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Recycling of epidermal growth factor in A431 cells

L.V. Teslenko, E.S. Kornilova, A.D. Sorkin and N.N. Nikolsky

Institute of Cytology of the Academy of Sciences of the USSR, Tichoretsky pr., 4, Leningrad 194064, USSR

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The fate of epidermal growth factor (EGF) after internalization by A431 cells was studied. First, cells containing ^{125}I-EGF-receptor complexes in endosomes were obtained. Subsequent incubation of the cells at 37°C resulted in the recycling of ^{125}I-EGF from endosomes to the cell surface in the receptor-bound state and the gradual release of recycled ligand into the medium. The excess of unlabeled EGF blocked both rebinding and re-internalization of recycled ^{125}I-EGF to produce enhanced accumulation of ligand in the medium. The rate of recycling was shown to be much higher than that of EGF degradation.

Epidermal growth factor; Internalization; Recycling; (A431 cell)

1. INTRODUCTION

EGF binds to specific receptors in various types of cells. EGF binding initiates the internalization of EGF-receptor complexes via the receptor-mediated endocytic pathway. A characteristic feature of the fate of internalized EGF and EGF receptor is their delivery to lysosomes and degradation by lysosomal enzymes [1,2]. In contrast, receptors of other ligands, e.g. α_2-macroglobulin and transferrin, are not targeted to lysosomes and recycled to plasma membrane [2]. The mechanism and role of EGF receptor degradation remain unknown.

Human epidermoid carcinoma A431 cells are the most common object for study EGF receptors [3]. The endocytic pathway of EGF and EGF receptor in A431 cells from plasma membranes through endosomes and multivesicular bodies to mature lysosomes has been studied in detail using electron microscopy [4,5]. The degradation of internalized EGF and its receptors in A431 cells has been well documented by means of biochemical methods [6]. To date, no evidence on EGF or EGF receptor recycling in A431 cells has been obtained.

In a previous paper [7], we demonstrated that most of the EGF remains associated with receptors for at least 1 h after internalization which suggests that only negligible amounts of unoccupied EGF receptors capable of recycling are present in endosomes. Here, we find that undissociated EGF-receptor complexes constantly recycle between endosomes and the plasma membrane. It is also shown that EGF degradation proceeds in parallel with recycling but at a much lower rate.

Correspondence address: A.D. Sorkin, Institute of Cytology of the Academy of Sciences of the USSR, Tichoretsky pr., 4, Leningrad 194064, USSR

Abbreviations: EGF, epidermal growth factor; BSA, bovine serum albumin; DME, Dublecco's modified Eagle's medium; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

EGF was purified from mouse submaxillary glands by chromatography on a Mono Q column (FPLC, Pharmacia) as described in [8] and iodinated using Iodo-Gen (Serva). The specific activity was about 40 000 cpm/ng.

Human epidermoid carcinoma A431 cells were obtained from the Cell Culture Collection (Institute of Cytology, USSR) and maintained as in [7,9].

For ^{125}I-EGF–cell interaction experiments cells

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were plated on 30–70 mm tissue culture dishes and used 2 days after plating. Cells were incubated with 40 ng/ml of ^{125}I-EGF in working medium (WM), containing DME, 0.1% BSA, 20 mM Hepes (pH 7.2) for 60 min at 4°C, rinsed free of unbound ligand and further incubated in WM for 10 min at 37°C. After rinsing with cold PBS cells were treated twice with acetate buffer solution (ABS), containing 0.2 M sodium acetate buffer, 500 mM NaCl (pH 5.0) at 4°C for 2.5 min and 15–20 s. Both extracts were combined to estimate the amount of surface-bound ^{125}I-EGF. Such acidic treatment allows the extraction of about 95% of the total surface-bound ^{125}I-EGF (not shown) as well as when a standard procedure was strictly followed [10].

The ABS-treated cells were rinsed with PBS and further incubated for a chase period in WM at 37°C. After the end of the case the incubation medium was collected and combined with subsequent rinsing in cold PBS to determine the amounts of intact and degraded ^{125}I-EGF. Surface-bound ^{125}I-EGF was then again extracted with ABS as described above. Finally, the acid-resistant intracellular radioactivity was removed by solubilizing the cells in 1 N NaOH.

The amount of low molecular mass products of ^{125}I-EGF degradation in the chase medium was estimated by precipitation of high molecular mass forms of ^{125}I-EGF with 5% trichloroacetic acid/1% phosphotungstic acid as described in [7].

Cells were processes for density gradient fractionation on 20% Percoll (Pharmacia) before and after the first ABS treatment, after a chase period and second ABS treatment. The procedures for gradient centrifugation and determination of marker enzymes activity for subcellular compartments have been described [9].

3. RESULTS AND DISCUSSION

Two lines of experimental evidence are required for demonstration of ligand or receptor recycling: (i) intracellular localization of labeled ligand/receptor; (ii) subsequent reappearance of the same ligand/receptor on the cell surface.

In our experiments the cells prelabeled with ^{125}I-EGF at 4°C were allowed to internalize the ligand for 10 min at 37°C followed by removal of the non-internalized surface-bound ^{125}I-EGF by mild acidic treatment. Usually, about 20–40% of the total cell-associated radioactivity was resistant to the first ABS treatment as well as to additional ABS treatments which indicates the intracellular localization of these ligand molecules. The short-time acidic treatment used did not change the viability of A431 cells, their morphology, the rate of endocytosis of EGF and transferrin (not shown), on the pH value in cytoplasm and organelles [11].

The subcellular fractionation experiments demonstrated that ABS removes ^{125}I-EGF from plasma membrane fractions (fig.1A). The remaining radioactivity was shown to be associated with two types of endosomes of low and high density, corresponding to that of Golgi membrane fractions and lysosomes (fig.1A). As shown earlier by

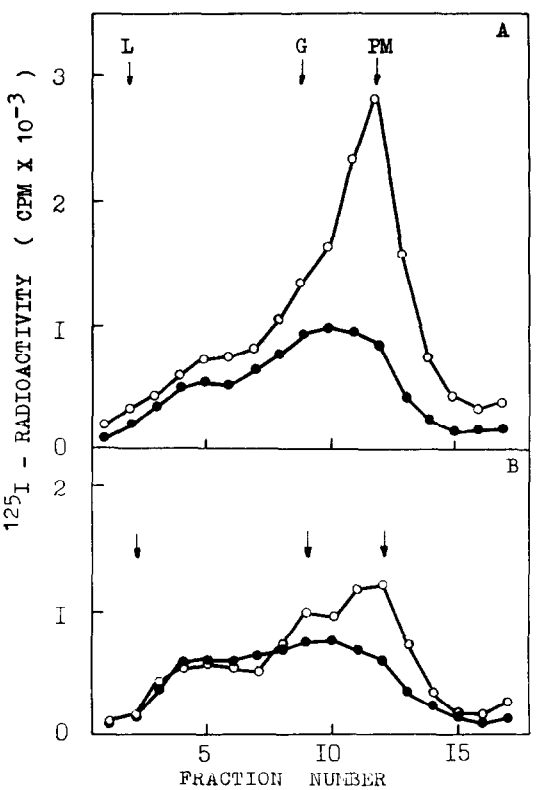

Fig.1. Distribution on Percoll gradient of ^{125}I-EGF in microsomal fractions obtained from cells prelabeled with ^{125}I-EGF at 4°C and successively incubated at 37°C for 10 min (A, ○—○), treated with ABS (A, ●—●), incubated at 37°C for 5 min (B, ○—○) and treated with ABS (B, ●—●). Arrows show the positions of marker enzymes of plasma membrane (PM), Golgi complex (G) and lysosomes (L).

electron microscopy [4,5], the majority of intracellular EGF and its receptors are localized at this stage of endocytosis within various electron-lucent vesicles (endosomes and multivesicular bodies) distributed mainly at the periphery of the cells.

Using detergent extraction technique [7], the bulk of the internalized EGF-receptor complexes was found to be undissociated 10–30 min after internalization. Hence, after ABS treatment we obtained cells 'injected' with endosomes containing ^{125}I-EGF-receptor complexes (^{125}I-EGF-'injected' cells).

The following incubation of ^{125}I-EGF-injected cells at 37°C produced the rapid reappearance of plasma membrane-bound ^{125}I-EGF as measured by the increase in ABS-extractable radioactivity (fig.2). The amount of surface ^{125}I-EGF increased linearly over the 10–15 min chase time (fig.2) and then gradually decreased to the steady-state level (fig.3). The reappearance of ^{125}I-EGF in plasma membrane fractions was detected as early as after 5 min incubation of ^{125}I-EGF-injected cells at 37°C (fig.1B). The amount of ^{125}I-EGF in the chase medium increased insignificantly over the 20 min incubation in comparison with the increase in surface ^{125}I-EGF, demonstrating that EGF recycles from intracellular sites to the plasma membrane in the receptor-associated state.

The following accumulation of non-degraded ^{125}I-EGF in the chase medium and decrease in surface ^{125}I-EGF (figs.2,3) indicate that recycled ^{125}I-EGF-receptor complexes can dissociate due to an

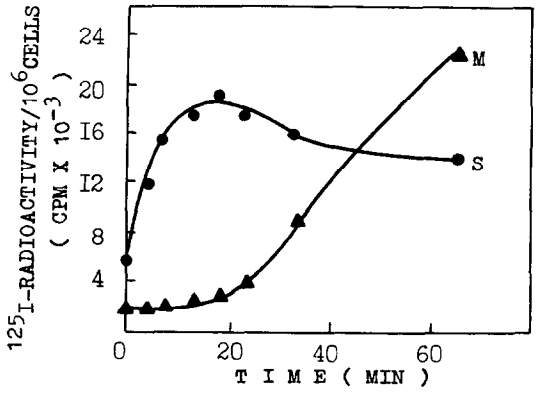

Fig.2. Time course of recycling of internalized ^{125}I-EGF from ^{125}I-EGF-injected cells to the cell surface (S) and incubation medium (M).

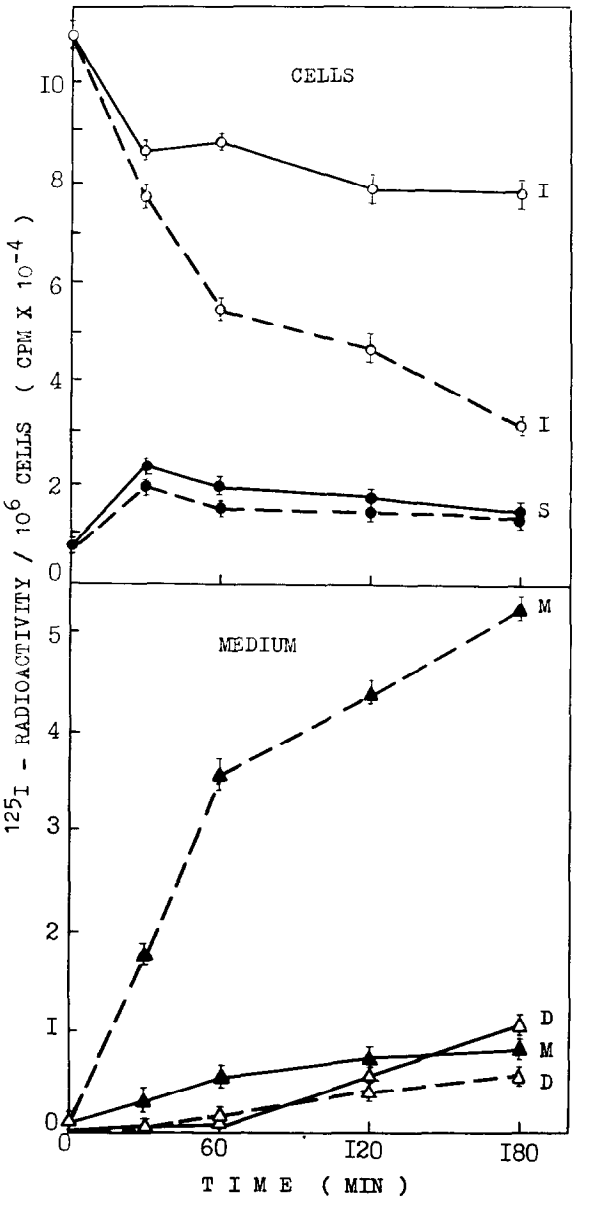

Fig.3. Time course of cell-associated and medium ^{125}I-EGF. ^{125}I-EGF-injected cells were incubated for a chase time at 37°C in the presence (dashed lines) or absence of 300 ng/ml of unlabeled EGF (solid lines). Then the amounts of surface-bound (S), intracellular (I), medium intact ^{125}I-EGF (M) and degradation products of ^{125}I-EGF (D) were determined.

equilibrium process or can be re-internalized by the cells. The excess of unlabeled EGF (300 ng/ml) blocks both rebinding of the released ^{125}I-EGF and

internalization of recycled ^{125}I-EGF-receptor complexes, resulting in significant accumulation of ^{125}I-EGF in the chase medium, thereby decreasing the cell-associated radioactivity (fig.3). The long-time accumulation of recycled ^{125}I-EGF in the medium shows that recycling takes place constantly during endocytosis, rather than being a single occurrence. Therefore, the rate of ^{125}I-EGF accumulation in the chase medium in the presence of an excess of unlabeled EGF can be taken as a measure of the rate of ^{125}I-EGF recycling.

Maximum accumulation of ^{125}I-EGF in the medium after 2 h incubation of ^{125}I-EGF-injected cells at 37°C occurred at a concentration of 300–1000 ng/ml of unlabeled EGF (fig.4), which is 500–1000-times higher than the effective concentration of released ^{125}I-EGF.

Much lower concentrations of unlabeled EGF (20–50 ng/ml) did not prevent the rebinding of medium ^{125}I-EGF and allowed recycled ^{125}I-EGF to accumulate on the cell surface, presumably owing to competetive inhibition of internalization of recycled ^{125}I-EGF-receptor complexes by unlabeled EGF-receptor complexes (fig.4).

The low molecular mass products of ^{125}I-EGF degradation were detected in the chase medium only after 60 min incubation of the ^{125}I-EGF-injected cells at 37°C (fig.3) as well as when endocytosis was not interrupted by ABS treatment [7]. The rate of ^{125}I-EGF degradation was much lower than that of recycling and was reduced additionally by excesss unlabeled EGF (fig.3).

The present data suggest that there are two parallel pathways for EGF-receptor compartmentalization in A431 cells: recycling and degradation. The kinetics of EGF-receptor complexes recycling is similar to that of transferrin-receptor complexes in A431 cells [12] and insulin-receptor complexes in adipocytes [13]. The secretion of internalized EGF into medium has previously been detected in pancreatic carcinoma cells [14], hepatocytes [15] and in the presence of chloroquine in 3T3 cells [16], but so far it remains as to what state EGF leaves these cells. Presumably, the ratio of the degradation and recycling pathways of EGF-receptor complexes is dependent on the cell type and the physiological state. The predominance of recycling in A431 cells is responsible for maintenance of the large pool of intact active EGF-receptor complexes even after prolonged exposure of these cells to EGF [17].

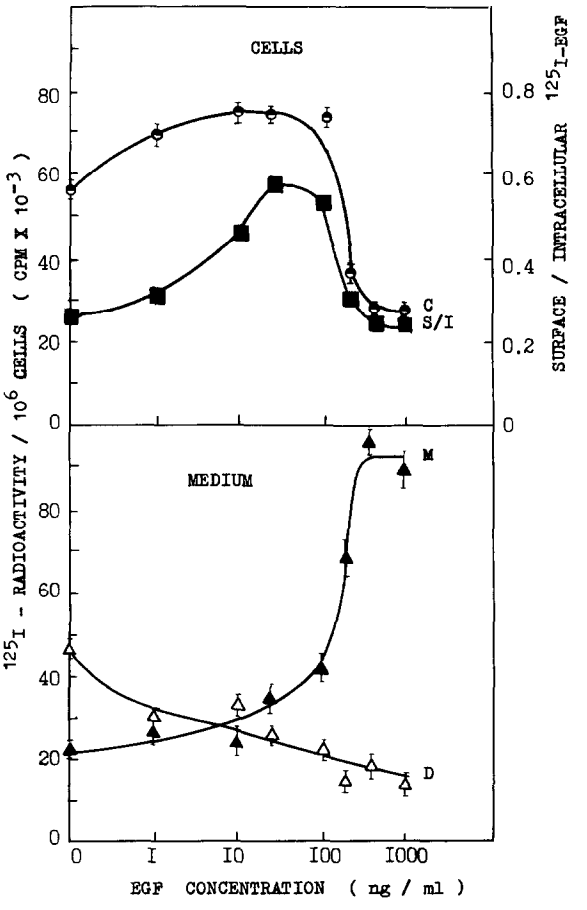

Fig.4. Effect of unlabeled EGF concentration on ^{125}I-EGF recycling and degradation. ^{125}I-EGF-injected cells were incubated in the presence of unlabeled EGF for 2 h at 37°C. Then the amounts of cell-associated (C), medium intact (M), and degraded ^{125}I-EGF (D) and the ratio of surface-bound to intracellular ^{125}I-EGF (*S/I*) were determined.

It is probable that two distinct types of endosomes are involved in recycling and degradation processes. The identification of these endosomal compartments is now in progress in our laboratory.

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