Recycling of Epidermal Growth Factor-Receptor Complexes in A431 Cells: Identification of Dual Pathways

Alexander Sorkin,*‡ Sergey Krolenko,* Natalia Kudrjavtceva,* Juri Lazebnik,* Ludmila Teslenko,* Ann Mangelsdorf Soderquist,‡ and Nikolai Nikolsky*

*Institute of Cytology, Academy of Sciences of the USSR, Leningrad 194064, USSR; and the ‡Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Abstract. The intracellular sorting of EGF-receptor complexes (EGF-RC) has been studied in human epidermoid carcinoma A431 cells. Recycling of EGF was found to occur rapidly after internalization at 37°C. The initial rate of EGF recycling was reduced at 18°C. A significant pool of internalized EGF was incapable of recycling at 18°C but began to recycle when cells were warmed to 37°C. The relative rate of EGF outflow at 37°C from cells exposed to an 18°C temperature block was slower ($t_{1/2} \approx 20$ min) than the rate from cells not exposed to a temperature block ($t_{1/2} \approx 5$–7 min). These data suggest that there might be both short- and long-time cycles of EGF recycling in A431 cells. Examination of the intracellular EGF-RC dissociation and dynamics of short- and long-time recycling indicated that EGF recycled as EGF-RC. Moreover, EGF receptors that were covalently labeled with a photoactivatable derivative of ^{125}I-EGF recycled via the long-time pathway at a rate similar to that of ^{125}I-EGF. Since EGF-RC degradation was also blocked at 18°C, we propose that sorting to the lysosomal and long-time recycling pathway may occur after a highly temperature-sensitive step, presumably in the late endosomes.

Many serum proteins, hormones, growth factors, and viruses are carried into the cell by receptor-mediated endocytosis. Ligand–receptor complexes formed at the cell surface are internalized via coated pits and transported to the membrane system of the endosomal compartment. This compartment represents several types of tubular-vesicular organelles that differ in morphology (12, 15, 16, 21, 29), biochemical composition (31), physical parameters (13, 23, 31), fusion capacity (13, 30), relative acidity (43), and subcellular localization (16, 19, 21, 29, 41). During traversal of the endosomal network, segregation (sorting) of ligands and receptors to different intracellular pathways can occur. Two major pathways exist for both ligand-occupied and free receptors: a "recycling" pathway that allows the internalized receptors to return back to the cell surface and possibly be involved in several rounds of endocytosis; and a "degradative pathway," the entrance to which appears to be related to the inability of some receptors to escape endosomes during their transformation into mature lysosomes (for review see reference 41). Many details of the intracellular trafficking of recycling receptors, typified by those for asialoglycoprotein and transferrin, have been demonstrated (11, 18, 19, 35). However, the general mechanism of sorting to the lysosomal pathway, which is known to be used for instance by the growth factor receptors, remains poorly understood.

EGF is internalized by a receptor-mediated mechanism in various receptor-bearing cells (for review see reference 6). Although the endocytic pathway for the EGF receptor and the transferrin receptor appears morphologically to be the same (1, 33), the final destination of the EGF receptor (as well as EGF itself) is thought to be mature lysosomes (2, 4, 10, 14, 27). The half-life of the EGF receptor is reduced dramatically when the internalization of receptors is induced by EGF (2, 17, 24, 36, 37). This rapid EGF-induced receptor degradation has been proposed to be dependent on the tyrosine kinase activity of the EGF receptor (17).

As a model system, we have studied EGF receptor endocytosis in human epidermoid carcinoma A431 cells, which express an extraordinarily high level of EGF receptors. Many details of EGF endocytosis in these cells have been demonstrated by using several methodological approaches (1, 7, 14, 20, 26, 27, 33, 34, 39). Although EGF-receptor complexes (EGF-RC)[1] have been reported to enter A431 cells via coated pits as well as by micropinocytosis (14, 20), no data indicate different intracellular processing of EGF-RC due to the different mechanisms of internalization (20, 27). Upon internalization the EGF-RC become distributed throughout the tubular cisternae and vesicular elements at the cell periphery (27). This peripheral endosomal compartment appears to be connected by carrier vesicles or an endosomal

1. *Abbreviations used in this paper*: ANBS-^{125}I-EGF, photoactivatable derivative of ^{125}I-EGF; anti-EGFR, antibody specific to the EGF-receptor; EGF-RC, EGF-receptor complexes; SAB, sodium acetate buffer; WM, working medium.

network with the pericentriolar endosomal complex, a region having a high concentration of radial incoming microtubules and different tubular–vesicular membrane organelles (27, 33, 39). The rate of delivery of EGF receptors to the degradative pathway appears to be relatively slow in A431 cells, since only a small proportion of EGF and the EGF receptors can be observed in lysosome-like compartments after 1 h of internalization (23, 27, 39).

In this report we demonstrate that EGF-RC can recycle in A431 cells. We propose that this recycling proceeds via two pathways that differ in relative rate, temperature sensitivity, and subcellular occurrence.

Materials and Methods

Mouse EGF was purified on a Mono Q HR5/5 column (FPLC, Pharmacia Fine Chemicals, Piscataway, NJ) and iodinated with Iodo-Gen (Serva Fine Biochemicals Inc., Garden City Park, NJ), according to Burgess et al. (5). The specific activity of ^{125}I-EGF was ∼50,000 cpm/ng. Rabbit anti-EGF receptor serum (anti-EGFR) was a gift from Dr. Graham Carpenter (Vanderbilt University, Nashville, TN). Formalin-fixed *Staphylococcus aureus* cells (Pansorbin) were obtained from Calbiochem-Behring Corp. (San Diego, CA). Reagents for chromatography and electrophoresis were purchased from Pharmacia Fine Chemicals. Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), Fluka AG (Buchs, Switzerland) or Serva Fine Biochemicals Inc.

Photoactivatable Derivative of ^{125}I-EGF

Photoactivatable ^{125}I-EGF (ANBS-^{125}I-EGF) was freshly prepared before each experiment. 1 μg of ^{125}I-EGF in 200 μl of 0.1 M sodium phosphate buffer (pH 7.6) was mixed with 15 μl of 0.2% *N*-(5-azido-2-nitrobenzoyloxy)-succinimide ester (Sigma Chemical Co.) in DMSO and held at 4°C overnight. The reaction was stopped by the addition of 30 μl of 100 mM lysine. All steps of the procedure were carried out under weak red light.

Cell Culture

Human epidermoid carcinoma A431 cells were obtained from Cell Culture Collection (Institute of Cytology, Leningrad, USSR) and maintained in basal Eagle's medium supplemented with 10% calf serum, as previously described (33).

^{125}I-EGF–Cell Interaction Experiments

The cells were plated on 35-mm-tissue culture dishes or 24-well plates and used 2–3 d after plating, in a confluent state. A working medium (WM), containing DME, 20 mM Hepes (pH 7.3), and 0.1% BSA, was used in most experiments. The cells were rinsed with cold WM and incubated with 20–40 ng/ml of ^{125}I-EGF in WM at 2°C for 1 h, followed by extensive washing of the cells to remove unbound ligand. The cells were incubated for 5 or 15 min in WM at 37°C to allow endocytosis of the ligand, and endocytosis was stopped by rinsing the cells with ice cold WM. The cells were then treated with 0.2 M sodium acetate buffer (pH 4.5) containing 0.5 M NaCl (hereafter referred to as SAB) at 2°C for 2 and 0.5 min, successively. The remaining SAB was then washed away by three rapid rinses with cold WM. Such mild acid/salt treatment removes ∼90% of the surface-bound EGF from A431 cells labeled with ^{125}I-EGF at 2°C. A subsequent rinse with 0.2 M acetic acid (pH 2.8), containing 0.5 M NaCl, or with SAB at pH 4.5 for 6 min removed not more than an additional 3–5% of the label. Cells subjected to the above protocol, including SAB treatment, are referred to as "^{125}I-EGF–loaded cells," and were used as the starting point in most experiments. These cells have a minimal amount of ^{125}I-EGF-RC on the surface and a relatively large pool of internalized ^{125}I-EGF-RC (34). The viability of the cells was evaluated by staining with a mixture of acridine orange and ethydium bromide and was ∼93–95% before and after SAB treatment.

The ^{125}I-EGF–loaded cells were chased in WM containing 250 ng/ml of unlabeled EGF at 2, 18, or 37°C for the times indicated in each experiment. At the end of each chase time, the medium was collected and used for determining the amount of intact ^{125}I-EGF and low molecular weight products of its degradation by the use of TCA precipitation, as described previously (33). The surface-bound ^{125}I-EGF was extracted by SAB treatment for 6 min. Finally, the cells were lysed in 1 N NaOH to determine the intracellular ^{125}I-EGF.

In some experiments the cells treated for 6 min with SAB were incubated in WM containing 0.5% Brij-58 (for 10 min at room temperature before being lysed in NaOH) to extract free intracellular ^{125}I-EGF. This mild detergent treatment has been used previously (33) to distinguish between intracellular, free (detergent-extracted), and receptor-bound (detergent-resistant) labeled ligand.

The nonspecific binding of ^{125}I-EGF routinely determined in the presence of 500-fold excess of unlabeled EGF was ∼1–2%.

Covalent Cross-Linking of EGF-RC

Cells were loaded with 40 ng/ml of ANBS-^{125}I-EGF, as described above for ^{125}I-EGF, and then incubated in WM containing 250 ng/ml of unlabeled EGF for 3 h at 18°C. The medium was replaced with fresh WM containing unlabeled EGF, and the cells were irradiated by using an UV lamp equipped with a 340-nm glass filter at 2°C for 10 min. All procedure steps before irradiation were carried out under weak red light.

The cells containing covalently linked ^{125}I-EGF-RC were then incubated in fresh WM with unlabeled EGF for a second chase at 37°C. At the end of each chase time point, the medium was collected and the cells were treated with SAB for 6 min, as described for ^{125}I-EGF binding experiments.

To separate surface from intracellular covalently linked ^{125}I-EGF-RC, the cells were incubated for 1 h in ice cold WM containing anti-EGFR diluted to a saturating concentration (1:50), as described by Soderquist and Carpenter (32). Then the cells were washed with PBS and solubilized in RIPA buffer (1% Nonidet-40, 1% deoxycholate, 50 mM Tris-HC, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 1 mM PMSF, 0.02% sodium azide) at 2°C for 15 min. An aliquot of the cell lysate, containing total cell-associated ^{125}I-EGF-RC, was saved, and the remaining lysate was immediately mixed with 20 μl of Pansorbin to precipitate the surface receptors recognized by anti-EGFR. After 30 min of shaking Pansorbin was pelleted in an Eppendorf centrifuge, an aliquot of supernatant (containing intracellular receptors not available to the antibody) was saved, and the pellet was washed four times with RIPA buffer and boiled in 40 μl of the sample buffer (25) for 10 min. The aliquots of lysate, supernatant, and immunoprecipitate were processed by SDS–PAGE (25). The gel was subjected to autoradiography at −70°C, and the 175- and 155-kD bands corresponding to the covalently linked EGF-RC were sliced from the dried gel and counted on a γ-counter.

In some experiments anti-EGFR was included in the chase medium. In these experiments, cells were solubilized at the end of the chase, and 20 μl of Pansorbin was added to the cell lysate to precipitate any ^{125}I-EGF-RC exposed to the antibody during the chase.

To determine the efficiency of cross-linking, two experimental procedures have been used. In one, cells labeled with ANBS-^{125}I-EGF at 2°C were either irradiated or kept in the dark. Surface-bound but not covalently linked ANBS-^{125}I-EGF was then removed by treatment with SAB for 6 min. The efficiency of cross-linking at the cell surface was ∼12–16%, as determined by the decrease in the amount of ANBS-^{125}I-EGF removed by SAB from irradiated cells.

In the other procedure, cells loaded with ANBS-^{125}I-EGF and exposed to 18°C, as described before, were not irradiated, but were solubilized in hot sample buffer in the dark before being processed for gel electrophoresis. Based on the decrease in the amount of free ANBS-^{125}I-EGF (that migrated with the front in 7.5% gels) from irradiated cells in comparison with that from nonirradiated cells, the cross-linking efficiency was 14–16%.

The protein content in the cell lysates was determined as described previously (23).

Results

To study the dynamics of ^{125}I-EGF recycling and degradation, ^{125}I-EGF–loaded cells (prepared as described in Materials and Methods) have been used as an experimental model. After ^{125}I-EGF loading, the cells were incubated in the presence of excess unlabeled EGF (250 ng/ml) according to the different experimental protocols outlined in Table I.

Table I. Experimental Protocols Used for ^{125}I-EGF–Cell Interaction Experiments*

Protocol No.	^{125}I-EGF cell-binding conditions	^{125}I-EGF cell-loading conditions‡	Chases in the presence of excess unlabeled EGF
1	1 h at 2°C	15 min at 37°C/SAB	Chase at 37°C
2	1 h at 2°C	15 min at 37°C/SAB	3-h chase at 37°C/2nd chase at 37°C
3	1 h at 2°C	15 min at 37°C/SAB	Chase at 18°C
4	1 h at 2°C	15 min at 37°C/SAB	3-h chase at 18°C§/2nd chase at 37°C
5	1 h at 2°C	5 min at 37°C/SAB	1-h chase at 2°C/2nd chase at 37°C
6	1 h at 2°C	SAB	1-h chase at 2°C‖/2nd chase at 37°C

* These protocols have also been used in experiments with photoactivatable ^{125}I-EGF.
‡ The cells loaded at 37°C and treated with SAB are referred to as "^{125}I-EGF–loaded" cells.
§ These cells are referred to as "18°C-exposed, ^{125}I-EGF–loaded" cells.
‖ These cells are referred to as "unloaded" cells.

Identification of Two Pathways for ^{125}I-EGF Recycling

In the first set of experiments, ^{125}I-EGF was allowed to internalize for 15 min at 37°C after labeling (protocol 1–4, Table I). Under these conditions ~40–50% (100–150,000 molecules/cell) of initially surface-bound ^{125}I-EGF was internalized. After mild SAB treatment to remove remaining ^{125}I-EGF on the surface, these ^{125}I-EGF–loaded cells were incubated for a chase time at 37°C (protocol 1) or 18°C (protocol 3) in the presence of unlabeled EGF. At 37°C rapid accumulation of ^{125}I-EGF into the medium (Fig. 1 *A*, ●) and a corresponding decrease of the amount of intracellular ^{125}I-EGF (Fig. 1 *B*, ▲) was observed. The high rate of ligand outflow (~1.3–1.5 × 10^3 molecules/cell per min in different experiments) measured during the first 20–40 min was followed by a slower exit during the next 2 h of the chase. The amount of free ^{125}I-EGF in the medium reached a maximal level after the 3-h continuous chase and did not increase with further incubation. If the medium was replaced with fresh medium after the 3-h time point (protocol 2), an additional small accumulation of labeled ligand in the medium occurred during the second chase at 37°C (Fig. 1 *A*, ●). However, this accumulation probably corresponds to dissociation of ^{125}I-EGF from the cell surface (Fig. 1 *B*, ★) rather than an additional outflow of internalized ligand (Fig. 1 *B*, ▲) due to recycling.

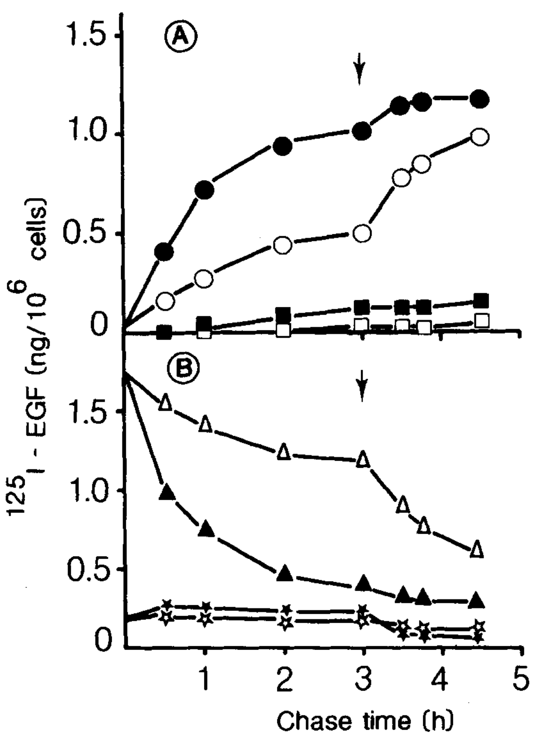

Figure 1. ^{125}I-EGF recycling and degradation in the presence of unlabeled EGF. A431 cells were loaded (as described in Materials and Methods) with 20 ng/ml ^{125}I-EGF as in protocols 1–4 (Table I). SAB treatment removed 1.69 ng ^{125}I-EGF/10^6 cells whereas 1.73 ng ^{125}I-EGF/10^6 cells were internalized. Cells were chased in the presence of excess unlabeled EGF at 37°C (*closed symbols*) or 18°C (*open symbols*) according to protocols 1 or 3, respectively. After a 3-h incubation, fresh medium was applied (*arrow*) and the cells were incubated for a second chase time at 37°C (protocols 2 or 4). At various time points, the medium (*A*) was assayed for intact ^{125}I-EGF (○,●) and low molecular weight products of its degradation (□,■), and the cells (*B*) were assayed for the surface-bound (☆,★) and intracellular ^{125}I-EGF (△,▲). Each data point was averaged from three values differing by <5%.

If the chase of ^{125}I-EGF–loaded cells was performed at 18°C (protocol 3), the rate of accumulation of free ^{125}I-EGF in the medium was three times lower than at 37°C and became negligible after 2–3 h of continuous incubation (Fig. 1 *A*, ○). The total amount of ^{125}I-EGF recycled to the medium during 3 h at 18°C was half that at 37°C. However, when the cells exposed at 18°C for 3 h (referred to as "18°C-exposed, ^{125}I-EGF–loaded cells") were chased a second time in fresh medium containing unlabeled EGF at 37°C (protocol 4), an additional outflow of intracellular ^{125}I-EGF was measured during the second chase (Fig. 1 *A*, ○). The total amount of ^{125}I-EGF leaving the cells during the 3-h chase at 18°C and the second chase at 37°C was similar to the amount exiting ^{125}I-EGF–loaded cells originally chased at 37°C for 3 h (Fig. 1 *A*, ●). These data suggest the existence of a temperature-sensitive pool of internalized ^{125}I-EGF incapable of recycling at 18°C but able to be recycled at 37°C.

When the 18°C-exposed, ^{125}I-EGF–loaded cells were incubated in the presence of unlabeled EGF at 37°C for a second chase period (Fig. 2 *A*, ●), free ^{125}I-EGF accumulated in the medium with an initial linear rate of outflow of ~700 ^{125}I-EGF molecules/cell per min. The accumulation was 90% complete after 70–90 min of continuous incubation at 37°C. The half-time for ^{125}I-EGF outflow from the 18°C-exposed, ^{125}I-EGF–loaded cells was measured in several experiments and was found to be ~20 min.

The degradation of internalized ^{125}I-EGF was blocked at

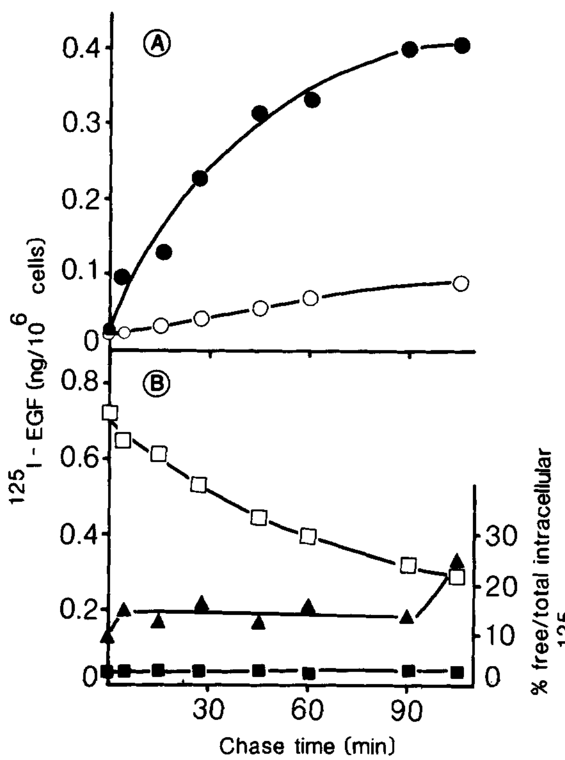

Figure 2. Long-time recycling of ^{125}I-EGF. Cells were loaded with ^{125}I-EGF as in Fig. 1. 1.23 ng ^{125}I-EGF/10^6 cells were removed by SAB and 1.08 ng ^{125}I-EGF/10^6 cells were internalized. ^{125}I-EGF–loaded cells were chased with unlabeled EGF for 3 h at 18°C and were incubated in fresh medium for a second chase time at 37°C (protocol 4, Table I). At various times during the second chase the amount of intact ^{125}I-EGF (●) and its degradation products (○) in the medium (*A*) as well as the surface-bound (■) and intracellular ^{125}I-EGF (□) in the cells (*B*) were determined. The amount of free intracellular ^{125}I-EGF (▲), expressed as a percent of total intracellular ^{125}I-EGF, was determined by using the Brij-extraction procedure described in Materials and Methods. The data points are averaged from three values differing by <5%.

18°C (Fig. 1 *A*, □) but was initiated after the cells were warmed to 37°C (Fig. 2 *A*, ○) during the second chase. The rate of degradation was found to be relatively slow, since only ~80% of the ^{125}I-EGF incapable of recycling was degraded even after a 7-h chase at 37°C in the presence of excess unlabeled EGF (data not shown).

Taken together, the data presented in Figs. 1 and 2 allow us to propose that ~25–30% of the total internalized ^{125}I-EGF recycles in A431 cells via both a slower ("long-time") pathway strongly inhibited at 18°C and a rapid ("short-time") pathway partially inhibited at this temperature. The remaining 40–50% of internalized ^{125}I-EGF fails to be recycled and undergoes gradual degradation. This proportion was found to be similar when the cells were treated as in protocol 4 with anywhere from 5 to 200 ng/ml of ^{125}I-EGF.

Examination of Internalized ^{125}I-EGF-RC Dissociation during Long-Time Recycling

In previous studies (34) no significant dissociation of intracellular EGF-RC was observed during rapid recycling of EGF in EGF-loaded cells. In these studies, we have used the Brij-58 treatment procedure described in Materials and Methods to determine how much ^{125}I-EGF dissociates from internalized receptors under conditions in which long-time recycling is observed (during a 37°C chase of 18°C-exposed, ^{125}I-EGF–loaded cells). As seen in Fig. 2 *B* (▲) the amount of Brij-extractable ^{125}I-EGF was between 10 and 15% of total intracellular ^{125}I-EGF after an 80-min chase at 37°C, by which time >90% of the ^{125}I-EGF capable of recycling has escaped the cells (Fig. 2 *A*, ●). The dissociation reached a maximal level of 50–60% after 4 h of chase at 37°C (data not shown). In control experiments ~7–10% of bound ^{125}I-EGF was released from the surface of cells labeled with ^{125}I-EGF at 2°C and then incubated at room temperature for 10 min. Approximately the same amount of ligand might be expected to dissociate from intracellular receptors during the Brij treatment. This suggests that at least 90–95% of internalized ^{125}I-EGF-RC do not dissociate during long-time recycling in 18°C-exposed, ^{125}I-EGF–loaded cells.

Comparison of Long- and Short-Time Recycling of ^{125}I-EGF

If ^{125}I-EGF recycles as ^{125}I-EGF-RC, the amount of surface ^{125}I-EGF present during the 37°C chase of ^{125}I-EGF–loaded cells should be dependent on the difference between the rates of ^{125}I-EGF-RC outflow and ^{125}I-EGF dissociation from cell surface receptors, since rebinding and reinternalization of ^{125}I-EGF is inhibited by an excess of unlabeled EGF. Therefore, in the next set of experiments the dynamics of long- and short-time recycling of ^{125}I-EGF and of dissociation of ^{125}I-EGF from cell surface receptors were examined in more detail.

To study the rapid recycling pathway, cells were treated as in protocol 5. ^{125}I-EGF was bound to the cells for 1 h at 2°C, and the cells were allowed to internalize ^{125}I-EGF for 5 rather than 15 min at 37°C before remaining surface-bound ^{125}I-EGF was removed with SAB. The initial rate of ^{125}I-EGF recycling in the cells loaded for 5 min should correspond to the rate of short-time recycling, since only a minimal contribution of long-time recycling ($t_{1/2}$ ≈20 min) would be expected in the early time point of the time-scale of endocytosis.

The ^{125}I-EGF–loaded cells were further incubated with unlabeled EGF for 1 h at 2°C to create an excess of unlabeled EGF-RC at the cell surface. As seen in Fig. 3, when the cells were then subjected to a second chase with unlabeled EGF at 37°C, both a rapid increase in the amount of surface-bound ^{125}I-EGF (Fig. 3 *A*, ○) and an accumulation of free ^{125}I-EGF in the medium (Fig. 3 *B*, ●) were observed. The same dynamics of rapid reappearance of EGF on the cell surface was seen if cells were allowed to internalize ligand at 37°C for anywhere from 2 to 15 min (data not shown).

To examine long-time recycling, cells were ^{125}I-EGF loaded and exposed to 18°C as in protocol 4. When these cells were chased at 37°C a second time, rapid accumulation of ^{125}I-EGF in the medium was observed (Fig. 3 *B*, ▲), but the amount of surface-bound ^{125}I-EGF showed a small initial decrease that then leveled off (Fig. 3 *A*, △).

To study specifically the rate of dissociation of ^{125}I-EGF from surface receptors, cells with the same, minimal surface pool of ^{125}I-EGF as in ^{125}I-EGF–loaded cells but with a negligible intracellular pool of ^{125}I-EGF were needed. To

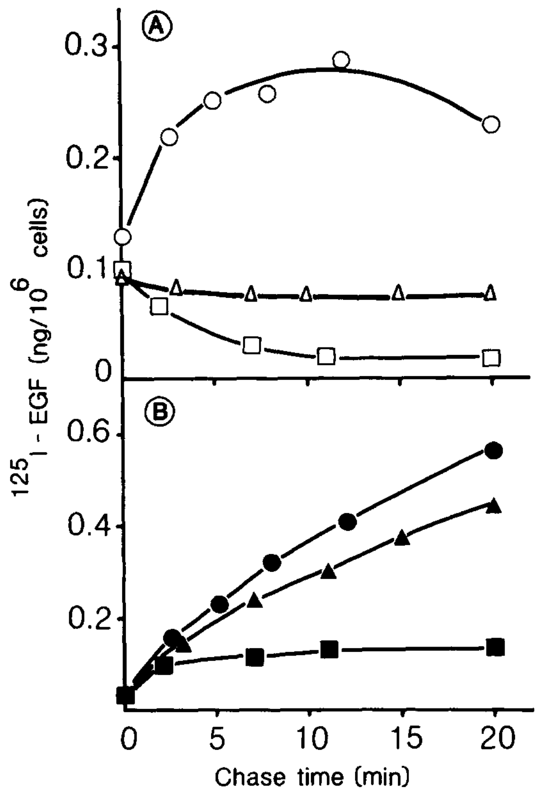

Figure 3. Dynamics of short- and long-time recycling and cell surface dissociation of ^{125}I-EGF. Cells were labeled with 40 ng/ml of ^{125}I-EGF and then treated as outlined in Table I: long-time recycling (△,▲) as in protocol 4, short-time recycling (○,●) as in protocol 5, and dissociation (□,■) as in protocol 6. During the procedure 1.79, 1.26, or 0.25 ng/10^6 cells of ^{125}I-EGF remained associated with cells after removal of 1.58, 2.25, or 2.90 ng/10^6 cells of ^{125}I-EGF from the cell surface when cells were treated according to protocols 4, 5, or 6, respectively. At various times during the second chase at 37°C, the amount of surface-bound (*A*) and free ^{125}I-EGF in the medium (*B*) was determined. Data represent the average value from two parallel dishes.

accomplish this, cells were labeled with ^{125}I-EGF at 2°C, treated with SAB without first being allowed to internalize the labeled ligand, and then chased with an excess of unlabeled EGF at 2°C for 1 h (protocol 6). When these "unloaded" cells were incubated for a second chase time at 37°C in fresh medium containing unlabeled EGF, a rapid release of ^{125}I-EGF from surface receptors to the medium (Fig. 3 *B*, ■), and a corresponding decrease in the amount of surface-bound ^{125}I-EGF (Fig. 3 *A*, □) were observed. The initial rate of ^{125}I-EGF release from surface receptors was similar whether the cells were ^{125}I-EGF loaded; 18°C-exposed, ^{125}I-EGF–loaded; or unloaded (Fig. 3 *B*).

The initial increase in the amount of surface-bound ^{125}I-EGF during short-time recycling indicates both that the bulk of ^{125}I-EGF recycles as ^{125}I-EGF-RC and that the initial rate of outflow of ^{125}I-EGF-RC is higher than the rate of ^{125}I-EGF dissociation from the cell surface. The rate of long-time recycling appears to be similar or slightly slower than the rate of dissociation, since dissociation was compensated for by the insertion of recycled ^{125}I-EGF-RC into the cell surface (Fig. 3 *A*). Furthermore, the decrease in the surface pool of ^{125}I-EGF in "unloaded" cells suggests that maintenance of a minimal level of surface ^{125}I-EGF during long-time recycling requires an intracellular pool of ^{125}I-EGF-RC.

The Long-Time Recycling of Covalently Linked ^{125}I-EGF-RC

The absence of a significant pool of free intracellular ^{125}I-EGF during long-time recycling (Fig. 2 *B*) and analysis of the kinetics of long-time recycling (Fig. 3) suggest that this recycling can occur as recycling of ^{125}I-EGF-RC. However, it is not possible to estimate from the data in Fig. 3 how much ^{125}I-EGF is recycled as ^{125}I-EGF-RC. Therefore, to directly examine the long-time recycling of EGF-RC, EGF receptors were covalently labeled with ANBS-^{125}I-EGF.

Cells were loaded with 40 ng/ml of ANBS-^{125}I-EGF and chased with unlabeled EGF for 3 h at 18°C according to protocol 4. UV irradiation of the cells after the 18°C chase resulted in covalent linking of ANBS-^{125}I-EGF to receptors with an efficiency of 14–16% (see Materials and Methods).

After irradiation 18°C-exposed, ANBS-^{125}I-EGF–loaded cells were incubated for a second chase time at 37°C in the presence of unlabeled EGF (250 ng/ml). At various times during the 37°C chase, covalently linked ^{125}I-EGF-RC present on the cell surface were separated from intracellular complexes by use of the cell surface immunoprecipitation method described in Materials and Methods. Besides the expected 175-kD EGF-RC band, a minor band of 155 kD, which represents the partially degraded EGF-RC, was found in immunoprecipitates from these cells. Its appearance, however, is dependent upon the time involved in the immunoprecipitation procedure, since only the 175-kD band was seen if cells were solubilized in hot sample buffer and subjected immediately to electrophoresis without immunoprecipitation.

As seen in Fig. 4 the intensity of the EGF-RC band immunoprecipitated from the cell surface was minimal in 18°C-exposed cells (lane *A*) and increased during the chase incubation at 37°C (lanes *B–E*). Intracellular receptors could be recovered from the supernatant of the cell surface immunoprecipitation. As expected, the intensity of the band corresponding to intracellular EGF-RC decreased with chase time (lanes *A'–E'*).

In Fig. 5 the amount of covalently linked ^{125}I-EGF-RC present at the cell surface (in cell surface immunoprecipitates) was expressed as a percent of the total amount of covalently linked ^{125}I-EGF-RC in the cells (in whole cell lysates). The redistribution of ^{125}I-EGF-RC from the intracellular pool to the surface pool during the chase at 37°C indicates that covalently linked EGF-RC were recycled by the long-time pathway.

In the experiments presented in Figs. 4 and 5, the total amount of covalently linked ^{125}I-EGF-RC (normalized to the protein content in each dish) decreased by 14% (SEM = 6.9%) during a 1-h chase at 37°C. Together with results presented in Fig. 2 *A*, this suggests a similar rate of degradation for ^{125}I-EGF in 18°C-exposed cells whether ^{125}I-EGF is covalently linked to receptors or not.

The rates of recycling and degradation of ANBS-^{125}I-EGF not covalently linked to receptors were found to be similar to the rates obtained for ^{125}I-EGF (data not shown). In Fig. 5 the apparent rate of ligand recycling is expressed as a percentage of the sum of ^{125}I-EGF found in the medium

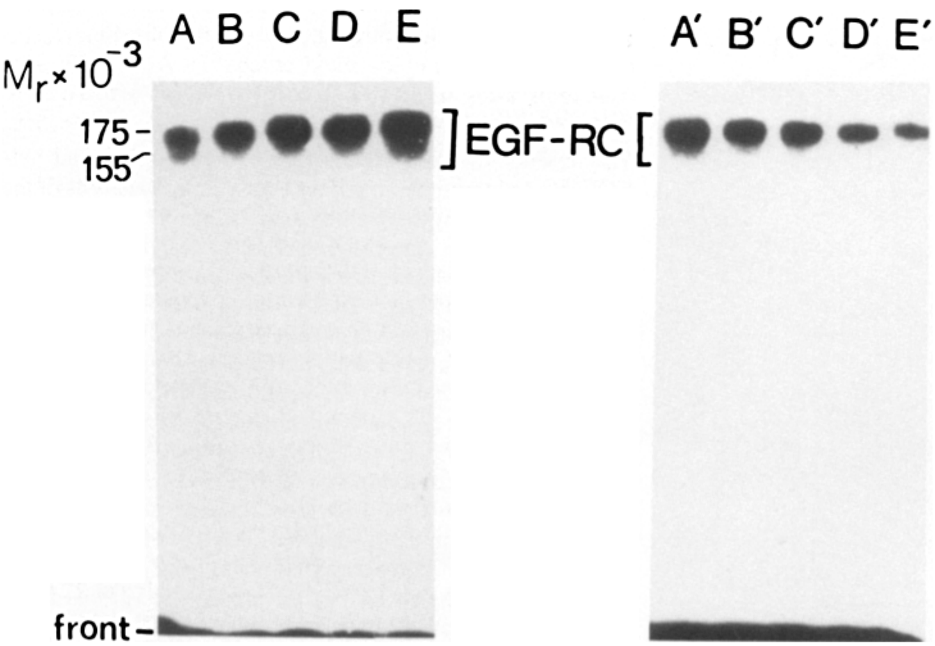

Figure 4. Long-time recycling of covalently linked ^{125}I-EGF-RC. Cells were labeled with 40 ng/ml of ANBS-^{125}I-EGF and treated according to protocol 4 (Table I) before being UV irradiated. 1.89 and 0.90 ng ^{125}I-EGF/10^6 cells were removed from cells during SAB treatment and the first chase, respectively, whereas 1.01 ng ^{125}I-EGF/10^6 cells remained intracellular. The second chase was for 0 (lanes *A* and *A'*), 20 (lanes *B* and *B'*), 30 (lanes *C* and *C'*), 45 (lanes *D* and *D'*), and 70 (lanes *E* and *E'*) min at 37°C. At each of these time points surface ^{125}I-EGF-RC were immunoprecipitated as described in Materials and Methods and subjected to SDS-PAGE. Lanes *A–E* are immunoprecipitates of cell surface ^{125}I-EGF-RC, while lanes *A'–E'* are supernatants of these immunoprecipitates, containing intracellular ^{125}I-EGF-RC.

and on the cell surface to the total ^{125}I-EGF associated with the cells and the medium. As seen in Fig. 5 the rate of reappearance of covalently linked ^{125}I-EGF-RC on the cell surface is slightly lower than the rate of ^{125}I-EGF recycling.

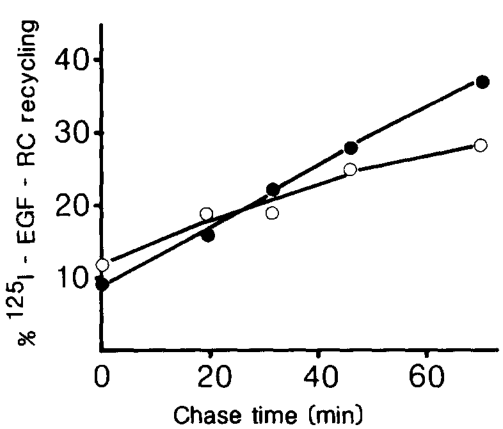

Figure 5. Time course of long-time recycling of ^{125}I-EGF and covalently linked ^{125}I-EGF-RC. The experiments on recycling of covalently linked EGF-RC were carried out as described in the legend of Fig. 4. The radioactivity from surface ^{125}I-EGF-RC (from cell surface immunoprecipitates) and total cell-associated ^{125}I-EGF-RC (from cell surface immunoprecipitates) was estimated by cutting out the appropriate bands from the gels and counting them in a γ counter. The recycling of covalently linked ^{125}I-EGF-RC (○) is expressed as a ratio of the surface to the total amount of the covalently linked ^{125}I-EGF-RC associated with cells at each time point during the second chase. The data are averaged from three separate experiments similar to those presented in Fig. 4. The recycling of noncovalently bound ^{125}I-EGF from 18°C-exposed, ^{125}I-EGF–loaded cells (●) is expressed as a ratio of the sum of medium and surface-bound ^{125}I-EGF relative to the total amount of ^{125}I-EGF associated with cells and medium at each time point. The data were averaged from two experiments carried out according to protocol 4 (Table I), as described in the legend of Fig. 2.

This might be due to some reinternalization of the recycled, covalently linked ^{125}I-EGF-RC during the second chase at 37°C, whereas the probability of reinternalization of the recycled, uncoupled ^{125}I-EGF (that can dissociate from the cell surface receptors) is much lower.

To demonstrate that covalently linked EGF-RC can, in the presence of excess unlabeled EGF, be reinternalized during the 37°C chase of 18°C-exposed cells, anti-EGFR was included in the chase medium, so that any recycled EGF-RC appearing on the cell surface would be bound by the anti-EGFR before reinternalization. As seen in Fig. 6, lane *C'*, more ^{125}I-EGF-RC was recovered in the immunoprecipitates when anti-EGFR was included in the chase medium than when anti-EGFR was added to the cells after the chase (lane *B'*). This result indicates that reinternalization of the recycled covalently linked complexes does take place.

The approximate amount of the covalently linked ^{125}I-EGF-RC capable of internalization during a 1-h chase at 37°C was determined in cells treated as in protocol 6 (Table I). The cells were first incubated with ANBS-^{125}I-EGF at 2°C, treated with SAB, chased with unlabeled EGF (250 ng/ml) at 2°C for an additional hour, and irradiated to couple the ligand to receptors. The intracellular pool of covalently linked ^{125}I-EGF-RC in these "unloaded" cells was negligible, whereas the surface pool was similar to that in 18°C-exposed, ^{125}I-EGF–loaded cells (Fig. 6 *B*, *A* and *A'*).

The unloaded cells were further incubated for a second chase with unlabeled EGF at 37°C in the presence or absence of anti-EGFR. The amount of covalently linked ^{125}I-EGF-RC present on the cell surface of these cells decreased more than two times after a 1-h chase (lane *B*), whereas a slight increase in the amount of covalently linked complexes is seen when complexes present on the cell surface anytime during the chase were recovered by including the antibody in the chase medium (lane *C*). Similarly, approximately half of the ^{125}I-EGF-RC present at the cell surface during a 1-h

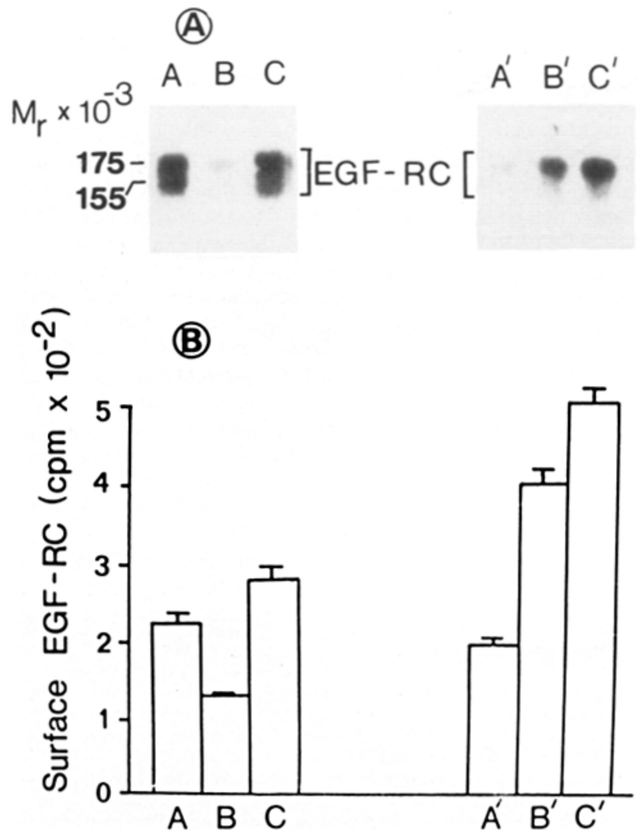

Figure 6. Long-time recycling and reinternalization of covalently linked ^{125}I-EGF-RC. "Unloaded" cells (lanes *A–C*) were obtained according to protocol 6 (Table I). Briefly, cells were labeled with 40 ng/ml of ANBS-^{125}I-EGF at 2°C, treated with SAB, and chased with unlabeled EGF at 2°C for 1 h before UV irradiation 2.97 ng/10^6 cells of ligand were removed from the surface whereas 0.26 ng/10^6 cells were still associated with the cells. 18°C-exposed, ANBS-^{125}I-EGF–loaded cells (lanes *A′–C′*) were obtained and irradiated as described in the legend of Fig. 4. During the procedure 1.75 ng/10^6 cells of the label were removed by SAB whereas 1.74 ng/10^6 cells were internalized. Both unloaded and 18°C-exposed cells were incubated for a second, 1-h chase at 37°C in fresh medium containing unlabeled EGF alone. The surface covalently linked ^{125}I-EGF-RC were immunoprecipitated before (lanes *A* and *A′*) and after the chase at 37°C (lanes *B* and *B′*). Covalently linked ^{125}I-EGF-RC exposed on the cell surface at anytime during the chase were identified by antiserum present throughout the chase (lanes *C* and *C′*). (*A*) Autoradiograms of the immunoprecipitates of the ^{125}I-EGF-RC. The gels were exposed with x-ray films at −70°C for 7 d (lanes *A–C*) or 2 d (lanes *A′–C′*) (*B*) The corresponding amount of radioactivity recovered from the imunopecipitates of ^{125}I-EGF-RC at each time point. The total amount of cellular covalently linked ^{125}-I-EGF-RC per dish did not change significantly during the chase in unloaded cells and decreased by 5–7% after the chase in 18°C-exposed, ^{125}I-EGF–loaded cells.

chase incubation of 18°C-exposed cells at 37°C can be internalized. Based on this estimation the rate of long-time recycling of covalently linked ^{125}I-EGF-RC would be ∼1.5 times higher than the apparent rate of reappearance of the complexes on the cell surface (Fig. 5, ○) and, therefore, similar to the rate of long-time recycling of ^{125}I-EGF-RC that are not covalently linked (Fig. 5, ●).

Discussion

We have studied the intracellular sorting of endocytosed EGF-RC in human epidermoid carcinoma A431 cells. Examination of the fate of internalized ^{125}I-EGF in these cells showed that ligand was rapidly recycled to the cell surface at 37°C in the early stages of endocytosis. The rate of this recycling was partially reduced at 16–18°C (Fig. 1 and reference 34). Moreover, a significant pool of internalized ^{125}I-EGF was unable to escape the cell at 18°C but was capable of recycling if cells were warmed to 37°C, albeit at a slower rate than if cells were not exposed to the temperature block. Recycling of this pool of internalized EGF seems to involve passage through a highly temperature-sensitive step, which suggests that there are different pathways for rapid ("short-time") and slower ("long-time") recycling.

The "slower" pool could be examined after 3 h of continuous incubation of ^{125}I-EGF-loaded cells at 18°C in the presence of excess unlabeled EGF. ^{125}I-EGF capable of recycling at 18°C escaped the cells during this incubation. Evaluation of the $t_{1/2}$ for ^{125}I-EGF outflow from 18°C-exposed cells warmed up to 37°C yielded a value of 20 min. This value for short-time recycling can not be determined correctly at 37°C since both types of recycling would occur at this temperature. However, it is likely that the component of long-time recycling would have only an insignificant influence on the initial linear rate of ^{125}I-EGF outflow measured during early stages of endocytosis. Therefore, based on the assumption that about half of the total pool of recycled EGF uses the long-time cycle (Fig. 1), a $t_{1/2}$ of 5–7 min for short-time ^{125}I-EGF recycling at 37°C was calculated from the data of several experiments similar to those presented in Figs. 1 and 3. Interestingly, similar values were obtained for rapid transferrin recycling in A431 cells (19) and insulin retroendocytosis in adipocytes (22).

The data obtained by using mild treatment of the cells with Brij-58 indicated that EGF-RC do not dissociate within the endosomal compartment in A431 cells (33). Electron microscopic studies with the use of EGF-ferritin (26) or anti-EGF serum (7) also indicate that ligand remains associated with endosomal membrane in these cells. No significant dissociation of internalized EGF-RC was observed during short- (34) or long-time recycling (Fig. 2 *B*).

Analysis of the dynamics of ^{125}I-EGF recycling (Fig. 3) supports a model of EGF-RC recycling, though these data do not demonstrate whether EGF recycles exclusively as EGF-RC, especially via the long-time pathway. However, examination of the long-time recycling of covalently labeled ^{125}I-EGF-RC revealed a similar rate to that observed for ^{125}I-EGF recycling (Figs. 4–6). Taken together, the data allow us to propose that EGF remains bound to the receptor during routing to the recycling and degradative pathways. However, a significant dissociation of EGF-RC appears to occur in the late stages of intracellular processing, within the lysosomal compartment (26, 33).

In our experiments short-time recycling could be observed in cells allowed to internalize receptors during a brief (2–5 min) exposure to 37°C. Morphologically EGF and EGF receptors have been demonstrated to localize in "early" endosomes after similar incubations (9, 14, 27). We have also observed phycoerythrin and peroxidase conjugates of EGF to be distributed throughout the peripheral endosomal compartment in A431 cells after similar treatments (data not shown). Therefore, it might be proposed that short-time recycling is due to the bi-directional movement of peripheral endosomes demonstrated by Nanovid microscopy in living A431 cells (9, 39) and/or to a rapid sorting process within the peripheral endosomal compartment that is similar to what has been described for the asialoglycoprotein and transferrin receptors in Hep G2 cells (35).

Long-time recycling of EGF-RC was highly inhibited at 18°C. Degradation of EGF (Fig. 1) and EGF receptor is also blocked at 16–20°C (10, 37). By EM EGF-RC have been demonstrated to accumulate within the pericentriolar endosomal compartment in A431 cells at 20°C (27). Similar accumulation of endocytosed ligand and receptors in "late" endosomes has been observed in several cell lines after relatively long incubations at 16–20°C (28, 30). Griffiths et al. (12) have observed that transport of endocytic and recycling markers through a so-called "sorting" compartment rich in mannose-6-phosphate receptor was blocked at 20°C. In our experiments a 3-h incubation at 18°C of cells loaded with fluorescent or peroxidase conjugates of EGF in the presence of unlabeled EGF resulted in concentration of the label exclusively within pericentriolar multivesicular endosomes (data not shown). Additionally, the initiation of long-time recycling and degradation of the labeled EGF by warming 18°C-exposed cells to 37°C was closely associated with redistribution of the EGF-containing endosomes from the pericentriolar endosomal complex. Therefore, we propose that intracellular sorting of EGF-RC to the long-time recycling or degradation pathway occurs within the pericentriolar compartment in A431 cells.

The juxtanuclear/pericentriolar endosomal compartment in A431 cells has been found to be a region of accumulation for transferrin receptor complexes (18, 33). A comparative analysis of the endocytic pathway of EGF and transferrin receptors in A431 cells shows a close similarity of localization throughout the endosomal compartment (1, 18–20, 33) and suggests common recycling pathways for these ligand-receptor complexes. In fact, the existence of rapid and slow transferrin recycling pathways in A431 cells has been proposed by Hopkins and Trowbridge (19).

Studies of the turnover of EGF receptor protein have revealed a relatively slow rate of EGF-induced receptor degradation in A431 cells compared with that in human fibroblasts (36, 37). Even saturating concentrations of EGF fail to downregulate the surface EGF receptors efficiently (24, 36). Wiley (42) has proposed that EGF internalization is a saturable process in A431 cells. This might explain the anomalous properties of the EGF receptor endocytic system in these cells. Our finding of recycling of EGF-RC raises the possibility that saturation in the routing of EGF-RC to the degradative pathway occurs and that recycling of EGF-RC contributes significantly to the inefficient downregulation of EGF receptors in A431 cells.

The molecular mechanism of intracellular sorting of EGF receptors so far remains unclear. Failure of EGF receptors to enter the degradative pathway has been demonstrated when internalization was initiated without activation of the EGF receptor kinase (3, 17, 38). Therefore, a correlation between the kinase activity of the receptors and their routing to the degradative pathway might be proposed. Although it has been demonstrated that internalized EGF receptors can display kinase activity in A431 cells (7), a significant pool of internalized, as well as surface, EGF receptors appears to lose its kinase activity because of protein kinase C-dependent phosphorylation of the receptors on Tre-654 (8, 40). Therefore, the presence of two pools of internalized EGF-RC, different in terms of the receptor kinase activity, might be responsible for the sorting of EGF-RC to recycling vs. degradative pathways in A431 cells. Isolation of the recycling pool of EGF-RC in A431 cells, together with subsequent examination of receptor kinase activity, would answer the question of whether tyrosine kinase activity is important for intracellular sorting of EGF receptors.

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