Immunoautoradiographic detection of epidermal growth factor receptors after electrophoretic transfer from gels to diazo-paper

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1. INTRODUCTION

Several groups have attempted to identify the EGF receptor by a variety of biochemical techniques. Das et al. [1] identified the EGF receptor on 3T3 cells as a radioactive band of M_r = 190 000 by photoaffinity labeling with a ^{125}I-EGF probe; Hock et al. [2] identified the EGF receptor in human placenta as a doublet of 160 000 and 180 000 M_r by covalent cross-linking to ^{125}I-labeled EGF. The EGF receptor on A-431 cells was identified [3] as a radioactive band of M_r 175 000 by 'direct labeling', which is accomplished by incubation of cells with ^{125}I-EGF. Cohen and coworkers [4] have partially purified the EGF receptor of A-431 cells by affinity chromatography and analyzed the denatured proteins of such preparations by SDS–polyacrylamide gel electrophoresis. They found numerous protein bands in the affinity-purified preparations and suggested that a major protein band of M_r 150 000 is the receptor for EGF in A-431 cells. More recently, Hunter and Cooper [5] isolated the EGF receptor from ^{32}P-labeled A-431 cells by immunoprecipitation with an antiserum raised against crude plasma membrane from A-431 cells. They have estimated the M_r of the EGF receptor present in the immunoprecipitates from A-431 cells as 155 000.

The interaction of EGF with its membrane receptor, present in both crude plasma membranes from A-431 cells and in affinity-purified preparations, results in specific stimulation of a protein kinase activity [4]. This activity phosphorylates the 150 000–170 000 M_r doublet, numerous endogenous membrane proteins and a variety of exogenous substrates [4]. It is not clear yet whether the receptor and the kinase activities are present in the same molecule or whether two separate entities are involved [4].

We report here the use of an immunoautoradiographic system for the detection and analysis of EGF receptors in complex protein mixtures. By utilizing this technique, we can visualize the interaction between EGF and its membrane receptor in A-431 cells. The procedure is simple and very sensitive, and it provides evidence that the 150 000 M_r protein is the predominant EGF receptor protein in A-431 membranes. This study, therefore, provides the basis for more detailed immunoautoradiographic studies of this important growth factor receptor.

2. MATERIALS AND METHODS

Mouse EGF (receptor grade) and anti-mouse EGF rabbit antiserum were obtained from Collab. Res. (Waltham MA). Sources of other materials were as in [6,7].

2.1. *Cell culture, membrane isolation, and preparation of soluble membrane proteins*

Human epidermoid carcinoma A-431 cells were provided by G. Todaro (NCI). A-431 cells were grown in 100-mm culture dishes containing Dulbecco-Vogt modified Eagle's medium supplemented with 10% calf serum as in [6]. Membranes were prepared by the method [8] as in [6]. The membranes were solubilized (4–6 mg protein/ml) in 20 mM

Abbreviations: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DBM, diazobenzyloxmethyl; TENG-N, 50 mM Tris–HCl (pH 7.4)–5 mM EDTA–150 mM NaCl–0.25% gelatin–0.05% Nonidet P-40

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Hepes buffer (pH 7.4) containing 1% (v/v) Triton X-100 and 10% glycerol [4]. After 30 min at 25°C the incubation mixture was centrifuged at 100 000 × *g* for 1 h at 2°C. The supernatant fraction contained the solubilized membrane proteins.

2.2. *Affinity chromatography*

2.2.1. Preparation of EGF-Affi-Gel

EGF (700 μg) was conjugated to Affi-Gel (500 μl packed beads) according to the instructions supplied by the manufacturer (Bio Rad, Richmond CA). Affi-Gel was then washed extensively as in [4].

2.2.2. Affinity purification of EGF receptor and protein kinase activity

The EGF receptor–kinase complex was purified by affinity chromatography as in [4]. In brief, solubilized A-431 membrane proteins (~ 15 mg protein in 2 ml) were incubated with packed EGF-Affi-Gel beads (300 μl) and the mixture was stirred at 25°C for 30 min. Then, the non-adsorbed material was removed, and the pelleted gel was washed 3 times with 1 ml 1% Triton X-100, 10% glycerol (pH 7.2) at 4°C. The material bound to the EGF-Affi-Gel was eluted with 300 μl cold elution buffer (10% glycerol, 1% Triton X-100, 5 mM ethanolamine (pH 9.7)) with gentle stirring for 30 min at 0°C. The resulting ethanolamine eluate was divided into portions and stored at – 70°C.

2.3. *Phosphorylation of membrane proteins*

The phosphorylation assay was performed at 0°C as in [7]. Standard conditions of phosphorylation were used for Triton X-100-solubilized membranes, except that EGF was allowed to interact with the solubilized membrane proteins for 10 min at 25°C prior to performing the phosphorylation assay at 0°C [4].

2.4. *Gel electrophoresis*

SDS–polyacrylamide gel electrophoresis was done in exponential gradient gels of 5–9% acrylamide using the buffer system in [9]. Unless otherwise noted, samples containing 5×10^5 cpm or 100 μg protein were applied/lane. After electrophoresis, gels were equilibrated for 1 h in transfer buffer (25 mM sodium phosphate at pH 6.5) as in [10]. The gels were then either dried and autoradiographed or immediately used for transfer to DBM-paper.

2.5. *Electrophoretic transfer of proteins from SDS–polyacrylamide gels to DBM-paper*

Aminobenzyloxymethyl (ABM)-paper was prepared as in [11] or obtained from Schleicher and Schuell (NH). ABM-paper was converted to diazobenzyloxymethyl (DBM) form exactly as in [11]. The apparatus utilized in the transfer procedure was from EC Apparatus (St Petersburg) or Bio Rad. The transfer conditions were similar to those in [10]. Transfer was performed at 0.6 A for 3 h. After transfer, the DBM-paper was placed in 250 ml 0.1 M Tris–HCl (pH 9.0), 0.25% gelatin, 10% (v/v) ethanolamine for 2 h at 37°C [12].

2.6. *^{125}I-Labeled protein A*

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

2.7. *Immunoautoradiography of DBM-paper containing gel protein transfers*

After washing as above, DBM-paper protein transfers were rinsed in water and blotted on Whatman 3 MM paper. Unless otherwise noted, the DBM-paper was incubated with EGF (100 ng/ml) in TENG-N [10], 50–100 μl/cm^2, in plastic boiling bags (Seal-N-Save, Sears) with gentle rocking for 1 h at 37°C. The paper was then washed for 3 h at 37°C with 800 ml same buffer (without EGF). Anti-EGF antiserum was diluted 1:16 in TENG-N, and 1.25 ml was added/lane DBM–protein transfer. The transfer was placed in plastic bags and incubated for 16 h with gentle rocking at 37°C. The transfer was rinsed in 800 ml TENG-N for 3 h at 37°C. It was then blotted and incubated with ^{125}I-labeled protein A (3×10^5 cpm/ml) in TENG-N in a plastic bag for 2 h at 37°C, rinsed briefly with water, and washed extensively in 250 ml 50 mM Tris–HCl (pH 7.4)–5 mM EDTA–1 M NaCl–0.25% gelatin–0.4% Sarkosyl (Sigma) with rocking at 37°C for ~ 24 h. The DBM-paper was rinsed with water, blotted, air dried and autoradiographed by using Kodak X-Omat R film and a Dupont Cronex® Quanta III intensifying screen at – 70°C.

2.8. *Other procedures*

Protein was determined in triplicate as in [13] using bovine serum albumin as standard. 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. These results were reproduced in ≥ 4 separate expt each. Several different batches of anti-EGF antiserum were used and all of them produced virtually identical results.

3. RESULTS

3.1. *Electrophoretic transfer of membrane proteins from SDS–polyacrylamide gels to DBM-paper*

To determine optimal conditions for the transfer of proteins from gels in DBM-paper, we analyzed A-431 membranes phosphorylated in the absence and presence of EGF. Duplicate samples were separated by electrophoresis in 5–9% SDS–polyacrylamide gradient gels. After electrophoresis, identical gels were either dried and autoradiographed or immediately used for transfer of proteins to DBM-paper which was then autoradiographed. The results of numerous experiments demonstrated that electrophoretic transfer of ^{32}P-labeled proteins from gels to DBM-paper was accomplished efficiently in 25 mM sodium phosphate buffer at pH 6.5.

We obtained transfer of proteins of all M_r-values to DBM-paper with excellent resolution (fig.1). As can be seen in fig.1B, the transferred proteins were representative of those in the polyacrylamide gels shown in fig.1A. For example, note that in lane 3 of fig.1B the 150 000 M_r protein is clearly defined on the transfer. Furthermore, lane 4 of fig.1B demonstrates that the enhancement of phosphorylation of the 150 000–170 000 M_r duplet induced by EGF replicates that observed in lane 2 of fig.1A. The results shown in fig.1B, lanes 1 and 2, also demonstrate that proteins of low M_r were transferred from gels to DBM-paper with excellent fidelity. As found in [10], autoradiographic analysis of DBM-paper was more sensitive than that of dried gels (fig.1A,B).

The extent of electrophoretic transfer of proteins to DBM-paper was a function of the concentration of proteins in the gels and the M_r-value. Visual inspection of gels and transfer indicate that transfer was virtually quantitative over the entire observable M_r-range (fig.1A,B). By densitometry of individual Coomassie blue-stained and ^{32}P-labeled protein bands in gels with and without transfer and ^{32}P-labeled bands bound to DBM-paper, we determine that, when 100 μg protein input were used, 30–75% of the protein was transferred, depending upon the M_r of the protein. The least transfer was observed with proteins > 200 000 M_r. The efficiency of transfer of the proteins of interest for this study (M_r 150 000–170 000) varied from 30–40%. Further experiments demonstrated (not shown) that 100 μg protein input were optimal for transfer under our conditions.

3.2. *Immunoautoradiographic detection of EGF receptors after electrophoretic transfer from gels to DBM-paper*

Having demonstrated excellent transfer of ^{32}P-labeled proteins from SDS–polyacrylamide gels to DBM-paper, we prepared equivalent transfers of unlabeled proteins from A-431 membranes for immunoautoradiographic analysis. DBM-paper transfers of SDS–polyacrylamide gels were incubated sequentially with EGF, anti-EGF antiserum and then with ^{125}I-labeled protein A, which binds to the Fc portion of IgG [14] (fig.2, lane 1). Among proteins from A-431 membranes treated with EGF, anti-EGF antiserum and ^{125}I-labeled protein A, only one protein band of 150 000 M_r was readily detected. No proteins were labeled in control experiments in which either EGF or anti-EGF antiserum were omitted from the incubation mixtures (fig.2, lanes 2,3). Furthermore, in control experiments in which anti-EGF antiserum was replaced by non-immune rabbit serum, the 150 000 M_r protein was not detected (not shown). Thus, the reaction between EGF and the 150 000 M_r membrane protein is selective.

The specificity of the interaction between EGF, anti-EGF antiserum, and the 150 000 M_r protein was further examined by the addition of a wide variety of peptide hormones and growth factors to the incubation mixture in place of EGF. When the DBM-paper transfers were reacted with luteneizing hormone, thyrotropion, prolactin, glucagon or transferrin (each added at final conc. 100 ng/ml) and then with anti-EGF antiserum and ^{125}I-labeled protein A, no bands were observed. Thus, the reaction between EGF, anti-EGF antiserum and the 150 000 M_r protein is specific.

The sensitivity of the immunoautoradiographic analysis of EGF receptors was determined by using increasing dilutions of membrane protein (6–100 μg) in the transfer procedure. We found that detection of EGF receptors was achieved efficiently when as little as 25 μg membrane protein were applied to the gel (not shown). Of course, the sensitivity varied with the amount of EGF, the titer of the serum, the specific activity of the protein A and the time of exposure.

From a comparison of the results with ^{32}P-labeled proteins (fig.1) and the data in fig.2, it can be inferred that the phosphoprotein of 150 000 M_r comigrates with the EGF receptor protein detected by immunoautoradiography. These results indicate that the EGF receptor is probably a phosphoprotein.

3.3. *Immunoautoradiographic analysis of EGF receptors in affinity-purified preparations*

In the following experiments, we compared phosphorylated components (detected by autoradiography) with EGF receptors (detected by immunoautoradiographic analysis) both present in the material eluted from the EGF-Affi-Gel by ethanolamine.

We conclude from the autoradiography data shown in fig.3 that:

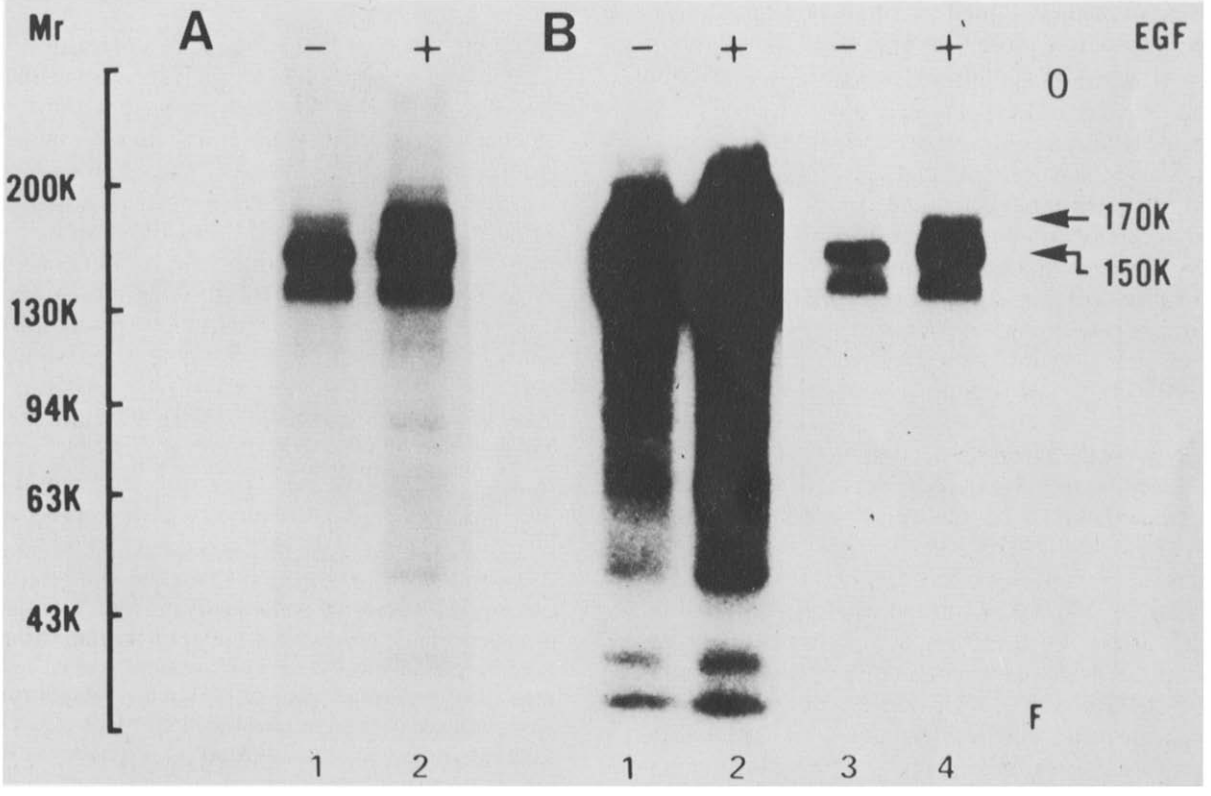

Fig.1. Electrophoretic transfer of ^{32}P-labeled A-431 membrane proteins from SDS–polyacrylamide gels to DBM-paper. Membranes were subjected to the standard phosphorylation procedures and the samples were separated by electrophoresis: (A) autoradiograph (24 h exposure) of membrane components phosphorylated in the absence (– , lane 1) or presence (+ , lane 2) of EGF resolved on a 5–9% SDS–polyacrylamide gel; (B) samples duplicating those in (A) were transferred electrophoretically to DBM-paper and autoradiographed; lanes (1,2), DBM-paper autoradiographs exposed for 24 h to determine fidelity of transfer in the low-M_r region of the gel; (3,4), same transfer as that shown in (1,2) but the film was exposed for 2 h to determine fidelity of transfer of the 150 000–170 000 M_r bands; (O) origin; (F) front. For details see text.

(i) The major phosphorylated component in the material eluted by ethanolamine was a doublet in the M_r 150 000–170 000 region (lane 1);
(ii) Addition of EGF to the ethanolamine eluate resulted in stimulation of phosphorylation of the 150 000–170 000 M_r duplet (lane 2);
(iii) The phosphoproteins transferred to DBM-paper (lanes 3,4) produced equivalent imprints of those in the SDS–polyacrylamide gels (lanes 1,2), except that trace quantities of other bands (not apparent in the autoradiographs of gels shown in lanes 1 and 2) were detectable on the transfers at the low-M_r region.

The immunoautoradiograph shown in fig.3, lane 5, illustrates the detection of EGF receptors by EGF, anti-EGF antiserum, and ^{125}I-labeled protein A in the material eluted by ethanolamine. The immunoautoradiographic analysis (fig.3, lane 5) show that under these conditions the anti-EGF antiserum reacts with a protein of M_r 150 000, confirming the results obtained by intact membrane analysis (fig.2). In addition, the results shown in fig.3, lane 5, demonstrate that the anti-EGF antiserum reacts with a protein band migrating close to the tracking dye front. When authentic EGF was transferred to DBM-paper and reacted with anti-EGF antiserum and ^{125}I-labeled protein A, the band of authentic EGF (fig.3, lane 6) comigrated with the low-M_r band present in the ethanolamine eluate (fig.3, lane 5). Furthermore, when identical DBM-paper transfers of ethanolamine eluates were reacted with

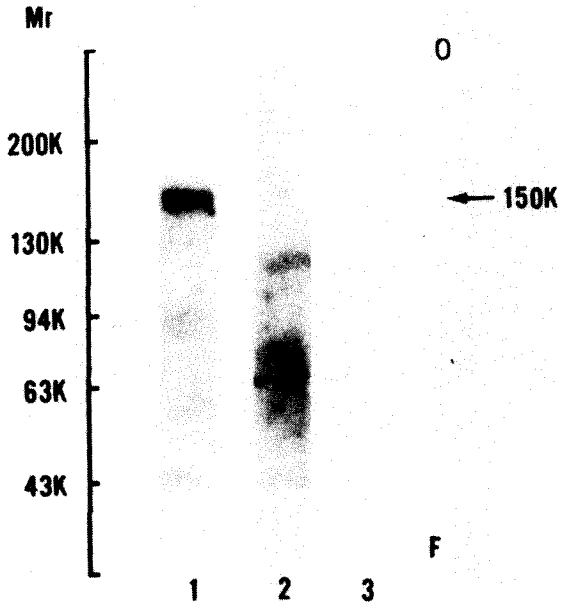

Fig.2. Immunoautoradiographic detection of EGF receptors transferred to DBM-paper. A-431 membrane proteins were separated on a 5–9% SDS–polyacrylamide gel and were electrophoretically transferred to DBM-paper. The lanes of DBM-paper transfer were treated as follows: (1) EGF, anti-EGF antiserum and ^{125}I-labeled protein A; (2) anti-EGF antiserum and ^{125}I-labeled protein A; (3) EGF and ^{125}I-labeled protein A. DBM-paper autoradiographs were exposed for 64 h: (O) origin; (F) front.

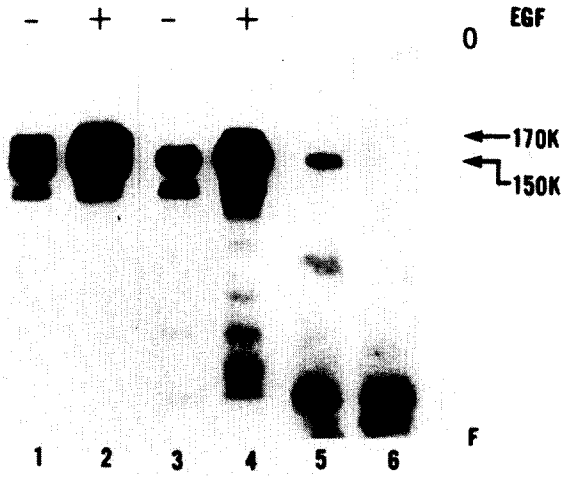

Fig.3. Immunoautoradiographic analysis of EGF receptors in affinity-purified preparations. Triton-solubilized A-431 membrane proteins were adsorbed to EGF-Affi-Gel beads. The beads were washed and the adsorbed material was eluted by ethanolamine. Aliquots of the ethanolamine eluate were prepared for phosphorylation, electrophoresis, and transfer to DBM-paper. For detail see section 2: lanes (1,2) Autoradiograph (24 h exposure) of ethanolamine eluate phosphorylated in the absence (–) or presence (+) of EGF resolved on a 5–9% SDS–polyacrylamide gel; (3,4) samples duplicating those in lanes (1,2) were transferred electrophoretically to DBM-paper and autoradiographed (24 h exposure); (5) immunoautoradiograph of ethanolamine-eluted proteins electrophoretically transferred to DBM-paper and treated with EGF, anti-EGF antiserum and ^{125}I-labeled protein A. The DBM-paper was autoradiographed for 24 h; (6) EGF (100 ng) was separated by electrophoresis and was electrophoretically transferred to DBM-paper. The DBM-paper transfer was treated with anti-EGF antiserum and ^{125}I-labeled protein A. The DBM-paper was autoradiographed for 24 h. (O) origin; (F) front.

anti-EGF antiserum alone and ^{125}I-labeled protein A, only the band migrating close to the tracking dye front yielded a clear signal (not shown). Results of these experiments indicate that the low-M_r band present in the ethanolamine eluate (fig.3, lane 5) is EGF. We conclude from the immunoautoradiographic data shown in fig.3 and other experiments that the 150 000 M_r protein is responsible for EGF binding activity in affinity-purified preparations.

4. DISCUSSION

The analysis of EGF receptors present in small amounts in cell membranes is important to elucidate the mechanism of action of EGF [15]. Here we present a system to identify such receptors by probing with EGF, specific antibodies against EGF and other reagents.

We have demonstrated that A431 membrane proteins immobilized on DBM-paper can be used to detect their respective EGF receptors. Because this method allows direct correlation of receptor activity to apparent protein M_r, information useful on receptors is immediately obtained. The system for the detection and identification of EGF receptors is very sensitive. As little as 22 ng receptor protein, present at ~ 0.3% of the intact A431 membrane protein [4], can be detected. A major advantage of this immunoautoradiographic technique is that it gives good ratios of signal to background, unlike that found when we attempted to identify the receptors directly by incubation of ^{125}I-labeled EGF with diazo–protein transfers (unpublished). In addition to the advantage that ^{125}I-labeled EGF is not required, purified anti-EGF antibody need not be used [12]. Since we have obtained quantitative transfer of A431 membrane proteins from gels to DBM-paper, the technique also provides a means of monitoring the relative abundance of membrane receptors for EGF. This technique, thereforse, should contribute additional information on the properties and specificities of EGF receptors in A431 cells.

These results conclusively demonstrate that the 150 000 M_r membrane-associated protein interacts with EGF in specific fashion, indicating that it is a subunit of the EGF receptor of intact A431 membranes. The data presented on EGF receptors in affinity purified preparations revealed a number of interesting points. On subjecting the receptor partially purified by affinity chromatography to SDS–polyacrylamide gel electrophoretic analysis, more than one protein band was observed. From these studies it is not clear which band, if any, corresponds to the EGF receptor activity. The most important conclusion that can be drawn from the data obtained by immunoautoradiographic analysis is that the protein responsible for a major proportion of the specific EGF binding activity in affinity-purified preparations is the 150 000 M_r protein. We have also noted that EGF appears to be present in the affinity purified preparations. Since a small amount of EGF may be present in intact A431 membranes, the possibility exists that EGF copurified with its receptor. A less attractive possibility is that the EGF detected in affinity-purified preparations was released from the EGF-Affi-Gel during the elution procedure.

In conclusion, to evaluate this system, we have analyzed membranes from A431 cells which possess an unusually high number of specific EGF receptor sites (2–3 × 10^6/cells) [3]. The transfer technique, however, has the potential for immunoautoradiographic analysis of EGF receptors in other cell lines which possess a few EGF receptors. Experiments with such cell lines are in progress.

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