IDENTIFICATION OF THE EGF RECEPTOR ON 3T3 CELLS BY SURFACE-SPECIFIC IODINATION AND GEL ELECTROPHORESIS

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1. Introduction

Epidermal growth factor (EGF), a low molecular weight polypeptide hormone isolated from mouse submaxillary glands or human urine, is a potent mitogen for human fibroblasts [1,2] and murine 3T3 cells [3,4]. EGF retains its biological activity after iodination, and 100 000 or more specific saturable binding sites per cell are displayed on human [1,2] and mouse [5,6] fibroblast cell surfaces. When EGF is incubated at high concentration with fibroblasts, 80% or more of the EGF binding activity is lost within a few hours at 37°C (receptor down regulation) [6–8]. Fluorescent and ferritin-labeled EGF derivatives have been used to demonstrate that specifically bound EGF is internalized into murine [9,10] and human cells [11,12], where it is degraded in the lysosomes [7]. The loss of EGF binding activity during down regulation may be correlated with receptor internalization and degradation, and with the appearance of receptor fragments in the lysosomes [13]. The process of receptor down regulation may be limiting for the mitogenic activity of EGF; both phenomena achieve half-maximal levels at the same EGF concentration [13–15].

Here we have compared gel electrophoretic patterns of surface radioiodinated proteins of untreated 3T3 cells and cells treated to down regulate EGF receptors. Detection of EGF receptors on surface radioiodinated cells was possible only when trypsin treatment was used to amplify the radioactivity present in receptor protein relative to other proteins. The molecular weight of the radioiodinated receptor protein is in good agreement with the value for the EGF–receptor complex formed by photoaffinity labeling [6] and that of the apparently covalent [^{125}I]EGF–receptor 'direct-labeled' complex which forms in low yield when cells are incubated with [^{125}I]EGF [16]. We also report that binding of EGF to its receptor may alter the site at which the receptor is cleaved by protease.

Abbreviations: EGF, epidermal growth factor; PBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; DME, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovin serum albumin; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. *Cell culture*

Swiss mouse 3T3 cells (clone 42 from George Todaro, National Cancer Institute) were grown in Dulbecco's modified Eagle's medium (DME, Gibco) containing 10% fetal calf serum (FCS, Gibco) and were subcultured after trypsinization. The cells were grown for at least 3 generations to achieve a confluent monolayer, and the medium was changed once prior to use.

2.2. *EGF binding*

EGF was purified from male mouse submaxillary glands (Pelco) [17] and [^{125}I]EGF was prepared [6] by published procedures. The specific activity varied from 1–4 X 10^5 cpm/ng EGF. Specific binding of [^{125}I]EGF to cells was determined after a 30 min incubation at 37°C in 1 ml of binding medium (DME containing 0.1% bovine serum albumin, 5 mM Hepes at pH 7.2) containing 50 ng [^{125}I]EGF/ml [6].

2.3. *Down regulation of EGF receptors*

Confluent monolayers were washed twice with binding medium and incubated further at 37°C in binding medium containing 50 ng unlabeled EGF/ml. After unlabeled EGF was removed by aspiration of the fluid, monolayers were washed twice with binding medium and [^{125}I]EGF binding determined [6].

2.4. *Surface iodination, gel electrophoresis and autoradiography*

These were performed as in [18]. Confluent cell monolayers in 35 mm plastic culture dishes were washed 4 times with PBS (Dulbecco's phosphate buffered saline), and 1.0 ml PBS containing 250 μCi Na ^{125}I (Amersham) was added to each dish. A 20 × 20 mm glass cover slip coated with 50 μg 1,3,4,6-tetrachloro-3,6α-diphenylglycoluril (Iodogen, Pierce) was placed into each dish and floated above the monolayer for 20 min at room temperature. After the coverslips were removed, 1 ml PBI (PBS containing NaI instead of NaCl) was added to each plate and the monolayers were washed 6 times with PBI. Washed cell monolayers were dissolved in 160 μl electrophoresis sample buffer (2% SDS, 0.68 M Tris/HCl (pH 6.8), 5% 2-mercaptoethanol), and the viscosity of the solution was reduced by extensive shearing through a syringe needle. The samples were boiled in a water bath for 3 min, and aliquots of 40 μl were applied to each lane of a 5–10% polyacrylamide gradient slab. Following electrophoresis [18], the gels were stained with Coomassie blue, destained, dried on filter paper and exposed to Kodak NS 2T X-ray film. The molecular weight standards used for the estimation of the molecular weights of the labeled protein bands were: ovalbumin, M_r = 43 000; bovine serum albumin, M_r = 68 000; α-phosphorylase, M_r = 100 000; *E. coli* β-galactosidase, M_r = 130 000; the spectrin bands from human erythrocyte ghosts, M_r = 215 000 and 240 000.

2.5. *Direct labeling of the EGF receptor*

Confluent cell monolayers were incubated with 50 ng [^{125}I]EGF/ml in binding medium at 37°C and unbound EGF was removed by 4 washes with DME. The washed cell monolayes were dissolved in electrophoresis sample buffer and processed for gel electrophoresis and autoradiography as described above.

2.6. *Trypsin treatment*

Confluent monolayers were incubated for 10 min at room temperature in 1.0 ml DME containing 40 μg trypsin/ml (Worthington, TPCK). The cells were then washed twice with DME, and the monolayers (the cells adhered to the dishes under these conditions) were incubated with 40 μg/ml of soybean trypsin inhibitor (Sigma) for 5 min at room temperature and washed twice again with DME.

2.7. *Preparation of 3T3 cell surface membranes*

Cell surface membranes from 3T3 cells were prepared by isopycnic banding of cell homogenates from cell grown on 10 cm diam. plastic dishes in DME containing 10% fetal calf serum [16].

3. Results

Previous approaches to identification of receptors for epidermal growth factor have relied on the linkage of [^{125}I]EGF to receptor [6,13,16]. These experiments revealed a small portion (1%) of the specifically bound [^{125}I]EGF covalently attached to a single high molecular weight radioactive receptor band. In this study we used surface specific radioiodination to compare untreated cells with cells down regulated for the EGF receptor. Due to the small number of receptors present on the surface of 3T3 cells, a comparison between untreated (10^5 sites/cell) and down regulated cells (2×10^4 sites/cell) required amplification of the radioactivity in the receptor band.

Most membrane proteins of cultured cells labeled by surface-specific radioiodination are removed by trypsin treatment [19]. We therefore first studied the effect of trypsin treatment on EGF binding and EGF-induced EGF receptor down regulation in 3T3 cells (fig.1). Neither process was influenced to any great extent by a trypsin treatment which extensively removed surface radioiodinated proteins from 3T3 cells (fig.2).

3.1. *Identification of a protein with the properties of the EGF receptor by surface specific labeling*

The experiment described in fig.3, lanes A, B incorporates two properties that optimize selective radioiodination of EGF receptor proteins. The data in fig.1, 2 suggested that treatment with trypsin

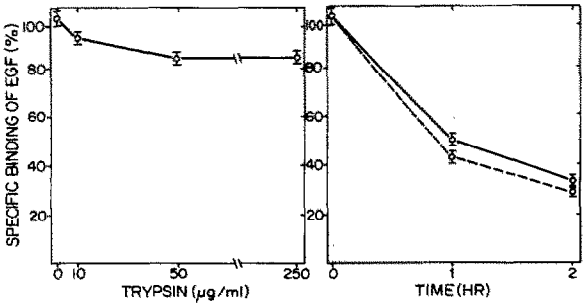


Fig.1. Effects of trypsin treatment on EGF binding and EGF-induced down regulation of the EGF receptor. Left. Effect of trypsin on EGF binding. Confluent monolayers were incubated at room temperature for 10 min with trypsin at the indicated concentrations. After a further 5 min incubation with 40 μg/ml of soybean trypsin inhibitor, the cells were washed 4 times with DME and [^{125}I]EGF binding was determined as in section 2. Right. Effect of trypsin treatment on EGF-induced down regulation of the EGF receptor. Confluent 3T3 cell monolayers were incubated for 10 min at room temperature with 40 μg trypsin/ml (- -) or no trypsin (—). After trypsin was removed by aspiration (section 2), the cells were incubated with 40 μg/ml of soybean trypsin inhibitor for 5 min at 22°C and washed 4 times with DME. The cells were then incubated with binding medium containing 50 ng/ml of unlabeled EGF for the times indicated to allow for down regulation of EGF receptors. After removal of unlabeled EGF, the cells were washed twice with DME and [^{125}I]EGF binding was determined (section 2).

followed by surface specific radioiodination would optimize the labeling of the receptor proteins, but other experiments (fig.3, lanes E–G and fig.4) showed that while EGF binding activity is not particularly sensitive to tryptic treatment, tryptic proteolysis reduced most receptor molecules to lower molecular weight forms. We therefore also had to take advantage of a second property of the system. In 3T3 cells the half time for receptor turnover is in the range of a few hours (M.W., C.F.F., unpublished data). Trypsin-treated cells were washed to remove trypsin and then incubated for a short time to allow for selective regeneration of EGF receptors. This amplified the quantity of EGF receptors relative to that of other surface proteins. In order to selectively increase the quantity of surface-displayed EGF receptor in one sample (lane A) relative to a second (lane B), EGF was included at high concentration in the second sample (lane B) to induce internalization and degradation of receptor proteins [13]. In lane A, the sample not treated with EGF, there is a 170 000 dalton band which can barely be detected in lane B,

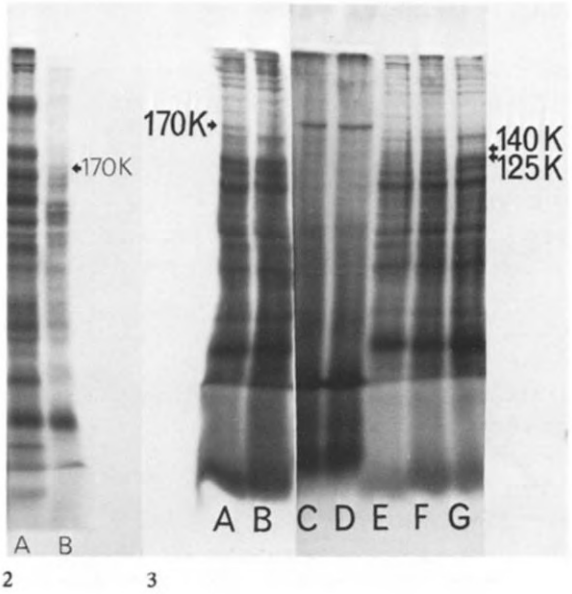


Fig.2. (left). Effect of trypsin on radioiodinated proteins displayed on surface labeled 3T3 cells. Confluent 3T3 monolayers were labeled by surface specific iodination. Sample B was incubated with 40 μg/ml of trypsin for 10 min as in section 2; sample A received no trypsin treatment. The monolayers were dissolved in electrophoresis sample buffer and processed for gel electrophoresis on a 5–10% gradient slab gel and autoradiography. The arrow indicates the position of the EGF receptor.

Fig.3. (right). The cells used in these experiment were grown and trypsin treated in parallel with those used for the experiments described in fig.1. (A,B) Effect of EGF on the appearance of proteins subject to surface specific iodination during the resynthesis of trypsin-sensitive surface proteins. 3T3 monolayers were treated with 40 μg trypsin/ml (section 2), incubated in binding medium containing 50 ng unlabeled EGF/ml (B) or no EGF (A) for 2 h at 37°C, labeled by surface specific iodination and processed for gel electrophoresis on a 5–10% gradient slab gel and autoradiography. (C,D) Direct labeling of EGF receptor. 3T3 monolayers were incubated for 30 min at 37°C in binding medium containing 50 ng [^{125}I]EGF/ml. After unbound [^{125}I]EGF was removed, the cells were processed for gel electrophoresis and autoradiography. The relatively high background in these samples is caused by unreacted ^{125}I which was not removed after the chloroglycoluril mediated iodination of EGF. (E,F,G) Effect of EGF-induced EGF receptor down regulation on the appearance of a 140 000 dalton protein band produced by trypsin treatment of surface radioiodinated cells. 3T3 monolayers were labeled by surface specific iodination, incubated for 0 (E) or 60 (F,G) min in binding medium containing 50 ng/ml of unlabeled EGF (G) or no EGF (F), treated with 40 μg trypsin/ml (section 2), washed twice, and processed for gel electrophoresis and autoradiography (section 2). Samples A–G were resolved on the same 5–12.5% polyacrylamide gradient slab gel, but samples A, B were photographed from a different autoradiograph than were samples C–G.

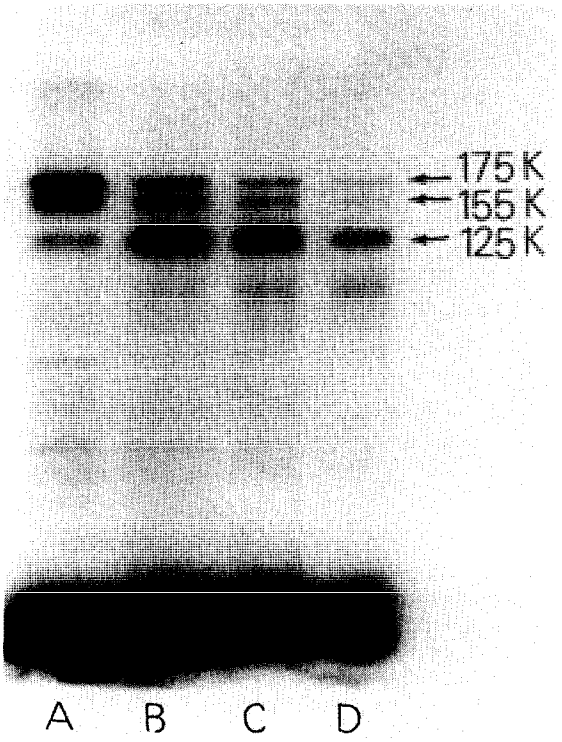


Fig.4. Tryptic degradation of the direct-labeled EGF receptor on isolated 3T3 membranes. Isolated surface membranes from 3T3 cells were incubated for 60 min at 23°C in binding medium containing 50 ng [^{125}I]EGF/ml to produce direct labeled EGF receptor [16]. The samples were layered above a 1 ml cushion of 10% (w:w) sucrose in binding medium and sedimented for 2 min in a Brinkman microfuge to remove unbound EGF. The pellets were washed with 1.5 ml binding medium and incubated for 30 min at 0°C with 100 μl DME containing 0 (A), 2.5 (B), 25 (C) or 250 μg/ml (D) of trypsin for 30 min. Trypsin was removed as described for the removal of unbound EGF, and the samples were washed twice with binding medium, dissolved in electrophoresis sample buffer and processed for gel electrophoresis on a 10% polyacrylamide slab gel. The labeled proteins were visualized by autoradiography (section 2).

the sample down regulated for EGF receptors. The molecular weight of this band is in good general agreement with the value of the EGF–receptor complex formed by photoaffinity labeling [6,13], and also with the molecular weight of the 'direct-labeled' EGF–receptor complex which forms in low yield when [^{125}I]EGF is incubated with cells ([16] and fig.3, lanes C, D).

3.2. *Tryptic degradation products of the EGF receptor proteins*

Lanes E–G of fig.3 describe tests for tryptic digestion products of EGF receptor proteins on 3T3 cells radiolabeled by surface-specific iodination. The sample in lane G was treated with unlabeled EGF after radioiodination to down regulate the EGF receptor. The samples not subjected to EGF-induced down regulation displayed a band of radioactivity at M_r = 140 000 which was absent in lane G. Lane G also differed in that the 170 000 dalton band was less visible than in lanes E and F, and the radioactivity in a band at 120 000–125 000 daltons appears to be increased. A 125 000 dalton band was the main product resulting from trypsin treatment of the covalent EGF–receptor covalent complex (fig.4).

When 3T3 cells are incubated with [^{125}I]EGF, the EGF–receptor complex which forms resists dissolution in boiling sodium dodecyl sulfate solution containing 2-mercaptoethanol ([16] and fig.3). Cell surface membranes were prepared from 3T3 cells and treated with [^{125}I]EGF to label the EGF recepotor. Three labeled bands were observed in the preparation:

(1) The 175 000 dalton EGF–receptor direct-labeled complex;
(2) A minor band at 125 000 daltons;
(3) A second minor band at 155 000 daltons.

The amount of the 125 000 dalton component was increased by trypsin treatment. The 125 000 dalton band probably has the same apparent molecular weight as the receptor-derived component which forms when cells with affinity-labeled EGF receptors are treated with trypsin or chloroquine [13]. The 155 000 dalton band has been observed only in broken cell preparations and may arise by action of a protease not normally accessible to the cell surface.

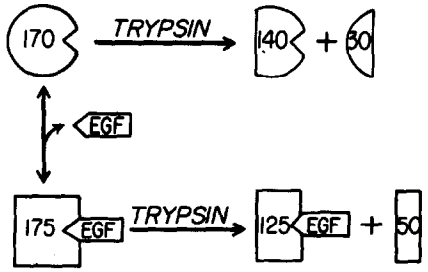
4. Discussion

A 170 000 dalton protein was detected when murine 3T3 cells were treated with trypsin and then incubated briefly in growth medium to amplify the relative quantity of cell surface proteins with a rapid turnover rate relative to other proteins. This protein band was absent in cells incubated with EGF to down regulate the EGF receptor. These data corroborate the affinity labeling studies reported by this laboratory and have the advantage that surface specific iodination is likely to detect a more sizable, and thus more

representative, fraction of receptor than is detected by the affinity labeling methods [6,16]. When cells were first surface labeled and then treated with trypsin, only a small amount of label appeared in the 170 000 dalton band, but a new band was detected at 140 000 daltons. Since this trypsin treatment of cells had little effect on specific binding of labeled EGF, the 140 000 dalton component is a likely candidate for the initial EGF receptor fragment produced by tryptic digestion of 'free' receptor.

When membranes containing 'direct-labeled' EGF–receptor, i.e., that which resists dissociation into EGF plus receptor in SDS solution [16], are treated with trypsin, a 125 000 dalton component is the major initial product. An increase in a 120 000-125 000 dalton radioiodinated band is also observed when surface labeled cells are treated with trypsin in the presence of EGF. These data indicate that the major tryptic digestion product of the 'bound' receptor is a 125 000 dalton EGF–receptor complex that arises when EGF is either covalently or noncovalently associated. A similar tryptic digestion product was observed when cells with an affinity labeled EGF receptor were treated with trypsin or incubated in the presence of chloroquine [13]. This last observation indicates the presence of an endogenous cell surface protease with trypsin-like specificity [13].

The results of our study are summarized in scheme 1 and indicate that EGF changes the conformation of EGF receptors, altering the position of trypsin sensitivity on the receptor protein. We have published evidence consistent with the view that EGF-induced internalization and proteolytic processing of the EGF receptor are steps in EGF-mediated signal transmission [13–15]. This 'endocytic activation' hypothesis [15] suffers from the possibility that nonspecific proteolysis might activate the receptor. This dilemma is averted if the hormone associated form of receptor is the only form sensitive to proteolytic activation, a requirement consistent with the properties of receptor reported here.



Scheme 1

Acknowledgements

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