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# NONFUNCTIONAL EPIDERMAL GROWTH FACTOR RECEPTOR IN CELLS TRANSFORMED BY KIRSTEN SARCOMA VIRUS

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The cell membrane receptor for epidermal growth factor (EGF) appears to be a glycoprotein of $M_r$ 170,000 and mediates the mitogenic and metabolic responses of cells with EGF receptors (EGF-R). Normal rat kidney (NRK) have about 3 x $10^5$ EGF-R per cell. Upon transformation of NRK cells by Kirsten sarcoma virus, the transformed derivative (KNRK) loses the ability to bind $^{125}$I-EGF. Membranes from NRK and KNRK cells were included in EGF-dependent phosphorylation reactions to search for evidence of the EGF-R. A phosphorylated protein of $M_r$ 170,000 was detected in both NRK and KNRK membranes. The $M_r$ 170,000 protein was identified to be EGF-R by immunoprecipitation with monoclonal antibody to the receptor. Furthermore, two-dimensional peptide mapping using trypsin and chymotrypsin digestions of the iodinated receptors from both NRK and KNRK cells showed essentially identical patterns. These data indicate that the EGF-R is present in KNRK cells with apparently the same protein structure as the NRK counterpart.

Epidermal growth factor (EGF)[1], a single polypeptide chain of $M_r$ 6045, is a potent mitogen whose effect is mediated by specific cell surface receptors (1). The biochemical and cellular responses of EGF can be divided into immediate and delayed stages (for review, see 2). The immediate responses occurring within seconds or minutes include the initiation of EGF binding (3,4), clustering of cell-bound EGF (5) and phosphorylation of membrane proteins (6-8). An EGF-stimulated protein kinase phosphorylates endogenous membrane proteins as well as EGF receptor (EGF-R) at tyrosine residues (9,10). The delayed responses

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include events related to cell proliferation, such as DNA replication, RNA and protein synthesis (11-13) and completion of cellular division (14).

The EGF-R on the plasma membrane has been characterized in a number of cell types and tissues (15-20) and seems to be a glycoprotein of $M_r$ 170,000 - 190,000. Most mammalian cells have about $10^5$ EGF-R per cell. Human epidermoid carcinoma cell line A431 contains as many as 2 x $10^6$ EGF-R per cell and has served as a model for the purification and extensive characterization of EGF-R (20-22).

We have demonstrated that normal rat kidney cells (NRK) have 3 x $10^5$ EGF-R per cell and upon transformation with Kirsten murine sarcoma virus (KiMSV), the transformed derivative (KNRK) lost the ability to bind $^{125}$I-EGF (23). This phenomenon appears to be a general effect of the RNA tumor viruses used in transformation (24-27). The mechanism of the loss of $^{125}$I-EGF binding remains unknown. It may reflect a complete or partial loss of the EGF-R complex or some structural alterations of the EGF-R domains. In this study, we used monoclonal antibody to EGF-R to identify receptor material and to immunoprecipitate EGF-R from both NRK and KNRK cells. KNRK cells were found to have EGF-R immunoprecipitable materials. Two-dimensional peptide mapping suggested that the EGF-R in KNRK cells had the same protein structure as the NRK counterpart.

## MATERIALS AND METHODS

### Cell Culture

Normal rat kidney cells (NRK) and KiMSV - transformed rat kidney cells (KNRK) were originally developed by Carchman et al (28) and subcloned (23). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Flow Laboratories) in 75 $cm^2$ tissue culture flasks (Costar). Cells were cultivated at one to ten split ratio every other day and were incubated at 37°C in humidified air containing 5% $CO_2$.

### Isolation of EGF

Mouse EGF was isolated from the submaxillary glands of male mice (Pel-Freeze Biologicals) by the method of Savage and Cohen (1).

### Ornithine Decarboxylase Assay

Subconfluent cultures of NRK and KNRK cells were washed twice with phosphate buffered saline (0.116 M NaCl, 1.5 mM $KH_2PO_4$, 12 mM $Na_2HPO_4$) and changed to 10 ml of serum-free DMEM for 24 h. Ten ml of fresh serum-free medium containing different amount of EGF was added and incubated at 37°C for 4 h. Ornithine decarboxylase (ODC) activity was measured by the release of $^{14}CO_2$ from

[1-$^{14}$C] ornithine according to the method of Bethell and Pegg (29). The cell extract (0.4 ml) was incubated in a final volume of 0.5 ml containing 0.08 mM pyridoxal phosphate, 2.5 mM dithiothreitol, 60 mM Tris-HCl, pH 7.5, 0.08 mM $Na_2$-EDTA and 0.5 µ Ci of DL-[1-$^{14}$C] ornithine (sp. act. 49.6 mCi/mmol, New England Nuclear). The reaction mixture was incubated at 37°C for 60 min and stopped by the addition of 0.6 ml 5M $H_2SO_4$. The $^{14}CO_2$ released was trapped in 0.5 ml of Hyamine hydroxide. Results were expressed as pmol of $^{14}CO_2$ released per mg protein added. The protein concentration was determined by the dye-binding method of Bradford et al (30) using bovine serum albumin (BSA) as standard.

EGF-dependent membrane phosphorylation

NRK and KNRK cell membranes were isolated by the method of Thom (31). Membranes were either untreated or solubilized with Triton X-100 by the method of Cohen et al (32). Phosphorylation of the original or the solubilized membranes was carried out as described by Fernandez-Pol (8). The reaction mixtures (50 µl) contained NRK or KNRK membranes (62.5 µg), Hepes buffer (20 mM, pH 7.4), $MnCl_2$ (2 mM), 0.125% BSA, [$\gamma$-$^{32}$P]-ATP (50 µCi, sp. act. 4285 Ci/mmole, ICN, Irvine, Ca) with or without EGF. The reaction was stopped by adding 50 µl of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.5% glycerol, 1.25% β-mercaptoethanol, 0.75% SDS), heated at 90°C for 3 min and loaded on an 8% SDS-polyacrylamide slab gel with a 4% stacking gel according to Laemmli (33). $^{14}$C-labelled molecular weight standards were purchased from Bethesda Research Laboratories, Inc. The gel was electrophoresed at constant current (50 mA per gel). The gel was fixed in 25% methanol and 10% acetic acid, dried on a Biorad filter paper and exposed to a Kodak X-Omat AR film at -70°C.

Metabolic Labeling

Subconfluent cultures of cells in 75 cm$^2$ flasks were rinsed three times with phosphate buffered saline (PBS) and incubated with [$^{35}$S]-methionine (50 µCi/ml) (specific activity 1017 Ci/mmole, New England Nuclear) in 10 ml of serum-free and methionine-free DMEM for 16 h. At the end of the labeling period, the cells were rinsed with ice-cold PBS and scraped into PBS with a rubber policeman. After a low speed centrifugation, the cell pellets were solubilized at 24°C for 1 h in 0.5 ml 50 mM Hepes buffer, pH 7.4 containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1000 units/ml aprotinin (Sigma). Insoluble material was removed by centrifugation at 100,000 x g for 90 min at 4°C.

Immunoprecipitation of the EGF-R

Immunoprecipitation was carried out with the phosphorylated protein mixture by adding monoclonal EGF-R antibody 2G2-IgM (34-36) or antibody 29 IgG (Schreiber, unpublished). Optimal amount of antibody was added to the phosphorylated protein mixtures or$^{35}$S-methionine labelled cell lysates and incubated at 37°C for 90 min in the presence of 100 mM NaCl, 50 mM Tris-HCl (pH 7.2), 0.5% Triton X-100, 0.5% deoxycholate, 0.15% SDS. A hundred µl of protein A-Sepharose (Pharmacia) was added for 90 min at 4°C. Immunoprecipitates were collected by centrifugation, washed, resuspended in 120 µl of SDS gel sample buffer and boiled for 3 min. Aliquots of the supernatant were analyzed by an 8% SDS-polyacrylamide gel.

Two-dimensional peptide mapping of the EGF-R

EGF-R band ($M_r$ 170,000) was excised from the immunoprecipitate gel and iodinated according to Elder et al (37). Two-dimensional peptide mapping with trypsin and chymotrypsin was carried out as described by Elder et al (37) with slight modifications. The gel samples were analyzed on cellulose-coated TLC plates (20 x 20 cm) (EM Laboratories). The samples were dissolved in 10 µl of

electrophoresis buffer (HAC:formic acid:$H_2O$, 15:5:80) and 2 µl was spotted onto each plate. Electrophoresis was carried out at 4°C on a high voltage electrophoresis apparatus (Savant Model TLE-20) in electrophoresis buffer at 1,000 V for 60 min. The plates were dried and subjected to chromatography in the second dimension in butanol:pyridine:HAC:$H_2O$ (32.5:25:5:20). The plates were dried and analyzed by autoradiography using Kodak XAR films.

## RESULTS

We previously have shown that KNRK cells have lost their ability to bind $^{125}$I-EGF (23). A number of other physiological assays were carried out to determine if EGF-R was functional in KNRK cells. EGF is known to rapidly induce ODC activity in responsive cells (38,39). Fig. 1 shows the dose-response curve of both NRK and KNRK cells after exposure to EGF for 4 h. The basal ODC activity in KNRK cells in the absence of EGF was substantially higher than that in the NRK cells. Addition of EGF to KNRK cells did not affect ODC activity. In contrast, the ODC activity in NRK cells increased dramatically and peaked at 25 ng/ml EGF.

We next measured the EGF-R mediated phosphorylation of NRK and KNRK membrane proteins. KNRK membranes did not respond to increasing concentration of EGF. However, there was an $M_T$ 170,000 phosphorylated protein which remained at the same level regardless of the amount of EGF added (Fig. 2, lanes 1-6).

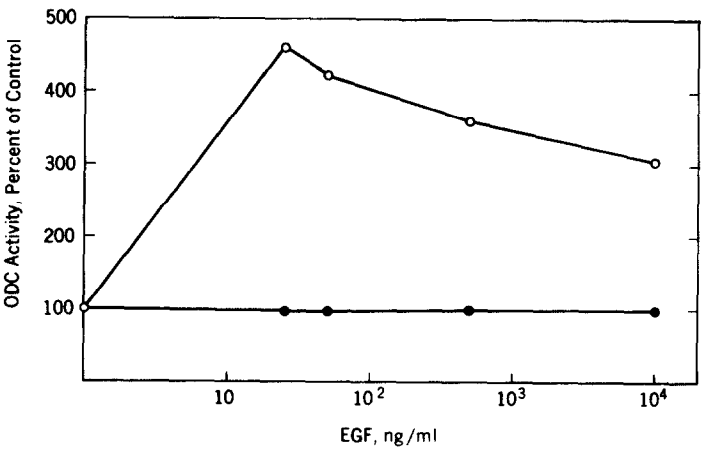


Fig. 1. The induction of ornithine decarboxylase activity in NRK and KNRK cells by EGF. NRK and KNRK cells were cultivated in 75 cm$^2$ flasks. ODC activity in the presence of increasing amount of EGF was determined as described in Materials and Methods. The activity for control cells in the absence of EGF was 18 pmol $^{14}CO_2$ released/mg/60 min for NRK cells (o--------o) and 560 pmol $^{14}CO_2$ released/mg/60 min for KNRK cells (•---------•).

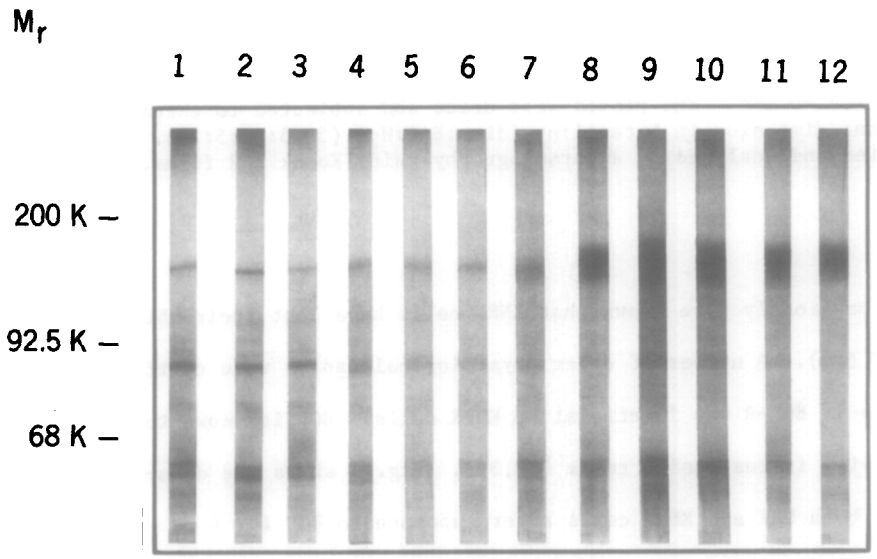


*Fig. 2.* *Effect of EGF on the phosphorylation of NRK and KRNK membrane proteins.* NRK and KNRK membranes were isolated from six 850 $cm^2$ roller bottles. EGF-stimulated phosphorylation was carried out in 50 µl reaction mixtures containing 65 µg of KNRK membranes (lanes 1-6) or 61 µg of NRK membranes (lanes 7-12). EGF level was 0 (lanes 1, 7), 0.67 µM (lanes 2, 8), 1.7 µM (lanes 3,9), 3.4 µM (lanes 4,10), 6.8 µM (lanes 5, 11), 17 µM (lanes 6, 12). The phosphorylated membrane proteins were analyzed on an 8% SDS-polyacrylamide gel. Molecular weight markers:myosin (200,000), phosphorylase b(92,500) and bovine serum albumin (68,000).

In NRK cells, phosphorylation of the $M_r$ 170,000 protein was EGF-dependent, reaching an optimal level at 1.7 µM (lanes 7-12).

In order to confirm that the $M_r$ 170,000 protein was EGF-R, the membranes were solubilized with Triton X-100, phosphorylated in the presence of [$\gamma$-$^{32}$P]-ATP and immunoprecipitated with monoclonal antibody 2G2-IgM to EGF-R. Fig. 3 shows the immunoprecipitates with and without the specific antibody. The $M_r$ 170,000 protein was present in the immunoprecipitate from both the NRK and KNRK cells (lanes 2,4) indicating that the EGF-R in KNRK cells retained the antigenic sites for monoclonal 2G2-IgM. The unsolubilized membranes showed similar immunoprecipitate pattern (results not shown).

We next labelled cells biosynthetically with $^{35}$S-methionine and immunoprecipitated NRK and KNRK cell lysates by another EGF-R antibody 29 IgG (Fig. 4). The specific immunoprecipitates were demonstrated in lanes 2 and 4 whereas the nonspecific binding was represented by lanes 1 and 3. The $M_r$ 170,000 band was again found in KNRK cell lysates.

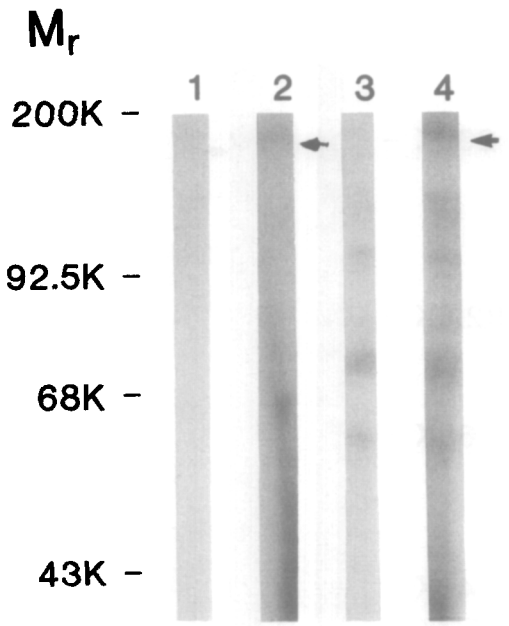


Fig. 3. Immunoprecipitation of EGF-R by monoclonal antibody 2G2. NRK and KNRK membranes were solubilized with Triton X-100 and centrifuged at 12,000 g for 10 min. The solubilized membranes were phosphorylated in 250 µl containing 5.4 µg EGF, 20 mM Hepes, 2 mM $MnCl_2$, 0.125% BSA, 278 µg NRK membranes or 259 µg KNRK membranes. The phosphorylation reaction was carried out as described in Materials and Methods. Reaction was stopped by adding 50 µl Laemmli sample buffer and heated for 3 min. Aliquots of 35 µl of the phosphorylated protein mixtures were immunoprecipitated with EGF-R monoclonal antibody 2G2-IgM in the presence of 100 mM NaCl-50 mM Tris-HCl (pH 7.2), 0.5% Triton X-100, 0.5% deoxycholate and 0.15% SDS. Protein A-Sepharose was used as the second antibody to bring the antigen-antibody complex down. The immunoprecipitates were washed, boiled and anlayzed on an 8% SDS-polyacrylamide gel. Molecular weight markers were: myosin (200,000), phosphorylase b (92,000), bovine serum albumin (68,000) and ovalbumin (43,000). Lanes 2 and 4 corresponded to immunoprecipitates of phosphorylated NRK and KNRK membranes in the presence of monoclonal antibody 2G2. Lanes 1 and 3 represented nonspecific binding of phosphorylated NRK and KNRK membranes to protein A-Sepharose in the absence of antibody.

The obvious question to follow is whether the EGF-R in KNRK cells is the same or different from the parent cells. The phosphorylated EGF-R was excised from the immunoprecipitate gel and iodinated according to Elder et al (37). Two-dimensional peptide mapping of tryptic (Fig. 5, panels A,B) and chymotrypic (panels C,D) digestion showed essentially identical patterns.

## DISCUSSION

Cells transformed by RNA tumor viruses (murine sarcoma virus and feline sarcoma virus) lose their ability to bind $^{125}$I-EGF, but the binding of other factors, such as insulin or multiplication-stimulating activity (MSA) may not be

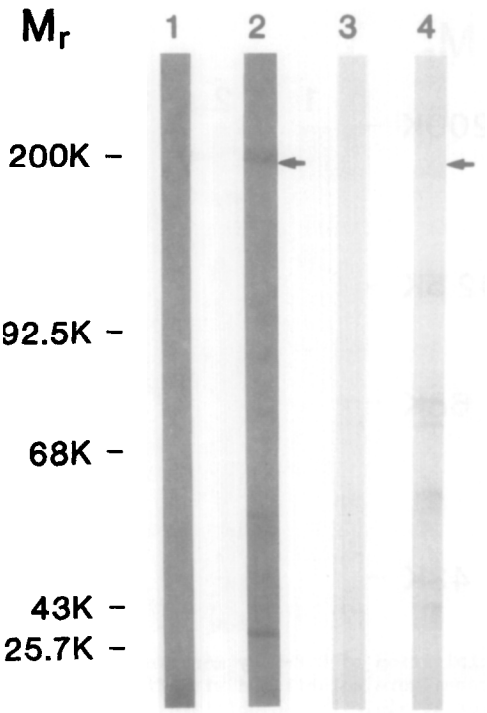


Fig. 4. Immunoprecipitation of EGF-R by monoclonal antibody 29 IgG. NRK and KNRK cells were labeled with $^{35}$S-methionine as described in Materials and Methods. Aliquots of 30 µl of cell lysates were immunoprecipitated with EGF-R monoclonal antibody 29 IgG. The conditions used were described in legend for Fig. 3.

altered (24-27). Previously we reported that KNRK cells showed no specific $^{125}$I-EGF binding (23), but, curiously, the level of non-specific $^{125}$I-EGF binding to KNRK was 10 to 15 fold greater than control NRK cells. This suggested to us that the loss of $^{125}$I-EGF binding by KNRK cells may not simply result from the loss of EGF-R but could be an indication of a complex membrane change or a structural alteration of EGF-R. Our findings indicate that EGF-R is not absent from KNRK membranes.

Fernandez-Pol et al (40) previously reported that phosphorylation of KNRK membranes in the absence of EGF resulted in the incorporation of radioactivity into a $M_r$ 130,000 protein, whereas the phosphorylation of $M_r$ 170,000 band was reduced. In the presence of EGF, the phosphorylation of the $M_r$ 130,000 band was greatly enhanced. These workers speculated that the $M_r$ 130,000 band might be a degradation product of the EGF-R in KNRK cells. The $M_r$ 130,000 band was not

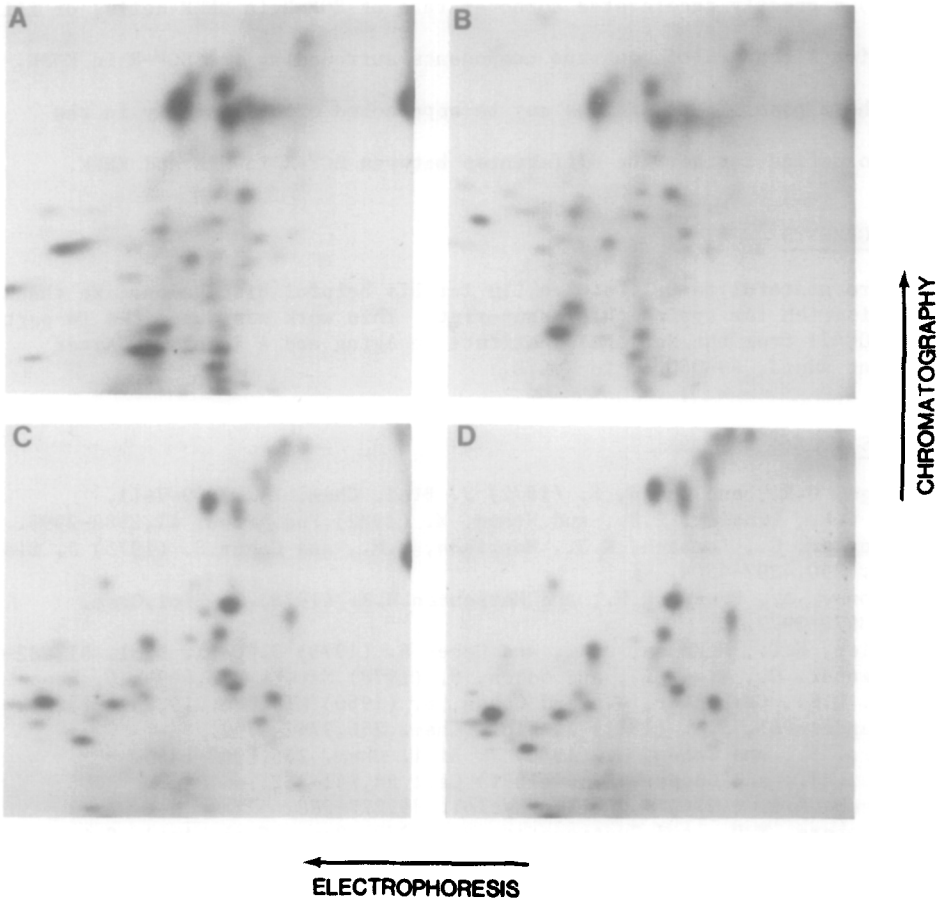


Fig. 5. Two-dimensional peptide mapping of iodinated EGF-R from NRK and KNRK cells. EGF-R band from the immunoprecipitate gel was excised and iodinated according to Elder et al. Two-dimensional peptide pattern of trypsin (panels A, NRK; B, KNRK) and chymotrypsin digestion (panels C, NRK; D, KNRK) were shown.

present under our experimental conditions. The reason for this discrepancy could be due to differences in membrane isolation procedures.

Most transforming DNA viruses do not affect the presence of EGF-R; however, a clone of SV40 transformed mouse embryo kidney fibroblast has been isolated which exhibited a 90% reduction in EGF-R (41). The EGF-R of this cell line was found to have an alteration in the carbohydrate moiety since it would no longer bind to concanavalin A.

The underlying mechanism for the apparent loss of $^{125}$I-EGF binding to KNRK cells remains unknown. A number of possibilities exist, including i) an alteration in the carbohydrate moieties specifically affecting the binding domain of

EGF-R; ii) a greatly accelerated turnover rate of EGF-R in KNRK cells, or iii) conformational changes of membrane components surrounding the EGF-R in KNRK. Each of these possible conditions may be approached experimentally in the attempt to define further the differences between EGF-R in NRK and KNRK.

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