VIRAL TRANSFORMATION INVOLVES LOSS OF EPIDERMAL GROWTH FACTOR-STIMULATED PHOSPHORYLATION OF TWO SPECIFIC MEMBRANE POLYPEPTIDES IN NORMAL RAT KIDNEY CELLS

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1. Introduction

EGF is a low molecular weight peptide of M_r 6045 which is a potent mitogen for a variety of non-transformed cell types including epidermal cells, fibroblasts, and glial cells [1]. The growth factor also enhances the multiplication of transformed cells such as SV40-3T3 and HeLa cells [1]. The initial event in EGF-mediated stimulation of DNA-synthesis involves an interaction between EGF and its specific plasma membrane receptor [1,6]. Specific EGF receptors have been detected in a wide variety of mammalian cells, including normal rat kidney cells [7]. The interaction of EGF with its membrane receptor in vitro also results in specific stimulation of phosphorylation of endogenous membrane proteins in the presence of [γ-^{32}P]ATP [2,3,10]. Phosphorylation of specific membrane-associated components may be one of the initial mitogenic signals generated as a consequence of interaction of EGF with its receptor [10]. Yet, the events mediated by EGF–receptor interaction which lead to cell multiplication have not been elucidated.

Cultured NRK cells respond to density-dependent regulation of growth, require EGF for cell proliferation, and have high levels of EGF receptors [7]. Viral transformation of NRK cells results in the loss of EGF requirements, reduction of EGF receptors, and enhanced proliferative activity [7]. These changes suggest that the loss of EGF receptors in transformed cells may be accompanied by qualitative or quantitative (or both) alterations in EGF-mediated events such as phosphorylation of specific membrane-associated proteins.

Here we investigate the EGF-dependent phosphorylation of specific membrane components of NRK cells and derivatives of these cells transformed by Kirsten murine sarcoma virus (K-NRK cells) and a temperature-sensitive mutant of the virus (Ts cells). We present evidence that the phosphorylation response to EGF of specific NRK membrane components is altered by viral transformation.

Abbreviations: EGF, epidermal growth factor; NRK, normal rat kidney; DME, Dulbecco-Vogt modified Eagle's medium; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

2. Materials and methods

2.1. *Materials*

Mouse EGF was obtained from Collaborative Research (Waltham, MA). [γ-^{32}P]ATP (1000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Sources of other materials were as in [5].

2.2. *Cell cultures*

Cells were grown in DME containing 10% (v/v) calf serum as in [5]. NRK-B C18 and K-NRK C132 were obtained from Dr R. Ting (Biotech Res., Rockville, MD). These cell lines were grown at 37°C. NRK and the transformed derivatives of these cells, K-NRK and Ts mutant cells, were developed by Dr E. Scolnick and co-workers [13]. These cell types were cultured at 32°C and 39°C, the permissive and non-permissive temperatures, respectively, for Ts cells transformed by the temperature-sensitive mutant. The clone of Ts cells studied here demonstrated stable characteristics on transfer between 32°C ⇌ 39°C. At the non-permissive temperature, Ts cells exhibited

a normal monolayer growth pattern. On the transfer of the Ts cells to the permissive temperature, a progressive change to a multilayered focal growth pattern was observed.

2.3. *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 phosphate-buffered saline 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 solution with a Teflon spatula and washed by pelleting at 3000 × *g* for 10 min at 0–4°C. The membranes were isolated as in [14].

2.4. *Phosphorylation of membrane proteins*

The assay used to investigate phosphorylation of membrane proteins was based upon the procedure in [3]. 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 50 μl final vol. 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 of 0°C was continued for 10 min. The reaction was terminated by addition of 50 μl SDS sample buffer [11] and heating at 95°C for 3 min.

2.5. *Gel electrophoresis and autoradiography*

Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was done in exponential gradient gels (5–9% acrylamide) using the buffer system in [11]. The protein M_r standards used were from BioRad (Richmond, CA), and New England Nuclear (Boston, MA). Gels were fixed, stained and destained as in [5]. Autoradiography was performed as in [5]. For quantitative studies care was taken to avoid overexposure of the film. 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). For each band of interest, the value obtained for each sample was expressed as a percentage of the largest stimulation over the control in the experiment.

2.6. *Other procedures*

Protein was determined in triplicate as in [12] using BSA as standard. Cells were counted with a Coulter counter.

3. Results

3.1. *SDS gel electrophoresis of phosphorylated and non-phosphorylated membrane polypeptides of NRK and K-NRK cells*

Crude membrane fractions of NRK or K-NRK cells were incubated with [γ-^{32}P]ATP with and without EGF, and the membrane polypeptides and the resultant [^{32}P]phosphopeptides analyzed by SDS gel electrophoresis, Coomassie blue staining and autoradiography (fig.1). Coomassie blue staining demonstrated the presence of a complex polypeptide pattern in the crude membrane preparations of both NRK and K-NRK cells. The major stained band which migrates at ~68 000 M_r is BSA used in the phosphorylation reaction mixtures to prevent non-specific adsorption. Fig.1A shows that the membrane polypeptide patterns of NRK cells were significantly different from those of K-NRK cells. The results shown in fig.1A indicate that the mobility of membrane components of both NRK and K-NRK membranes was not affected by the presence of EGF in the incubation mixture. Fig.1B shows that incubation of both NRK

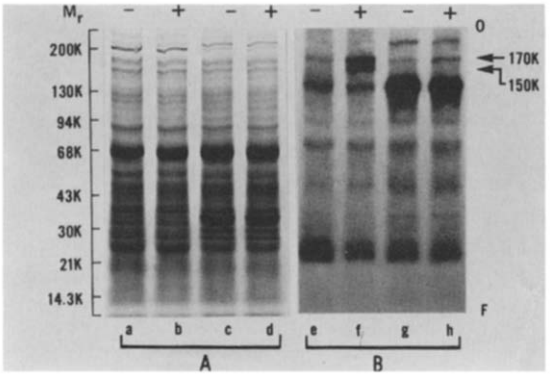

Fig.1. EGF-dependent phosphorylation of NRK and K-NRK membrane components. The phosphorylation assays were performed in the absence (–) or presence (+) of 120 nM EGF. The samples were subjected to SDS gel electrophoresis, Coomassie blue staining (A) and autoradiography ((B) 24 h exposure). The samples were analyzed in exponential 5–12% acrylamide gels; 50 μg protein was applied to each lane; (O) origin; (F) tracking dye front. (a,b,e,f) NRK-B membranes; (c,d,g,h) K-NRK C132 membranes. See text for details.

and K-NRK membranes with [γ-^{32}P]ATP resulted in the phosphorylation of numerous membrane components whose phosphorylation was enhanced by EGF. Inspection of fig.1 A,B shows that there was no apparent correlation between the intensity of protein staining and the intensity of protein phosphorylation in both NRK and K-NRK membrane preparations. In NRK membrane preparations, two phosphoproteins of $M_r \sim 150\,000$ and 170 000, which were either undetectable or weakly phosphorylated in the basal state, respectively, were primarily affected by exposure to EGF. In NRK membranes the ^{32}P-labeled 170 000 and 150 000 M_r bands comigrated with barely detectable Coomassie blue-stained bands. Thus, in NRK membranes the phosphorylated forms of 170 000 and 150 000 M_r proteins are minor components.

In K-NRK membranes, one phosphoprotein of $\sim$170 000 M_r which was weakly phosphorylated in the basal state, was particularly affected by EGF. The level of EGF-dependent phosphorylation of the 170 000 M_r protein in K-NRK membranes, however, was much lower than that of the 170 000 M_r protein in NRK membranes. Since in K-NRK membranes the 170 000 M_r phosphoprotein comigrated with a weakly stained band (fig.1 A), the phosphorylated form of the 170 000 M_r protein is a minor membrane component. Inspection of fig.1 shows that the 150 000 M_r phosphoprotein was undetectable in K-NRK membranes in both the basal and EGF-stimulated state. A prominent feature of K-NRK membranes is that phosphorylation in the absence of EGF resulted in a large incorporation of radioactivity into a component of $\sim$130 000 M_r (fig.1 B). The addition of EGF to the phosphorylation mixtures of K-NRK membranes resulted in a large stimulation of phosphorylation of the 130 000 M_r band (fig.2C,D). The EGF-stimulated phosphorylation of the 130 000 M_r band is not apparent in fig.1 because in this experiment the film was overexposed to detect minor components of K-NRK membranes such as the 170 000 M_r band.

3.2. *Effects of cell density on phosphorylation of NRK and K-NRK membrane components*

Since various cellular functions are dependent on cell density [4,8,9], the possibility exists that some of the observed effects (e.g., loss of the EGF-dependent phosphorylation of the 170 000 M_r protein in K-NRK membranes) may be due to cell density effects. Therefore, we have examined the effects of EGF on ^{32}P incorporation into specific membrane components derived from both NRK and K-NRK cells at various cell densities. Fig.2A shows that the EGF-dependent phosphorylation of the 170 000 and 150 000 M_r proteins in NRK membranes is dependent upon cell density. Fig.2A,B demonstrates that the phosphorylation of the 170 000 M_r membrane component is lower at lower cell densities. The phosphorylation of the 170 000 M_r protein was $\sim$3-fold higher at nearly maximal cell density than at low cell density. Intermediate phosphorylation of the 170 000 M_r protein was observed at intermediate cell densities. We observed that conditions non-permissive for growth also influenced the EGF-dependent phosphorylation of the 170 000 M_r protein in NRK membranes. Fig.2A,B shows that NRK cultures in stationary phase for $\sim$18–24 h prior to cell collection (fig.2A, lanes h and j, respectively) showed reduced EGF-dependent phosphorylation of the 170 000 M_r protein in comparison to cells that were not yet fully confluent at the time of collection (fig.2A, lane f). The basal levels of phosphorylation of the 170 000 M_r protein in NRK membranes appeared to be relatively independent of both cell density and conditions non-permissive for growth (fig.2A,B).

The results shown in fig.2C demonstrate that the EGF-dependent phosphorylation of the 170 000 M_r K-NRK membrane component was independent of cell density. The phosphorylation of the 130 000 M_r K-NRK membrane protein in both the basal and EGF-stimulated state was also independent of cell density (fig.2D). Thus, the loss of the EGF-dependent phosphorylation of the 170 000 M_r protein and the appearance of the heavily phosphorylated 130 000 M_r band in K-NRK membranes are not due to cell density effects.

3.3. *Phosphorylation of the Ts membrane components*

We have examined the effects of EGF on ^{32}P incorporation into specific membrane components from Ts cells when cultures were cycled between 32°C $\rightleftharpoons$ 39°C. Fig.3 shows that in Ts membranes the EGF-dependent phosphorylation of the 170 000 M_r protein was undetectable at 32°C and they exhibited just detectable levels of this phosphoprotein at 39°C. The level of EGF-dependent phosphorylation of the 170 000 M_r protein in Ts membranes at 39°C, however, was much lower than that of the 170 000 M_r protein in NRK membranes at 37°C (fig.1 B) or at

39°C (not shown). Fig.3 shows that in Ts membranes the 170 000 M_r band was undetectable in the basal state at both 32°C and 39°C. Switching the Ts cells from 32°C to 39°C produced a detectable increase in the EGF-dependent phosphorylation of the 170 000 M_r protein within 6 h (fig.3A). When Ts cells were switched from 39°C to 32°C, EGF-dependent phosphorylation of the 170 000 M_r protein disappeared in ~3 h. A prominent feature of Ts membranes at both 32°C and 39°C is that phosphorylation in the absence of EGF resulted in a large incorporation of radioactivity into a component of 130 000 M_r. The addition of EGF to the phosphorylation mixtures of Ts membranes at both temperatures resulted in a several-fold stimulation of phosphorylation of the 130 000 M_r band. The EGF-dependent phosphorylation of the

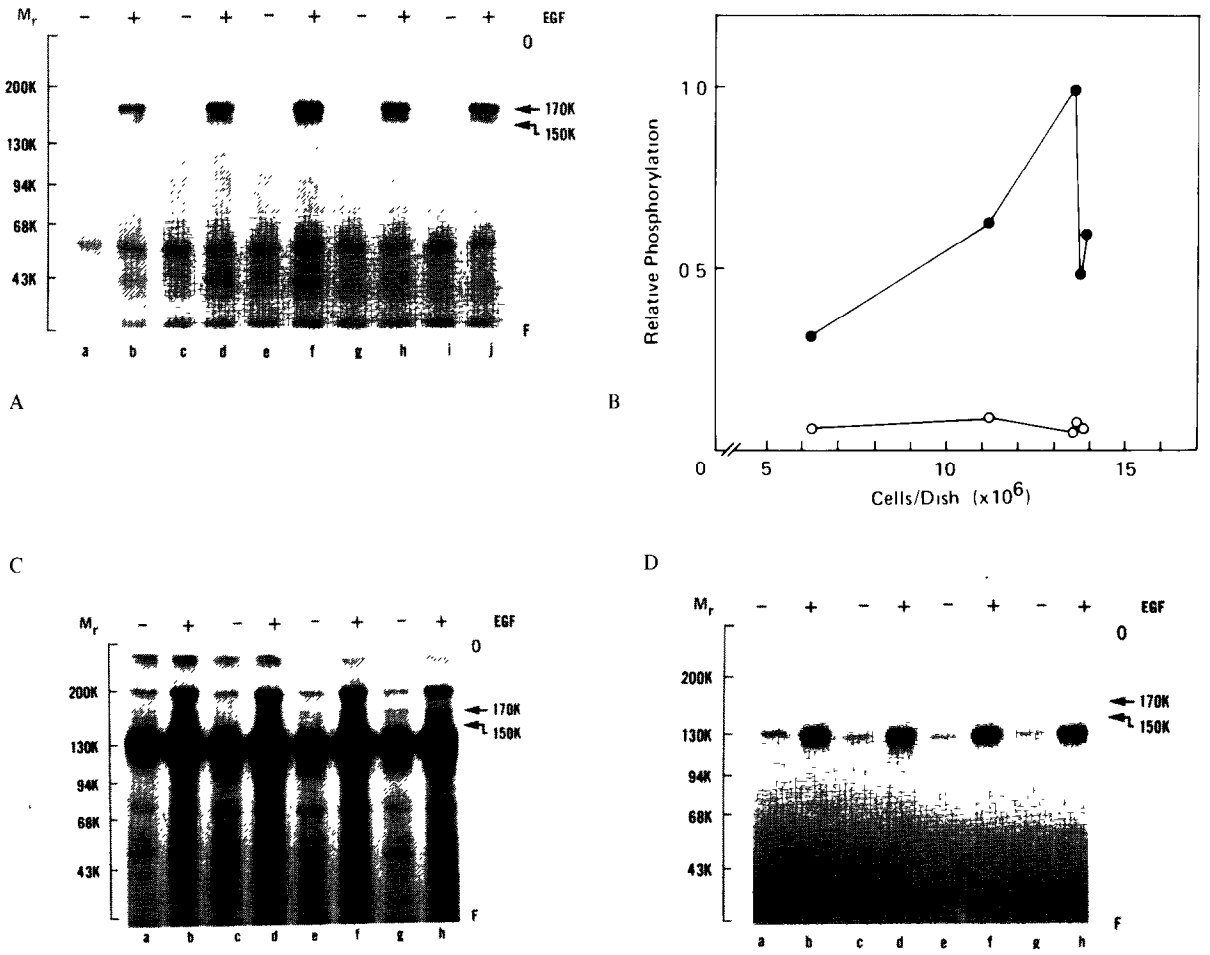

Fig.2. Effect of cell density on basal and EGF-stimulated phosphorylation of membrane components of NRK and K-NRK cells. Cells were plated at densities varying from 1500–10 000 cells/cm². After 26 h the medium was removed and new medium was added. After 36 h the cells were collected and the membranes were isolated. Membrane samples were prepared for phosphorylation, electrophoresis, Coomassie blue staining, and autoradiography as in section 2. The cell densities are for 10 cm plates. The phosphorylation assays were performed in the absence (–,○) or presence (+,●) of 120 nM EGF. The samples were analyzed in exponential 5–9% acrylamide gels. (A) Autoradiographs (7 h exposure) of NRK membrane samples. The cellular densities at the times of harvest were: 6.27 (a,b); 11.2 (c,d); 13.5 (e,f); 13.7 (g,h); 13.8 (i,j) cells/dish. (B) The relative phosphorylation of the 170 000 M_r protein of NRK membranes, corresponding to the experiment shown in (A), is plotted as a function of cell density. (C) Autoradiographs (72 h exposure) of K-NRK membrane samples. The cellular densities at the time of harvest ranged from ~2.0 × 10^6 (a,b) to 8.0 × 10^6 (g,h) cells/dish. (D) Same experiment as that shown in (C) but the film was exposed for 7 h to determine the extent of phosphorylation of the 130 000 M_r band. For details see text.

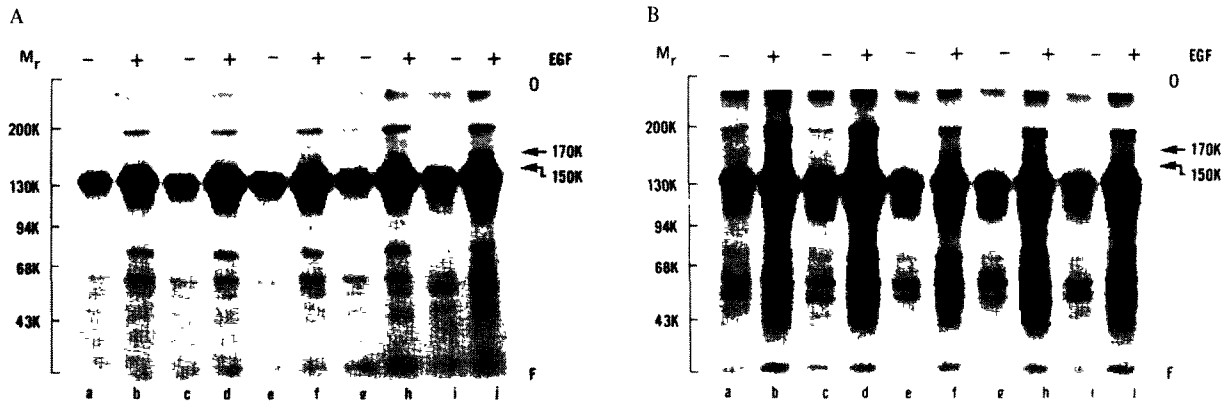

Fig.3. EGF-dependent phosphorylation of Ts membane components at permissive (32°C) and non-permissive (39°C) temperatures. Samples were prepared for phosphorylation, electrophoresis, Coomassie blue staining and autoradiography (72 h exposure) as in section 2. The samples were analyzed in exponential 5–9% acrylamide gels. See text for details. (A) Autoradiographs of samples from Ts cells switched from 32°C to 39°C and incubated at 39°C for 0 h (a,b), 1 h (c,d), 3 h (e,f), 6 h (g,h), and 48 h (i,j). (B) Autoradiographs of samples from Ts cells switched from 39°C to 32°C and incubated at 32°C for 0 h (a,b), 1 h (c,d), 3 h (e,f), 6 h (g,h), and 24 h (i,j).

130 000 M_r protein was more prominent at 39°C than at 32°C. The basal level of phosphorylation of the 130 000 M_r protein was also slightly higher at 39°C than at 32°C. Inspection of fig.2C and 3A,B revealed that the phosphorylation patterns of Ts membranes at both temperatures were similar to those of K-NRK membranes at 37°C (fig.2C) or at both 32°C or 39°C (not shown).

4. Discussion

Here we report in NRK membranes there are numerous components whose phosphorylation can be stimulated by EGF. Among these phosphoproteins, two components of M_r 170 000 and 150 000 were primarily affected by EGF. Additionally, we found that the phosphorylation of the 170 000 and 150 000 M_r proteins differed significantly between proliferating and density-inhibited cells. These results suggest that the 170 000 and 150 000 phosphoproteins may be involved in regulatory events associated with cell proliferation. These data are consistent with data and suggestions in [10] that phosphoproteins of M_r 170 000 and 150 000 may be components of the EGF receptor of a variety of mammalian cells. Therefore, the doublet of M_r 170 000 and 150 000 we have observed in the NRK membranes may represent components of the EGF receptor of NRK cells which are substrates of the phosphorylation reaction.

These findings establish an association between Kirsten sarcoma virus-induced transformation and reduction of EGF-dependent phosphorylation of the 170 000 M_r membrane component. From a comparison of the results with K-NRK membranes (fig.1) and the data in [7], it can be inferred that the loss of EGF-dependent phosphorylation of the 170 000 M_r protein is associated with a loss of EGF receptors. It is interesting to note that despite the major loss of the EGF-dependent phosphorylation response of the 170 000 M_r protein, addition of EGF still can extensively stimulate phosphorylation of numerous membrane components. This finding suggests that if the 170 000 M_r protein is the receptor for EGF in K-NRK membranes, the concentrations of receptors remaining after viral transformation are sufficient to elicit an extensive phosphorylation of numerous membrane components. Alternatively, the presence of a defective EGF receptor in K-NRK membranes is also possible and may account for the enhanced membrane phosphorylation induced by EGF.

The mechanism leading to reduced EGF-dependent phosphorylation of the 170 000 M_r protein in K-NRK membranes is as yet a matter for speculation. The decrease phosphorylation response of the 170 000 M_r protein to EGF could be due to an absolute decrease in the number of 170 000 M_r molecules present in K-NRK membranes. The presence in K-NRK membranes of an active endogenous protease may result in proteolytic processing of the 170 000

M_r phosphoprotein [15]. If this is the case, the 130 000 M_r component is a likely candidate for the initial fragment produced by proteolytic digestion of the 170 000 M_r phosphoprotein. Of course, other factors, such as the relative concentrations or activities of protein kinases or phosphatases in K-NRK membranes, may also be important.

An attempt was made to correlate phenotypic changes produced by switching Ts cells to permissive and non-permissive temperatures with changes in both the basal and EGF-dependent phosphorylation of specific membrane components. Although many changes in protein phosphorylation were observed, the changes in phosphorylation of the 170 000 M_r protein after downshift of Ts cells to the permissive temperature suggest a correlation between the transformed phenotype, the decrease in EGF receptors [7], and the decrease in EGF-dependent phosphorylation of the 170 000 M_r protein. Since the phosphorylation patterns of Ts membranes at the permissive and non-permissive temperatures were similar to that of K-NRK membranes, it seems possible that the reduced phosphorylation response of the 170 000 M_r protein and the presence of a prominent EGF-sensitive 130 000 M_r phosphoprotein band may be characteristic of cells transformed by murine sarcoma virus.

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