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Epidermal Growth Factor: Relationship between Receptor Down Regulation in Cultured NRK Cells and Epidermal Growth Factor Enhancement of Phosphorylation of a 170 000 Molecular Weight Membrane Protein in Vitro†

J. A. Fernandez-Pol

ABSTRACT: Incubation of confluent nondividing NRK cells in serum-free media with unlabeled epidermal growth factor (EGF) leads to a reduction in the specific binding capacity for ^{125}I-labeled EGF. This modulation of the binding capacity for ^{125}I-labeled EGF by unlabeled EGF, termed receptor down regulation, was dependent on EGF concentration and time. Membranes from untreated NRK cells have a phosphorylating system which catalyzed in vitro the phosphorylation of numerous membrane components; this phosphorylating system was stimulated by EGF. Although EGF enhanced the phosphorylation of many membrane proteins, one major component with M_r 170K and a minor band of M_r 150K were primarily affected. A comparison of the membrane phosphoproteins of untreated and down-regulated cells by in vitro phosphorylation and $NaDodSO_4$ gel electrophoresis revealed that down regulation of EGF receptors results in a specific decrease in ^{32}P phosphorylation of the 170K- and 150K-dalton components to subsequent stimulation with EGF in vitro. We further characterized the modulation of phosphorylation of the 170K protein by down regulation with EGF and found it to be dependent on EGF concentration and time. These studies demonstrated a correlation between the loss of ^{125}I-labeled EGF binding activity by the cells and the loss of the vitro EGF-dependent ^{32}P phosphorylation of the 170K-dalton membrane protein. In addition, the results suggest that the major 170K M_r phosphoprotein band is a component of the receptor for EGF which is a substrate of the phosphorylation reaction.

Epidermal growth factor (EGF),[1] a low molecular weight peptide hormone isolated from mouse submaxillary glands, is a potent mitogen for a variety of cell types including fibroblasts and epidermal cells (Carpenter & Cohen, 1979). The initial event in EGF-mediated stimulation of DNA synthesis is the interaction between EGF and its specific plasma membrane receptor (Carpenter & Cohen, 1979; Fox & Das, 1979; Fox et al., 1979). Specific saturable binding sites have been found on cell surfaces of a wide variety of mammalian cells, including NRK cells (Carpenter & Cohen, 1979; Guinivan & Ladda, 1979). Specifically bound EGF is internalized by the cells and subsequently degraded in the lysosomes (Fox & Das, 1979). Incubation of high concentrations of EGF with cells induces a loss in the binding capacity for the hormone; this phenomenon has been termed receptor down regulation (Fox et al., 1979; Wrann & Fox, 1979). The loss of binding activity which parallels EGF internalization and degradation has been attributed to internalization and degradation of EGF receptors (Carpenter & Cohen, 1979; Fox & Das, 1979). This phenomenon has been shown to correlate with the mitogenic activity of EGF (Fox et al., 1979). The interaction of EGF with its membrane receptors in vitro also results in specific stimulation of phosphorylation of endogenous membrane proteins in the presence of [γ-^{32}P]ATP (Carpenter et al., 1979; King et al., 1980). Although EGF stimulated the phosphorylation

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[1] Abbreviations used: EGF, epidermal growth factor; DME, Dulbecco–Vogt modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid.

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of numerous membrane proteins, two components which appear to be glycoproteins of M_r 170K and 150K were primarily affected in A-431 cells (King et al., 1980). It has been suggested that these proteins may represent the EGF receptor which is phosphorylated as an early result of the binding of EGF to its receptor and that phosphorylation of membrane-associated components may be one of the initial mitogenic signals generated as a consequence of interaction of EGF with its receptor (King et al., 1980).

The knowledge that EGF-induced internalization of its receptor parallels down regulation of the EGF receptor led us to test for a possible connection between EGF-induced down regulation and EGF-induced stimulation of [γ-^{32}P]ATP phosphorylation of specific NRK cell membrane proteins in vitro. Here we have compared gel electrophoretic patterns of in vitro phosphorylated membrane proteins in the presence and absence of EGF of untreated NRK cells and cells treated to down-regulate EGF receptors. We found that EGF-induced down regulation of EGF receptors results in a specific decrease in the EGF-dependent phosphorylation of a major membrane phosphoprotein of M_r 170K. Thus, we have demonstrated for the first time the regulation of phosphorylation of a specific protein in a cell-free membrane system as function of down regulation of EGF–receptor complexes in vivo.

Materials and Methods

Materials. EGF and ^{125}I-labeled EGF were obtained from Collaborative Research (Waltham, MA). [γ-^{32}P]ATP (1000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Ham's F12 was from Microbiological Associates (Rockville, MD). Insulin was from Lilly (Indianapolis, IN). Transferrin was from Behring Diagnostics (Somerville, NJ). Sources of other materials were as described previously (Fernandez-Pol, 1979; Fernandez-Pol & Klos, 1980).

Cell Culture. Cells were grown in DME containing 10% (v/v) calf serum as previously described (Ferandez-Pol et al., 1977). NRK cells were developed by Dr. E. Scolnick (NIH); the clone of NRK cells utilized in this study was designated NRK-A. NRK-B C18 were provided by Dr. R. Ting (Biotech Research Inc, Rockville, MD).

^{125}I-Labeled EGF Binding Assays. For determination of ^{125}I-labeled EGF binding, confluent cell monolayers grown in 60-mm dishes were washed twice with serum-free DME medium, incubated with 1.5 mL of binding medium (DME medium containing 0.1% BSA, 25 mM Hepes, pH 7.2, and ^{125}I-labeled EGF at 1×10^5 cpm/mL) for 60 min at 37 °C, washed 4 times with cold PBS, pH 7.2, and dissolved in 3 mL of 1% Triton X-100 and 1% $NaDodSO_4$. Specific binding was calculated by the difference in binding observed in the absence and presence of 10 μg of unlabeled EGF. Nonspecific binding was <2% of total binding.

Down Regulation of EGF Receptors. NRK cells were grown in 60- or 100-mm culture dishes containing DME supplemented with 10% calf serum. After 48 h, confluent monolayers were washed twice with serum-free DME and were incubated with 1.5 mL (60-mm dishes) or 5 mL (100-mm dishes) of defined medium containing EGF at 37 °C in the tissue culture incubator. The defined medium is referred to as DME/F12+F and was based upon one published by Cherington et al. (1979). The formulation of DME/F12+F was DME and Ham's F12 mixed 1:1 and supplemented with glutamine (4 mM), penicillin (100 units/mL), streptomycin (100 μg/mL); insulin and transferrin were added at 10 and 5 μg/mL, respectively. EGF was added at 1–300 ng/mL. After set incubation periods, EGF was removed by aspiration, and the monolayers grown in 60-mm dishes were washed twice with serum-free DME medium prior to determination of ^{125}I-labeled EGF binding. The cells grown in 100-mm dishes were processed for membrane isolation as indicated below.

Membrane Isolation. The cells grown in 100-mm dishes were placed on a bed of ice, the culture medium was decanted, and the cells were rinsed 3 times with PBS and once with Earle's balanced salt solution containing 10% sucrose at 0–4 °C. The cells, corresponding to 10–20 pooled dishes, were scraped into 10 mL of this solution with a Teflon spatula and washed by pelleting at 3000g for 10 min at 0–4 °C. The membranes were isolated by the method of Thom et al. (1977). The cells were collected by centrifugation, resuspended in harvesting solution, and extracted at 25 °C with hypotonic borate–EDTA buffer, pH 10.2. After addition of borate solution, the lysed cells were filtered through a nylon gauze (mesh size 900 μm). The suspension was centrifuged at 450g for 10 min at 2 °C, and the supernatant was recentrifuged at 25000g for 30 min at 2 °C. The membrane-rich pellet was resuspended in PBS, layered over 35% (w/w) sucrose solution in PBS, and centrifuged at 40000g for 45 min at 2 °C in a swinging bucket rotor (Beckman Model SW41). The membrane fraction at the buffer–sucrose interface was collected, resuspended in 10 mM Hepes, pH 7.4, and recentrifuged at 75000g for 30 min at 2 °C. The membranes were resuspended in 10 mM Hepes buffer, pH 7.4, divided into portions, frozen on dry ice, and stored at −70 °C. The membrane suspension contained approximately 5–10 mg of protein/mL.

Phosphorylation of Membrane Proteins. The assay used to investigate phosphorylation of membrane proteins was based upon the procedure of Cohen et al. (Carpenter et al., 1979; King et al., 1980). Unless otherwise noted, the reaction mixtures contained the following: NRK membranes (62.5 μg); Hepes buffer (20 mM, pH 7.4); $MnCl_2$ (2 mM); 0.125% BSA, [γ-^{32}P]ATP (0.5 μM, 1×10^6 cpm); EGF (35 ng, 120 nM) in a final volume of 50 μL. The reaction tubes were placed on ice and preincubated for 10 min in the absence or presence of EGF. The reaction was initiated by the addition of labeled ATP, and the incubation at 0 °C was continued for 10 min. The reaction was terminated by addition of 50 μL of $NaDodSO_4$ sample buffer (Laemmli, 1970) and heating at 95 °C for 3 min.

Gel Electrophoresis and Autoradiography. Polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ was carried out in exponential gradient gels ranging from 5 to 9% acrylamide with the buffer system described by Laemmli (1970). The protein molecular weight standards used were from Bio-Rad (Richmond, CA) and New England Nuclear (Boston, MA). Gels were fixed, stained, and destained as described elsewhere (Fernandez-Pol & Klos, 1980). Autoradiography was performed as indicated previously (Fernandez-Pol, 1979; Fernandez-Pol & Klos, 1980). Radioactive bands were quantitated by scanning with an ISCO densitometer. The amount of radioactivity in the bands of interest was determined by an Elographic Digitizer (Elographics, Oak Ridge, TN).

Other Procedures. Protein was determined in triplicate by the method of Lowry (Lowry et al., 1951) using BSA as standard. Cells were counted with a Coulter counter. The results reported here were reproduced in at least three separate experiments each.

Results

Binding of ^{125}I-Labeled EGF to Cultured NRK Cells. NRK cells have $\sim 2.5 \times 10^5$ binding sites/cell for ^{125}I-labeled EGF (Guinivan & Ladda, 1979). They lose more than 70% of their

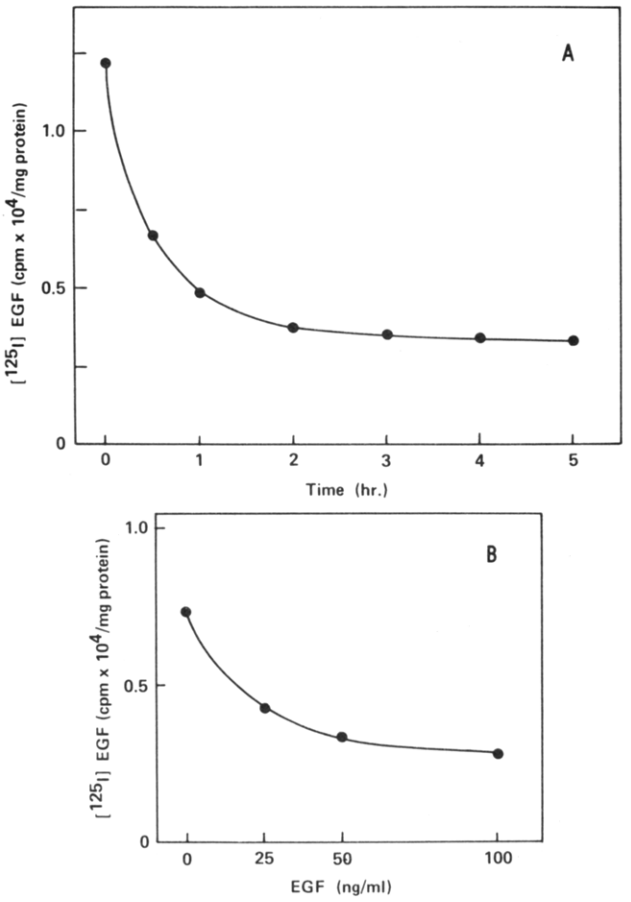

FIGURE 1: Effect of time and EGF concentration on extent of EGF receptor down regulation. Confluent NRK-B cells were washed twice with serum-free DME medium. The cells were then incubated with defined DME/F12+F medium containing unlabeled EGF. After removal of unlabeled EGF, specific binding of ^{125}I-labeled EGF was determined as described under Materials and Methods. Each point is the average from three cultures. The standard deviation did not exceed 5% of the mean. (A) Cells incubated with DME/F12+F medium containing 50 ng/mL unlabeled EGF for the indicated times at 37 °C; (B) cells incubated with DME/F12+F medium containing the indicated concentrations of EGF for 5 h at 37 °C.

^{125}I-labeled EGF specific binding activity when incubated with 50 ng/mL of unlabeled EGF for 5 h at 37 °C (Figure 1A). As shown in Figure 1A, the loss of ^{125}I-labeled EGF binding activity occurs rapidly; 50% inhibition of specific binding occurred in 1 h. The rate of loss of ^{125}I-labeled EGF binding activity between 2 and 5 h was slower and not too much different. Figure 1B shows that the ^{125}I-labeled EGF binding activity decreased in a nonlinear manner as the concentration of unlabeled EGF was varied between 0 and 100 ng/mL. With increasing incubation times of untreated NRK cells in serum-free medium, there was a progressive reduction in ^{125}I-labeled EGF binding, which at 5 h was <40% of the ^{125}I-labeled EGF binding activity at 0 h (Figure 1A). The decrease in binding of ^{125}I-labeled EGF to NRK cells may reflect inactivation of EGF receptors in response to EGF exposure (Carpenter & Cohen, 1979; Fox & Das, 1979) or could indicate presistent binding of unmodified EGF (receptor saturation), or both.

NaDodSO$_4$ Gel Electrophoresis of Phosphorylated and Nonphosphorylated Membrane Components of Untreated and Down-Regulated NRK Cells. Membranes from untreated cells and cells exposed to EGF were phosphorylated in vitro in the presence and absence of EGF and analyzed by NaDodSO$_4$ gel electrophoresis, Coomassie Blue staining, and autoradiography to determine if incubation of cells with EGF would result in

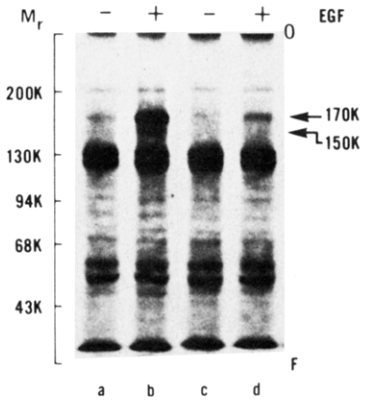

FIGURE 2: Selective effects of EGF-induced down regulation on in vitro phosphorylation of NRK membrane components incubated in the absence [basal phosphorylation (–)] or presence (+) of EGF. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated for 5 h at 37 °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 [γ-^{32}P]ATP, and the samples were subjected to NaDodSO$_4$ gel electrophoresis and Coomassie Blue staining. The ^{32}P-labeled membrane components, shown in this figure, were visualized by autoradiography (24-h exposure). The samples were analyzed in exponential 5–9% acrylamide gels; 50 μg of protein was applied to each lane: O, origin; F, front. In the phosphorylation assays, EGF was present at a final concentration of 120 nM. See text for details. (a, b) Cells incubated in the absence of EGF; (c, d) cells incubated with medium containing EGF at 100 ng/mL.

an alteration of the in vitro [γ-^{32}P]ATP phosphorylation of specific membrane proteins. Coomassie Blue staining indicated the presence of many proteins in the crude membrane preparation. The migration or intensity of stained protein components was not altered by incubation of cells with EGF in defined medium at all times and concentrations of EGF examined (data not shown). The data shown in Figure 2, lanes a and b, demonstrated that in membranes from untreated cells (1) in the absence of EGF many membrane components are phosphorylated, (2) EGF increased the phosphorylation of numerous membrane proteins, and (3) one component of M_r 170K and a minor band of M_r 150K were primarily affected. The patterns of radioactivity shown in Figure 2, lanes c and d, demonstrated that incubation of cells with EGF produced a dramatic decrease in the radioactivity of the 170K and 150K M_r bands.

To study this phenomenon further, we examined the effects of increasing concentrations of EGF in the incubation media on the subsequent phosphorylation of the 170K M_r membrane protein in the presence and absence of EGF (Figure 3). At the lowest concentration of EGF used (25 ng/mL), 50% of the EGF-dependent phosphorylation of the 170K M_r protein was lost within 5 h (Figure 3B). Approximately 90% reduction in the EGF-dependent phosphorylation of the 170K dalton protein occurred in the presence of 300 ng/mL EGF (Figure 3B). Twenty percent reduction in phosphorylation of the 170K dalton protein was noted with as little as 1 ng/mL EGF for 5 h (data not shown). The reduction in ^{32}P incorporation in the 170K M_r protein was accompanied by a parallel decrease in the radioactivity of a minor band of 150K daltons (Figure 3A).

The patterns of radioactivity in Figures 2 and 3 demonstrate that only two phosphoprotein bands in down-regulated cells have drastically 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 3C shows that the radioactivity in one

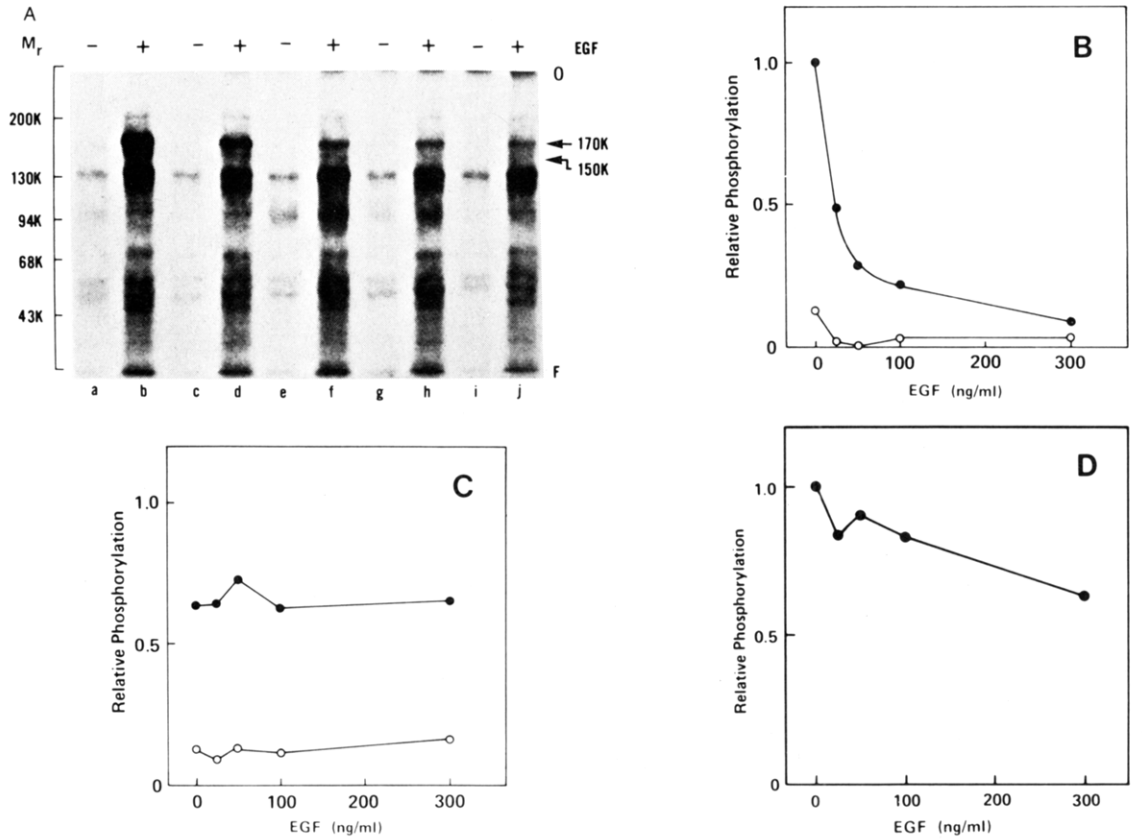

FIGURE 3: Effect of EGF concentration on extent of in vitro phosphorylation of NRK membrane components incubated with [γ-^{32}P]ATP in the absence (−) or presence (+) of EGF. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated for 5 h at 37 °C with DME/F12+F medium containing various concentrations 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-h 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 25, 50, 100, and 300 ng/mL, respectively. The relative phosphorylation of the 170K M_r protein (B) and the 130K M_r protein (C) and phosphorylation of all protein bands, except the 130K, 150K, and 170K bands (D), are plotted as a function of EGF concentration in the culture medium. The phosphorylation assays were performed in the absence (○) or presence (●) of 120 nM of EGF.

prominent band of 130K daltons, which in some experiments was resolved as a duplet, for reasons that are not clear, was unaffected by down regulation with concentrations of EGF up to 300 ng/mL. Furthermore, the radioactivity present in all protein bands of down-regulated cells, except the 130K, 150K, and 170K bands, was determined, and the results are presented in Figure 3D. At low concentrations of EGF (25–50 ng/mL) there was a small decrease (<20%) in the EGF-dependent phosphorylating capacity of these membrane components (Figure 3D). With increasing concentrations of EGF there was a dose-dependent decline in the EGF-dependent phosphorylation of most membrane components (Figure 3D). This reduction, however, did not exceed 36% of control levels (0 ng/mL EGF) at all concentrations of EGF examined (Figure 3D). Thus, preincubation of cells with EGF results in a selective reduction in the EGF-dependent phosphorylation of the 170K-dalton protein band.

For comparison purposes, the gel patterns of membranes from down-regulated cells phosphorylated in the absence of EGF are also shown (Figure 3A). As expected from the much lower phosphorylation of membrane components in the absence of EGF, the 170K-dalton band was weakly phosphorylated in the basal state (Figure 3A). Addition of EGF to the cells decreased the basal phosphorylation of the 170K M_r band (Figure 3B, open circles), with maximal reduction observed at 50 ng/mL EGF. This was followed by a progressive increased toward control basal levels despite the increasing concentrations of hormone.

The experiment shown in Figure 4 tests the effects of time during down regulation on the subsequent accessibility of the 170K M_r protein to phosphorylation by [γ-^{32}P]ATP in the presence and absence of EGF. The data show that the addition of 50 ng/mL EGF to the cells caused a time-dependent decline of the levels of phosphorylation of the 170K M_r membrane protein in the presence of EGF in vitro (Figure 4A). During the first 10 min of incubation of the cells with EGF there was a rapid decline in the phosphorylation of the 170K M_r protein which was followed by a recovery of 170K protein phosphorylation at 15 min. The phosphorylation of the 170K M_r protein gradually decreased between 0.5 and 3 h. Fifty percent reduction of phosphorylation of the 170K M_r protein occurred in 3 h in comparison to control at 0 h. The phosphorylation of 170K M_r protein reached a minimal level of ~26% of control levels by 5 h (Figure 3B). Incubation of cells with EGF also resulted in a time-dependent decline of the phosphorylation of the 150K-dalton protein band (Figure 4C). At all time points examined (Figure 4A), the EGF-stimulated phosphorylation of other membrane proteins of cells exposed to 50 ng/mL EGF either remained constant or was only reduced by 5–10%. The results also showed that the phosphorylation of the 170K-dalton protein in the basal state decreased as a function of the time of incubation of cells with EGF (Figure 4A). With increasing incubation times of untreated NRK cells in defined medium, there was a decline in the phosphorylation of the 170K M_r protein in the EGF-stimulated state (Figure 4A,B); this reduction did not exceed 8% of the EGF-stimulated

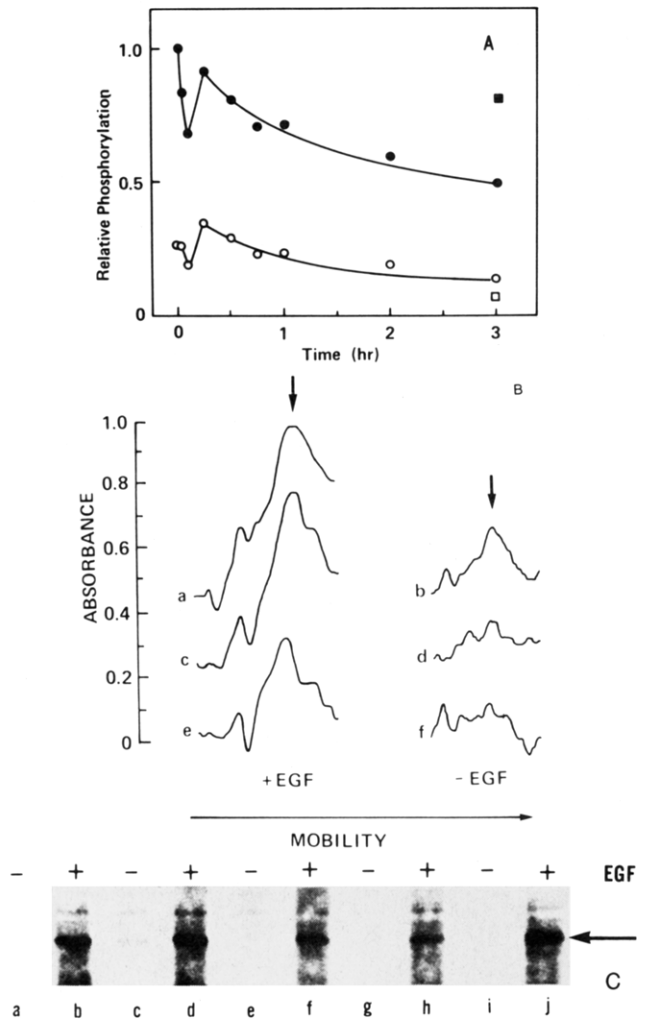

FIGURE 4: Time dependent effects of EGF-induced down regulation on extent of phosphorylation of 170K M_r membrane component incubated with [γ-^{32}P]ATP in the absence (–) or presence (+) of EGF. NRK cells were grown to confluence in normal medium. The monolayers were washed twice with serum-free DME medium and incubated with DME/F12+F medium containing 50 ng/mL EGF for the indicated times. After removal of EGF, the cells were collected, the membranes isolated, and the phosphorylated membrane components analyzed as indicated in Figure 3. See the text for details. (A) The relative phosphorylation of the 170K M_r protein is plotted as a function of the time of incubation of cells with EGF; the phosphorylation assays were performed in the absence (○, □) or presence (●, ■) of 120 nM of EGF; (○, ●) cells exposed to EGF; (□, ■) control, untreated cultures. (B) Densitometric scans of the 170K M_r protein band: (a, b) cells incubated in the absence of EGF at $t = 0$; (c, d) cells incubated in the absence of EGF for 3 h; (e, f) cells incubated with EGF at 50 ng/mL for 3 h. The absorbance scale is the same for all densitometric tracings. The small arrows point to the 170K M_r protein. (C) Photographs of the region of interest of representative gels are shown; the arrow points to the 170K M_r protein; (a, b) cells incubated in the absence of EGF at $t = 0$; (c, d), (e, f), (g, h) cells incubated with EGF at 50 ng/mL for 2.5 min, 2 h, and 3 h, respectively; (i, j) cells incubated in the absence of EGF for 3 h.

phosphorylation of the 170K protein of untreated cells at 0 h (Figure 4A,B). Figure 4A,B also shows that with increasing incubation times of untreated NRK cells in defined medium, there was an appreciable decrease in the extent of basal phosphorylation of the 170K-dalton protein. This time-dependent decrease in the phosphorylation of the 170K-dalton protein of untreated cultures was not specific since it was accompanied by a parallel decrease of similar extent in total membrane phosphorylating capacity (data not shown). Results of experiments presented here show that there is a correlation between down regulation of specific EGF receptors and the specific decrease of phosphorylation of a 170K M_r membrane protein.

Discussion

Loss of EGF receptors from cell surfaces has been shown to occur in a variety of cell lines when the cells are exposed to unlabeled EGF (Carpenter & Cohen, 1979). In vitro phosphorylation of two membrane proteins of M_r 170K and 150K which may represent the EGF receptor has been reported for A-431 cells (King et al., 1980). We have characterized the EGF-stimulated phosphorylation reaction of a 170K-dalton membrane protein in isolated NRK membranes, the details of which will be presented elsewhere (our unpublished data). We subsequently tested to determine if prior down regulation of EGF receptors in NRK cells would decrease the amount of a specific protein subject to EGF-induced stimulation of phosphorylation in the presence of [γ-^{32}P]ATP. We found that the loss of ^{125}I-labeled EGF binding activity by NRK cells during down regulation under serum-free conditions closely paralled the dramatic decrease in the EGF-induced phosphorylation of the 170K M_r membrane protein. These findings are consistent with the data and suggestions of King et al. (1980) that the 170K M_r protein may be a component of the EGF receptor of a variety of mammalian cells.

The correlation between the decreased phosphorylation of the 170K M_r protein and the down regulation process suggests that both of these activities share some limiting step or alternatively that down regulation is itself the limiting EGF-induced step in the reduction of phosphorylation of the 170K M_r protein. The specificity of this phenomenon is suggested by the fact that concomitant selective alterations in the phosphorylation of other proteins have not been observed with physiological doses of EGF. The mechanism leading to the selective reduction in EGF-dependent phosphorylation of the 170K M_r protein after down regulation is as yet a matter for speculation. The reduction of ^{32}P phosphorylation of the 170K-dalton membrane protein following incubation of cells with EGF may result from the prior saturation of phosphate acceptor sites with unlabeled phosphate during the cell incubation procedure. It is conceivable that the 170K M_r phosphoprotein band is a component of the receptor for EGF which is a substrate of the phosphorylation reaction (King et al., 1980). Thus, it is possible that as a result of the interaction of EGF with its membrane receptor, the 170K M_r protein disappears from the cell surface by cellular internalization. The observed net decrease in ^{32}P phosphorylation of the 170K M_r protein may be an even more indirect result of the formation of the EGF–receptor complex.

Incubation of cells with high doses of EGF for a few hours did not completely abolish the phosphorylation of the 170K M_r protein. This could result from the short incubation times tested in the present study which were not sufficient to allow complete inhibition of the EGF-dependent phosphorylation of the 170K M_r protein. Failure to completely abolish phosphorylation of the 170K M_r protein in vitro could be due to washing off some of the prebound EGF which would result in the subsequent availability of EGF binding sites in the membrane. It could also be due to the presence in the 170K M_r band of proteins that neither bind EGF nor participate in EGF–receptor complex formation but are accessible to phosphorylation. The observed partial decrease in phosphorylation of 170K protein may be an even more complex result of down regulation.

It is interesting to note that despite the major loss of the EGF-dependent phosphorylation response of the 170K-dalton protein, EGF still can extensively stimulate phosphorylation

of numerous membrane components. These findings suggest that if the 170K dalton protein is the receptor for EGF, the concentrations of membrane receptors remaining after down regulation are sufficient to produce near maximal phosphorylation of numerous membrane components. Thus, additional studies are required to elucidate this aspect of the mechanism of action of EGF alteration of hormonal responsiveness in vitro.

The results presented here, taken together with published reports (Fox & Das, 1979; Fox et al., 1979; Carpenter & Cohen, 1979; Greengard, 1978; Heldin et al., 1979), suggest that modulation of receptor number and membrane phosphorylation may be a general mechanism for regulation of cellular responsiveness to hormones. Whether down regulation and reduction of phosphorylation of a protein of M_r 170K represent different aspects of the same phenomenon remains to be determined. It seems possible that the specific apparent reduction of phosphorylation of the 170K M_r phosphoprotein mediated by EGF may regulate the hormonal sensitivity of NRK cells to EGF. Finally, it is conceivable that the 170K-dalton phosphoprotein is a component of the EGF receptor of NRK cells which is a substrate of the phosphorylation reaction.

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Conformations of Oxidized Cytochrome *c* Oxidase†

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ABSTRACT: Oxidized cytochrome *c* oxidase is shown to exist in three conformations in addition to the transient "*g*5" conformation previously reported [Shaw, R. W., Hansen, R. E., & Beinert, H. (1978) *J. Biol. Chem. 253*, 6637–6640]. The "resting" and "*g*12" conformations are distinguished by an NO-induced cytochrome a_3 electron paramagnetic resonance (EPR) signal and an EPR signal at $g' = 12$, respectively. The "oxygenated" conformation exhibits an unusual EPR signal in the presence of fluoride and is identical with the "oxygenated" state first discovered by Okunuki et al. [Okunuki, K., Hagihora, B., Sekuzu, I., & Horio, T. (1958) *Proc. Int. Symp. Enzyme Chem., Tokyo, Kyoto*, 264]. It is proposed that when the reduced enzyme is reoxidized by dioxygen, the *oxidized* enzyme first relaxes from the "*g*5" into the "oxygenated" conformation after which a percentage of the molecules slowly relax into the "*g*12" conformation. The "resting" conformation is not formed when the enzyme is reoxidized. On the basis of the EPR observations, it is proposed that these various conformations of the oxidized enzyme differ in the structure of the cytochrome a_3–Cu_{a_3} site. Structures for the cytochrome a_3–Cu_{a_3} site are proposed for each conformation, and a mechanism by which these conformations undergo interconversion among themselves is described.

Oxidized cytochrome *c* oxidase can exist in more than one conformation (Muijsers et al., 1971; Antonini et al., 1977; Rosén et al., 1977; Shaw et al., 1978; Petersen & Cox, 1980). Two classes of conformations have been recognized: those that are present in the oxidized enzyme as isolated and those that are transiently formed when the reduced enzyme is reoxidized with O_2. At this time these two classes of conformations have been collectively referred to as the "resting" and "oxygenated" (also "pulsed") conformations, respectively. The distinction between the two classes is that the resting conformation reacts slowly with O_2 when mixed with both reductant and O_2, whereas the pulsed enzyme reacts rapidly (Antonini et al., 1977; Rosén et al., 1977). However, the question of the

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