) cells incubated with EGF at 1, 5, 10, and 25 ng/ml, respectively. The phosphorylation assays were performed in absence (−) or presence (+) of 120 nM EGF. 0, origin; F, front. (B) Densitometric scans of the 170K-, 150K-, and 130K-dalton protein bands corresponding to the experiment shown in (A). The absorbance scale is the same for all densitometric tracings. The small arrow points to the 170K-dalton band. (C) The relative EGF-dependent phosphorylation of the 170K- (●) and 150K- (○) Mr protein bands are plotted as a function of EGF concentration in the culture medium.

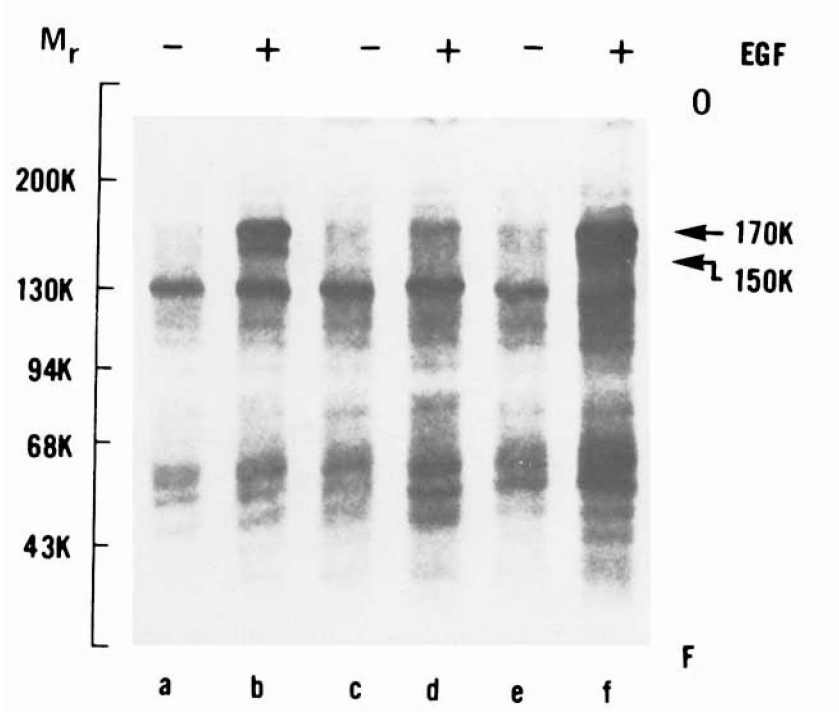



Fig. 4. Effect of incubation of NRK cells with growth hormone on extent of in vitro EGF-dependent phosphorylation of membrane components. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated for 5 hr at 37°C with DME/F12+F medium without or with added hormones. After removal of the hormones, the cells were collected and the membranes were isolated. Standard conditions of phosphorylation, electrophoresis, and autoradiography (24-hr exposure) were as described under Methods and Materials. 0, origin; F, front. (a,b) Cells incubated in the absence of hormones; (c,d) cells incubated with EGF at 10 ng/ml; (e,f) cells incubated with growth hormone at 25 ng/ml. The phosphorylation assays were performed in the absence (−) or presence (+) of 120 nM of EGF.

preparations of rat growth hormone (data not shown) and with highly purified preparations of human growth hormone (Fig. 4). Further experiments (not shown) demonstrated that the in vitro EGF-dependent $^{32}$P-phosphorylation of the 170K and 150K Mr proteins was not significantly affected by any of the following hormones each added at a final concentration of 25 ng/ml for 5 hr: glucagon, follicle-stimulating hormone, prolactin, luteinizing hormone, or thyrotropin.

We have also examined the effects of increasing concentrations of transferrin in the incubation media on the subsequent phosphorylation of the 170K Mr membrane protein in the presence and absence of EGF (Fig. 5). At the lowest concentration of transferrin tested (5 $\mu$g/ml), there was a significant increase (approximately 55%) in the EGF-dependent phosphorylation of the 170K Mr membrane protein (Fig. 5, lane b). Maximal stimulation of EGF-dependent phosphorylation of the 170K-dalton membrane protein occurred in the presence of 50 $\mu$g/ml transferrin (Fig. 5, lane d, and densitometry, not shown). With high concentrations of transferrin (500 $\mu$g/ml) there was a decline in the EGF-dependent phosphorylation of the 170K-dalton membrane

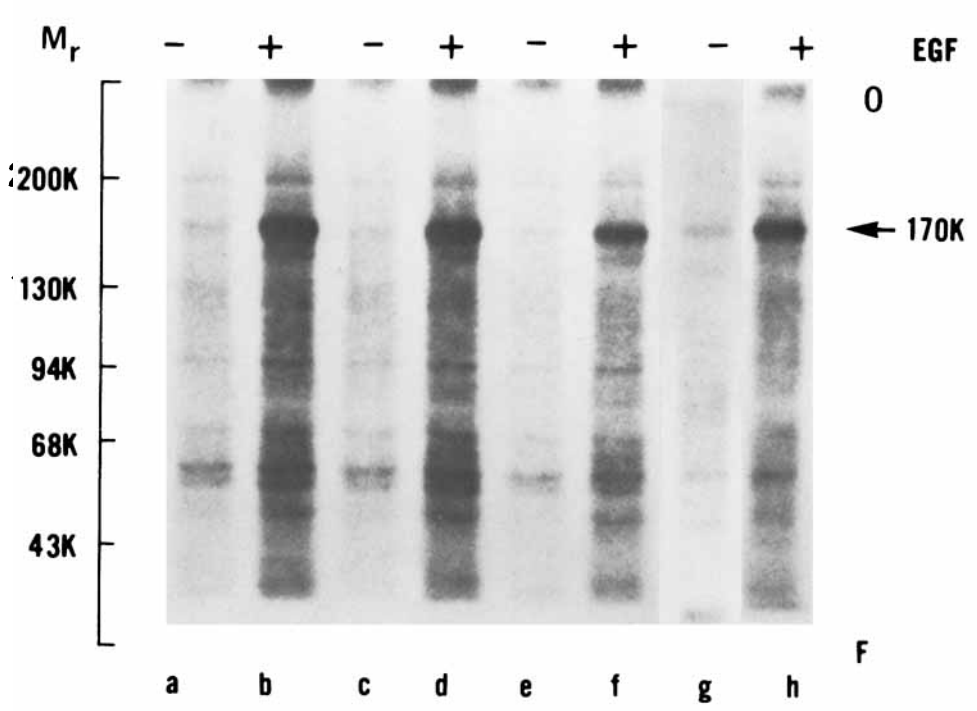



Fig. 5. Effect of transferrin concentration in the culture medium of NRK cells on the extent of in vitro phosphorylation of NRK membrane components incubated with [$\gamma$-$^{32}$P] ATP in the absence (−) or presence (+) of EGF. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated for 5 hr at 37°C with DME/F12+F medium containing various concentrations of transferrin. After removal of transferrin, the cells were collected and the membranes were isolated. Samples were prepared for phosphorylation, electrophoresis, (5–9% exponential gradient gel), autoradiography, (24-hr exposure), and densitometry as described under Materials and Methods. Photographs of representative autoradiographs are shown. O, origin; F, front. (a,b;c,d;e,f) Cells incubated with transferrin at 5, 50, and 500 $\mu$g/ml, respectively. (g,h) control, cells incubated in the absence of transferrin. The phosphorylation assays were performed in the presence or absence of 120 nM EGF.

protein (Fig. 5, lane f) which did not significantly exceed control levels (Fig. 5, lane h). The increase in the EGF-dependent phosphorylation of the 170K-dalton membrane protein induced by transferrin was unselective since it was accompanied by a parallel increase of similar extent in the radioactivity of numerous other protein bands, including the 150K-dalton component (Fig. 5). Figure 5, lanes a and c, also shows that incubation of NRK cells with transferrin (5-50 $\mu$g/ml) resulted in an appreciable increase in the extent of phrosphorylation of numerous membrane proteins in the absence of EGF.

### Immunoautoradiographic Analysis of EGF Receptors After Electrophoretic Transfer From Gels to DBM-Paper

The results of numerous experiments demonstrated that $^{32}$P-labeled A431 membrane proteins of all molecular weights were transferred to DBM-paper with excellent fidelity (Fig. 6A) [17]. For example, note that in lane 1 of Figure 6A, the 150K-dalton

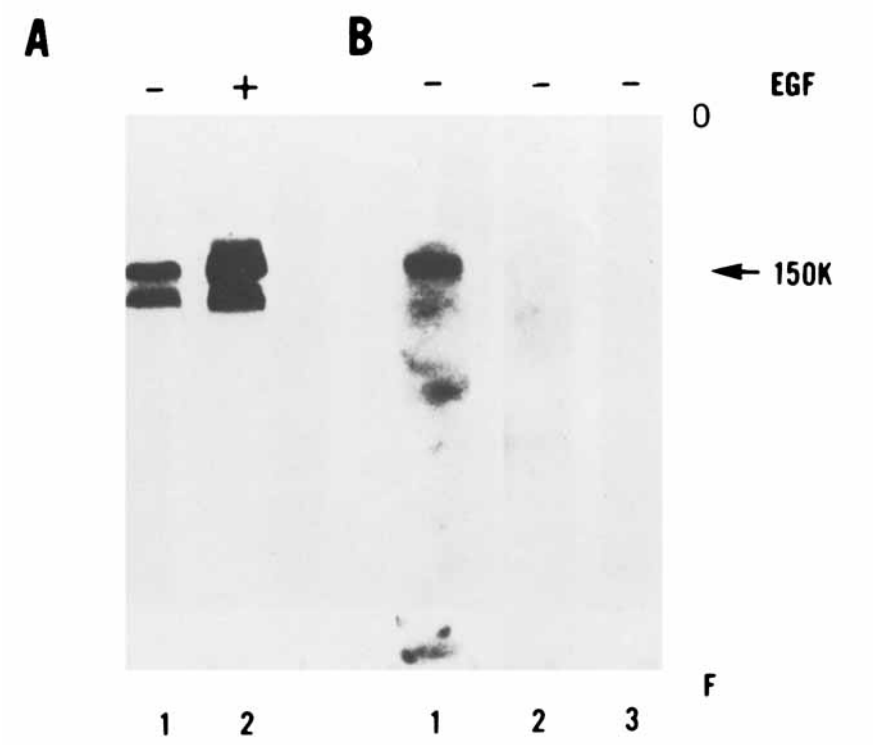



Fig. 6. Immunoautoradiographic analysis of EGF receptors transferred to DBM-paper. $^{32}$P-labeled (A) and unlabeled (B) A431 membrane proteins were separated on 5–9% $NaDodSO_4$/polyacrylamide gel and were electophoretically transferred to DBM-paper. (A) DBM-paper autoradiograph (3-hr exposure) of membrane components phosphorylated in the absence (−, lane 1) or presence (+, lane 2) of EGF. DBM-paper autoradiograph was underexposed to determine fidelity of transfer of the 150K-dalton band. (B) The lanes of DBM-paper transfer were treated as follows: lane 1: EGF, anti-EGF antiserum, and $^{125}$I-labeled protein A; lane 2: anti-EGF antiserum and $^{125}$I-labeled protein A; lane 3: EGF and $^{125}$I-labeled protein A. DBM-paper autoradiographs were exposed for 60 hr. 0, origin; F, front.

phosphoprotein band is clearly defined on the transfer. Furthermore, lane 2 of Figure 6A demonstrates that the enhancement of phosphorylation of the 150K-dalton protein induced by EGF is detected on the transfer.

Having demonstrated a high degree of fidelity for the transfer of $^{32}$P-labeled A431 membrane proteins from $NaDodSO_4$/polyacrylamide gels to DBM-paper, we prepared equivalent transfers of unlabeled proteins from A431 membranes for immunoautoradiographic analysis. The first lane of Figure 6B shows the localization of the major EGF-receptor protein band in A431 membranes by immunoautoradiography. DBM-paper transfers were incubated sequentially with EGF, anti-EGF antiserum, and then with $^{125}$I-labeled protein A, which binds to the Fc portion of IgG [18] (Fig. 6B, lane 1). Among proteins from A-431 membranes treated with EGF, anti-EGF antiserum, and $^{125}$I-labeled protein A, only the 150K-dalton component of the EGF receptor was readily detected (Fig. 6B, lane 1); and the 170K-dalton component was faintly visible after a long exposure time (data not shown). No proteins were labeled in control

experiments in which either EGF or anti-EGF antiserum were omitted from the incubation mixtures (Fig. 6B, lanes 2 and 3).

We conclude from the data shown in Figure 6 that the major protein responsible for EGF-receptor activity in membrane preparation is the 150K-dalton protein, in agreement with data of Cohen et al [19]. In addition, these results suggest that both the 150K- and 170K-dalton membrane proteins are substrates for the phosphorylation reaction.

## Immunoautoradiographic Analysis of EGF Receptors of Untreated and Down-Regulated A431 Cells

The detection of EGF receptors by immunoautoradiography in A431 membrane preparations derived from cells grown in serum-supplemented medium and cells grown in defined serum-free medium is shown in Figure 7A,B, lanes 2 and 1, respectively. It may be seen that the EGF receptor of both membrane preparations is a band of Mr 150K. A comparison of lane 2 of Figure 7A and lane 1 of Figure 7B indicates that the abundance of EGF receptors is greater in confluent cells incubated in serum-free medium than in cells incubated with serum-supplemented medium.

We have examined the effects of incubation of confluent cells in defined serum-free medium with various concentrations of EGF on the abundance of EGF receptors detected by immunoautoradiography. We found that incubation of A-431 cells with 400 ng/ml EGF produced a loss of approximately 25–30% of their EGF receptors (Fig. 7B, lanes 3 and 4) when compared with control untreated cells (Fig. 7B, lanes 1 and 2). The extent of EGF-receptor loss in down-regulated cells was not increased to over 40%, even at an EGF concentration of 1,200 ng/ml (data not shown).

For comparison purposes, the immunoautoradiographs of membranes from untreated and down-regulated cells both exposed to EGF in vitro prior to $NaDodSO_4$/polyacrylamide gel electrophoresis are also shown in Fig. 7B, lanes 2 and 4, respectively. As expected from the fact that down regulation of EGF receptors occurs in intact cells (1,4), addition of EGF in vitro to membranes derived from untreated (Fig. 7B, lane 2) or down-regulated (Fig. 7B, lane 4) cells did not significantly change the intensity of the signal from the 150K-dalton band.

The experiment shown in Figure 7B, lane 3 revealed that, in addition to the EGF receptor band of 150K-dalton, membranes from down-regulated cells yielded a band migrating close to the tracking dye front. We conclude from the immunoautoradiographic data shown in Figure 7B and other data that: 1) the low Mr band is absent in membranes from control untreated cells (Fig. 7B, lane 1); 2) incubation of membranes from untreated cells with EGF in vitro prior to electrophoresis produced a low Mr band (Fig. 7B, lane 2) which comigrated with both authentic EGF (data not shown) and the low Mr band present in membranes from down-regulated cells (Fig. 7B, lane 3); 3) addition of EGF in vitro to membranes from down-regulated cells increased the signal from the low Mr band (Fig. 7B, lane 4); and 4) when the transfers shown in Figure 7B were erased [15] and subsequently reacted with anti-EGF antiserum alone, only the low Mr band present in lanes 2, 3, and 4 gave a clear signal. Thus, the low Mr band present in membranes derived from down-regulated cells (Fig. 7B, lane 3) most likely is EGF.

Since various cellular functions are dependent on cell density [8], we have examined the effects of down regulation on the abundance of the 150K-dalton EGF-receptor protein derived from cells at various cell densities. We found that the loss of

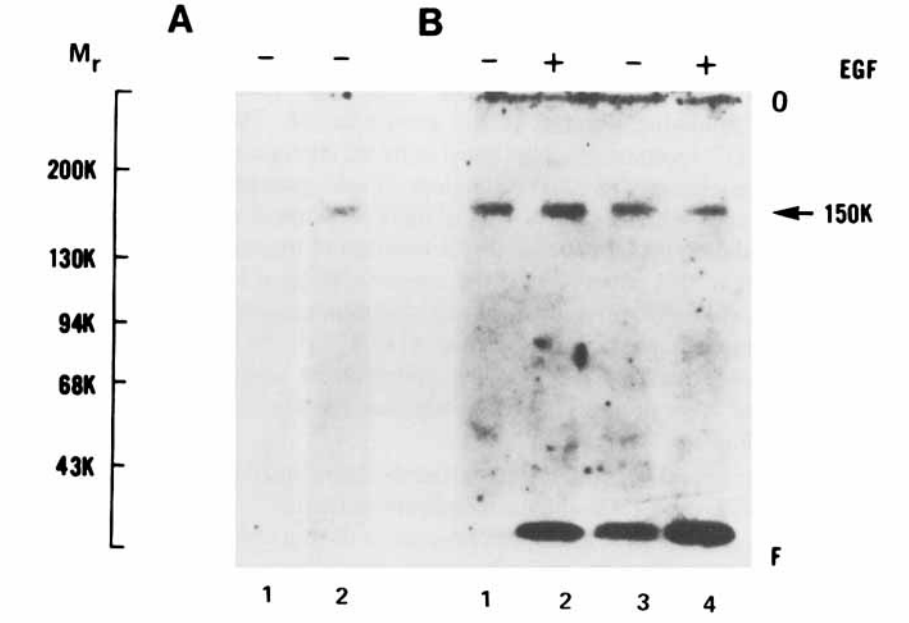
EGF binding activity of the 150K-dalton protein induced by down-regulation was much more prominent at intermediate cell densities (0.8–1 × $10^5$ cells/$cm^2$) than at high cell densities (approximately 2 × $10^5$ cells/$cm^2$). As shown in Figure 7C, A-431 cells at intermediate cell density lost approximately 60% of their EGF receptors when incubated with 400 ng/ml EGF for 5 hr at 37°C. Thus, the magnitude of EGF-induced down regulation of EGF receptors is dependent upon cell density.

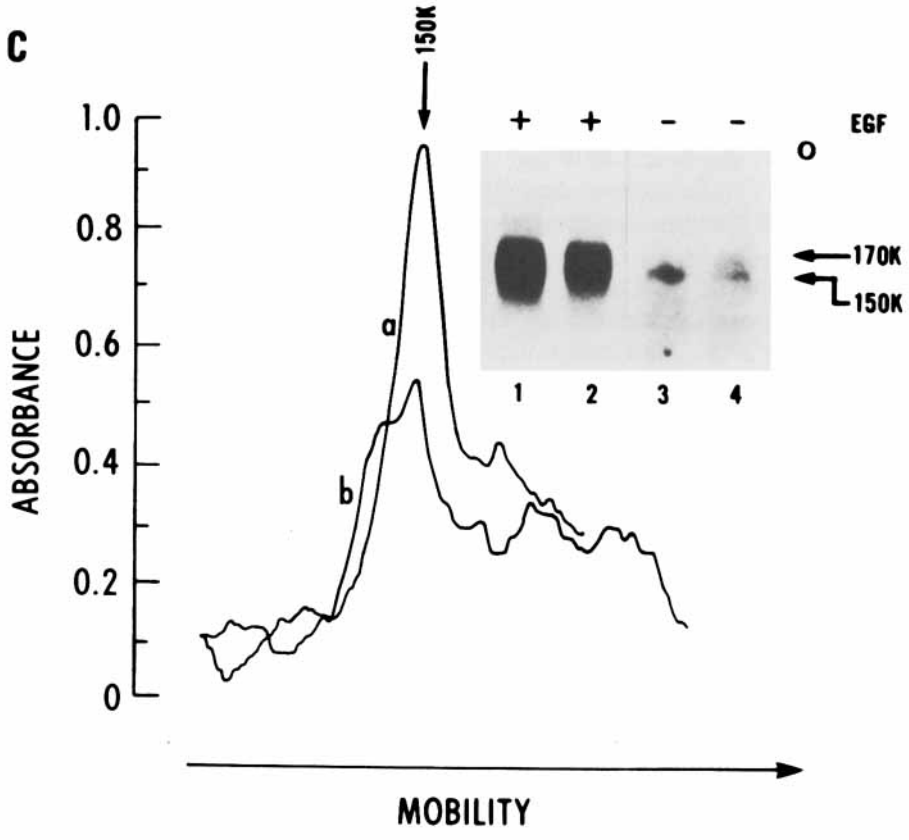
In Figure 7 (inset) we have compared EGF receptors detected by immunoautoradiography with the extent of EGF-dependent $^{32}$P-phosporylation of the 150–170K-dalton duplet phosphorylated in the presence of EGF using membranes derived from untreated and down-regulated cells. It may be seen that the decrease (approximately 60%) in EGF receptors in down-regulated cells (inset, lane 4) was accompanied by a decrease (approximately 50%) in the EGF-dependent $^{32}$P-phosphorylation of the 150–170K-dalton duplet (inset, lane 2). These findings suggest an inherent close relationship between EGF-binding activity and EGF-stimulated phosphorylation activity.

## DISCUSSION

Incubation of cultured NRK cells with EGF results in a decrease in the in vitro EGF-dependent phosphorylation of two membrane proteins of Mr = 170K and Mr = 150K. The characterization of this phenomenon here and elsewhere [6] revealed a number of interesting points: 1) This decrease was induced by EGF in proportion to its ability to occupy the EGF receptor [6]. 2) The loss of EGF-dependent phosphorylation on the 170K- and 150K-dalton proteins was temperature-sensitive. Continued incubation of NRK cells with EGF at 37°C resulted in loss of EGF-dependent phosphorylation of the 170K- and 150K-dalton components. In contrast, the loss was not observed when the cells were incubated with EGF at 4°C. These observations are

Fig. 7. Immunoautoradiographic analysis of EGF receptors of A431 cells incubated with serum-supplemented medium, cells incubated with serum-free medium, and cells subjected to down regulation with EGF. (A) Immunoautoradiographs of A-431 membrane proteins of confluent cells (approximately 2 × $10^5$ cells/$cm^2$) grown in serum-supplemented DME medium. Lane 1, control: DBM-paper transfer treated with anti-EGF antiserum and $^{125}$I-labeled protein A; lane 2: DBM-paper transfer treated with EGF, anti-EGF antiserum, and $^{125}$I-labeled protein A. The DBM-paper autoradiographs were exposed for 64 hr. (B) Immunoautoradiographs of A-431 membrane proteins of confluent cells (approximately 2 × $10^5$ cells/$cm^2$) incubated for 5 hr at 37°C with defined DME/F12+F medium without (lanes 1 and 2) or with (lanes 3 and 4) 400 ng/ml EGF. After removal of EGF, the cells were collected and the membranes were isolated. Prior to $NaDodSO_4$/polyacrylamide gel electrophoresis membranes were incubated in the absence (−) or presence (+) of 120 nM EGF. The DBM-paper transfer (lanes 1,2,3, and 4) was treated with EGF, anti-EGF antiserum, and $^{125}$I-labeled protein A. The DBM-paper autoradiographs were exposed for 64 hr. 0, origin; F, front. (C) Immunoautoradiographic analysis of A-431 membranes of subconfluent cells (approximately 1 × $10^5$ cells/$cm^2$) subjected to down regulation with EGF as indicated in (B). Densitometric scans of the 150K Mr protein band detected by immunoautoradiography (inset, lanes 3 and 4) are shown: a) Cells incubated in the absence of EGF for 5 hr. b) Cells incubated in the presence of EGF for 5 hr. The absorbance scale is the same for both densitometric tracings. Immunoautoradiographic detection of EGF receptors was performed as indicated in A, lane 2. Inset: Photographs of the region of interest of representative $^{32}$P gels (lanes 1 and 2) and immunoautoradiographs (lanes 3 and 4) are shown for comparison. Lane 1 and 3) Cells incubated in the absence of EGF. Lane 2 and 4) Cells incubated with medium containing EGF at 400 ng/ml. Lane 1 and 2) Membranes were incubated with [$\gamma$−$^{32}$P] ATP in the presence (+) of 120 nM EGF. The $^{32}$P-labeled membrane components were visualized by autoradiography (24-hr exposure). The DBM-paper autoradiographs were exposed for 64 hr. For details see text.


A
B
$M_r$
200K
130K
94K
68K
43K
– – – + – +
EGF
O
150K
F
1 2 1 2 3 4


C
150K
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
a
b
+ + – –
EGF
O
170K
150K
1 2 3 4
MOBILITY

consistent with the fact that down regulation of EGF receptors occurs at 37°C but not at 4°C [1]. 3) When EGF was removed from the medium, the EGF-dependent phosphorylation was restored rapidly; one-half of the loss was replaced by 3 hr and the full apparent $^{32}$P-phosporylation of the 170K- and 150K-dalton proteins was restored by 6 hr following removal of the growth factor. Thus, it appears that the combination of EGF receptor complex must activate continuously one or more steps in order to induce changes in EGF-dependent $^{32}$P-phosporylation of the 170K- and 150K-dalton proteins. 4) The rate of loss of EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins was a direct function of the concentration of ambient EGF. The rapidity of this effect [6] and the concentrations of EGF required to induce this phenomenon (Fig. 3) are compatible with conditions needed to demonstrate the EGF receptor-mediated physiologic effects of EGF [1]. 5) The specificity of this phenomenon is suggested by the fact that concomitant selective alterations in the phosphorylation of other proteins have not been consistently observed with physiological doses of EGF.

At least two processes could explain the decrease in EGF-dependent phosphorylation of the 170K- and 150K-dalton membrane proteins: 1) the prior saturation of phosphate acceptor sites with unlabeled phosphate during the cell incubation procedure; 2) cellular internalization and subsequent degradation of the 170K- and 150K-dalton membrane proteins; or 3) a combination of both. We have performed an additional series of experiments which do not support the first possibility (unpublished data). It is conceivable that the 170K- and 150K-dalton phosphoprotein bands are components of the receptor for EGF which is a substrate of the phosphorylation reaction [19–21]. Thus, it is possible that as a result of the interaction of EGF with its membrane receptor, the 170K- and 150K-dalton phosphoproteins disappears from the cell surface by cellular internalization. This interpretation is consistent with the hypothesis [1,4] that bound EGF and receptor are first internalized by a process of absorptive endocytosis and then degraded by lysosomal enzymes.

Loss of EGF-dependent phosphorylation of the 170K- and 150K-dalton proteins following exposure of NRK cells to EGF was a specific effect on the EGF receptor. Thus, incubation of NRK cells with EGF causes the effect, but incubation with several other growth factors did not significantly alter the EGF-dependent phosphorylation of the 170K–150K-dalton duplet. In contrast, we found that incubation of cells with growth hormone or transferrin induced a significant increase in the EGF-dependent phosphorylation of numerous membrane proteins, including the 170K- and 150K-dalton proteins. Regardless of the mechanism by which growth hormone and transferrin preparation modulate EGF-dependent phosphorylation, these results indicate that the EGF-dependent phosphorylation of the 170K–150K-dalton EGF receptor proteins can be modified not only by its homologous growth factor but also by an effect of a heterologous growth factor. These "heterologous" effects on the EGF-dependent phosphorylation of the 170K-150K-dalton proteins are most likely indirect because neither growth hormone nor transferrin added to NRK cell-free membrane system significantly affected the $^{32}$P-phosphorylation of the 170K- and 150K-dalton proteins [5]. These results, taken together with published reports [1,4,9], suggest that modulation of receptor number and membrane protein phosphorylation by homologous and heterologous growth factors may be a general mechanism for regulation of cellular responsiveness to growth factors.

The 170K–150K-dalton EGF receptor-kinase complex possesses at least three functions which may reside in one molecule: 1) EGF binding activity, 2) basal and

EGF-dependent protein kinase activity, and 3) a substrate for the protein kinase activity [19,21]. Therefore, it was of interest to determine whether the decrease in the EGF-dependent phosphorylation of the 170K–150K-dalton duplet observed in the present studies was accompanied by a concomitant decrease in the EGF binding activity of the EGF receptor protein. We have approached this problem by using A431 cells, which possess an unusually high number of specific EGF receptor sites ($2 - 3 \times 10^6$/cell) [2], and immunoautoradiography to detect the binding activity of the EGF receptor protein.

We have previously demonstrated [17] and extensively confirmed in this report (Figs. 6, 7) that membrane proteins immobilized on DBM-paper can be used to detect their respective EGF receptors by probing with EGF, specific antibodies against EGF, and other reagents. This procedure is simple and very sensitive [14,17] and it allows direct correlation of receptor activity to apparent protein Mr. Since we have obtained quantitative transfer of membrane proteins from gels to DBM-paper [17], the technique also provides a means of monitoring the relative abundance of membrane receptors for EGF. This technique, therefore, was utilized to obtain information on EGF receptors in untreated and down regulated A431 cells.

We found that in cells exposed to EGF the loss of EGF-dependent $^{32}P$ phosphorylation of the 170K–150K-dalton A431 membrane duplet correlated with a decrease in the EGF binding activity of the 150K-dalton protein, which presumably is a proteolytic degradation product of the 170K-dalton EGF receptor protein [19,20]. These results show that the two activities remain associated following down regulation with EGF and support the contention that these activities reside in the same molecule [19]. It is not clear whether the decrease in the EGF binding activity of the 150K-dalton protein reflects a decrease in the concentration of the 150K-dalton molecules in membrane preparations, a decrease in the affinity for EGF of individual receptor molecules, or both. These results are in essential agreement with those of Wrann and Fox [22] who reported, using EGF-induced down regulation, surface-specific iodination and "direct labeling," that the loss of EGF binding activity by A431 cells during down regulation closely paralleled the decrease in the labeling of the 170K-dalton EGF receptor protein band.

One additional interesting observation we have made by immunoautoradiography is the difference in the down regulation of the 150K-dalton EGF receptor protein observed in high density cells versus A431 cells at intermediate cell densities. In the former cells, although loss of EGF binding activity of the 150K-dalton protein occurs as it does in cells at intermediate cell densities, the reduction in the EGF binding activity of the 150K-dalton protein is much less pronounced. The increase in down regulation observed in subconfluent cells may be a result of the larger number of EGF receptors in these cells, as compared to confluent cells, as has been reported for other cell types [8]. Both the mechanisms and significance of this contrast between subconfluent and confluent cells remain to be elucidated.

## ACKNOWLEDGMENTS

The author wishes to thank P. Hamilton and D. Klos for expert technical assistance and J. Daly for the computer programs necessary to evaluate densitometric scans. Valuable secretarial assistance was provided by J. Becker and M. Gacioch.

This study was supported by VA MRISS 657/2620-01.

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