Separate Endocytic Pathways of Kinase-defective and -active EGF Receptor Mutants Expressed in Same Cells

A. M. Honegger,* A. Schmidt,* A. Ullrich,~ and J. Schlessinger*

* Rorer Biotechnology, Inc., King of Prussia, Pennsylvania 19406; and ~Max Planck Institut fur Biochemie, Federal Republic of Germany

Abstract. Ligand binding to the membrane receptor for EGF induces its clustering and internalization. Both receptor and ligand are then degraded by lysosomal enzymes. A kinase defective point mutant (K721A) of EGF receptor undergoes internalization similarly to the wild-type receptor. However, while internalized EGF molecules bound to either the wild-type or mutant receptors are degraded, the K721A mutant receptor molecules recycle to the cell surface for reutilization. To investigate the mechanism of receptor trafficking, we have established transfected NIH-3T3 cells coexpressing the kinase-negative mutant (K721A) together with a mutant EGF receptor (CD63) with active kinase. CD63 was chosen because it behaves like wild-type EGF receptor with respect to biological responsiveness and cellular routing but afforded immunological distinction between kinase active and inactive mutants. Although expressed in the same cells, the two receptor mutants followed their separate endocytic itineraries. Like wild-type receptor, the CD63 mutant was downregulated and degraded in response to EGF while the kinase-negative mutant K721A returned to the cell surface for reutilization. Intracellular trafficking of EGF receptor must be determined by a sorting mechanism that specifically recognizes EGF receptor molecules according to their intrinsic kinase activity.

The growth stimulatory signal of EGF is mediated by the EGF receptor (EGFR), a 170-kD transmembrane glycoprotein with intrinsic protein tyrosine kinase activity (for reviews, see references 1, 19, 23). Ligand binding to the extracellular domain of EGFR leads to receptor oligomerization, which plays a crucial role in the activation of the cytoplasmic protein tyrosine kinase (for reviews, see references 19, 20). This leads to autophosphorylation of tyrosine residues within the carboxy terminus of EGFR and the phosphorylation of a variety of cellular substrate proteins (for reviews, see references 14, 15, 25). The kinase activity of the receptor is indispensable for signal transduction. Mutant receptors with inactive kinase are unable to elicit any of the effects associated with EGF-stimulated mitogenesis. These include EGF-induced increases in intracellular pH and free Ca^2+ concentration, stimulation of glucose and amino acid transport, phosphoinositol turnover, c-myc and c-fos expression, DNA synthesis, and cell transformation (2, 8, 9, 16, 18). Besides ligand activation, a variety of intrinsic and external mechanisms modulate the activity of the EGFR kinase (19). The intrinsic autophosphorylation sites of EGFR provide a control mechanism by competing with endogenous substrates for the active site, thus buffering receptor activity by increasing the activation threshold for biological response (10, 11, 22). Binding of EGF induces a negative feedback response by stimulating receptor internalization and degradation (6, 7, 21). An EGFR point mutant (K721A) with inactive kinase undergoes ligand-induced internalization, but fails to be downregulated. Unlike wild-type receptors that are degraded after internalization, the kinase-defective mutant recycles to the cell surface for reutilization (8).

To study the mechanisms regulating receptor trafficking after ligand stimulated internalization, we have established transfected cells that coexpress kinase negative receptor mutant together with EGFR bearing an active, ligand-regulated tyrosine kinase. Detailed analysis of ligand internalization, surface receptor downregulation, and degradation of these two receptor species coexpressed at different ratios in the same cell indicated that the kinase defective mutant and the active EGFR follow separate endocytic pathways even when expressed in the same cells.

Materials and Methods

Transfection

NIH 3T3 cells (clone 2.2), lacking endogenous EGFR (8) were grown in DME (Gibco Laboratories, Grand Island, NY) with 10% FCS. Cells grown in 10-cm dishes were transfected with 10–20 μg of plasmid DNA per dish using the calcium phosphate precipitation technique (24). The plasmid contained dihydrofolate reductase (DHFR) and neomycin resistance genes as
Cells were plated at a density of 100,000 cells/well in fibronectin-coated 24-well dishes. After 2 d, the cells were washed and incubated with 1, 10, or 100 ng/ml EGF and 15 mM of the chemical cross-linking agent 1-ethyl-3(3-dimethylaminopropyl) carbodiimide EDAC in DME containing 2% BSA and 50 mM Hepes pH 7.5 for 30 min at 37°C. To compare the relative contributions of surface bound versus internalized EGF in different cell lines, the curves were expressed as a percent of total cell associated radioactivity after 90 min incubation on ice ($t = 0$).

**Scatchard Analysis of $^{125}$I-EGF Binding Data**

Cells were plated at a density of 100,000 cells/well in fibronectin-coated 24-well dishes. After 2 d, the cells were incubated for 2 h at 37°C with 50 ng/ml EGF in DME with 0.5% FCS. The cells were washed twice with PBS and incubated in DME, 0.5% FCS, 25 nM Hepes without any added EGF for 30 min at 37°C to dissociate or internalize residual unlabelled EGF. After an additional wash with PBS, the cells were incubated with $^{125}$I-EGF in DME, 50 mM Hepes, 200 ng/ml BSA for 60 min at room temperature. After washing and solubilization the cells were counted in a gamma counter to determine the total amount of ligand bound to the cells. The results were presented in the form of Scatchard curves and fitted by a model for either one or two binding sites to calculate the apparent affinity and number of binding sites.

**Chemical Covalent Cross-linking Experiments**

Cells were grown to confluence in fibronectin-coated 10-cm dishes. They were washed and starved overnight in DME containing 0.5% FCS. 500 ng/ml EGF were added to each dish, and after different times up to 8 h, the cells were lysed in 0.5 ml of lysis buffer per plate (lysine buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl$_2$, 10% glycerol, 1% Triton X-100; protease inhibitors: 4 g/ml PMSF, 10 g/ml leupeptin, 10 g/ml aprotinin) and the lysates were frozen at -70°C. The EGF receptor was immunoprecipitated from the lysates with protein A-Sepharose-bound RK2 antibodies, which recognize all mutant receptors described in this report. After washing with HNTG (25 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) two equal samples were analyzed by SDS-PAGE and transferred to nitrocellulose. One of the blots was analyzed with anti-C antibodies (which recognize wt and K721A), the other with RK2 antibodies. After incubation with $^{125}$I-protein A, the two blots were autoradiographed. Radioactive bands were cut out and quantitated in a $\gamma$-counter.

**Determination of Receptor Half-Lives**

Cells were grown in fibronectin-coated 10-cm dishes and labeled overnight with [35S]methionine in DME containing 0.5% dialyzed FCS. The cells were washed to remove excess methionine and chased in complete medium containing 500 ng/ml EGF. After different chase times, the cells were lysed and the EGF receptor immunoprecipitated with either protein A-Sepharose-mAb680 complex (monoclonal antibody against the extracellular domain of the EGF receptor) or with protein A-Sepharose anti-C antibodies. The immunoprecipitates were washed and analyzed by SDS-PAGE. The fixed gels soaked in ENHANCE® (Amersham Corp., Arlington Heights, IL) were dried and autoradiographed to detect $^{35}$S-methionine-labeled protein bands. For quantitation of labeled EGF, the densities of the bands were measured with a densitometric scanner and their integrals compared.

**Results**

We have generated NIH-3T3 cells expressing wt EGFR (HER), kinase-negative mutant (K721A), and COOH-terminally truncated EGFR (CD63) as well as cells coexpressing K721A and CD63 (K721A/CD63 cells). The covalent cross-linking agent EDAC was used to demonstrate that EGF binding induces rapid oligomerization of EGFR in vitro and in living cells (3). Transfected cells expressing either wt or K721A mutant were incubated with the covalent cross-linking agent EDAC in the absence or presence of EGF. Neither receptor showed dimer formation in the absence of EGF (Fig. 1, A and B). At room temperature wt and K721A mutant were cross-linked with similar efficiency. At 37°C, however, cross-linking of K721A was impaired, unless internalization was blocked by pretreating the cells with 10 mM phenylarsine oxide (PAO) for 15 min at 37°C. At 37°C, both wt and K721A receptor are rapidly internalized upon ligand stimulation. While wt and CD63 remain in endocytic compartment, kinase-negative mutants are stripped of their ligands, recycled to the cells' surface and redispersed. This process is fast compared with the cross-linking reaction at 37°C but long enough to enable quantitative cross-linking at 23°C. This explains the reduced cross-linking efficiency of K721A under conditions that permit normal receptor trafficking. We have previously demonstrated that EGF-stimulated receptor oligomerization in vitro allowed the immunoprecipitation of CD63 along with K721A when antibodies against carboxy terminus (anti-C) were used for the immunoprecipitation (12). The formation of hetero-oligomers of the two mutants in living cells was confirmed by using a covalent cross-linking agent. EGF was added to K721A/DC63 cells, and after incubation with the cross-linking agent EDAC, the cells were washed, lysed, and the EGFR immunoprecipitated with anti-C antibodies (which recognize K721A but not CD63) and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1, EGF induced the formation of receptor dimers. Moreover, the fact that kinase activity can be detected in the im-
Figure 1. Receptor cross-linking in intact cells. Control untransfected cells (NIH). Cells expressing wt EGFR (HER), a COOH-terminally truncated active mutant (CD63), kinase-negative receptor (K721A), and cells coexpressing CD63 and K721A were treated with the covalent crosslinker EDAC (3) in the presence or absence of EGF (500 ng/ml). Monomeric EGF receptor and cross-linked products were immunoprecipitated and separated by SDS-PAGE. For detection, the receptor was either allowed to autophosphorylate in the presence of [γ-32P]ATP before electrophoresis or was immunoblotted and labeled with 125I-protein A. (A and B) HER and K721A cells were incubated for 15 min at 20°C (A) or for 60 min at 37°C (B) in the absence or presence of 500 ng/ml EGF and with 15 mM EDAC. The cross-linked receptors were immunoprecipitated with mAb108 and immunoblotted with RK2. (C and D) HER, K721A, CD63 and K721A/CD63 cells were treated for 15 min at 37°C in the absence or presence of 500 ng/ml EGF and with 15 mM EDAC. The cross-linked receptors were immunoprecipitated with RK2 and immunoblotted with RK2 (C) or autophosphorylated with [γ-32P]ATP (D). (E and F; next page) HER, CD63 and two different clones of K721A/CD63 cells were treated for 15 min at 37°C in the absence or presence of 500 ng/ml EGF and with 15 mM EDAC. The cross-linked receptors were immunoprecipitated with mAb108 (E) or anti-C (F), then autophosphorylated with [γ-32P]ATP.

munoprecipitate with anti-C antibodies indicates that heterodimers of K721A and CD63 were formed.

Ligand Internalization and Receptor Downregulation
Cells expressing wt EGFR (HER), COOH-terminal truncation mutant (CD63), or kinase-negative mutant (K721A) were allowed to bind 125I-EGF for 90 min at 4°C. The cells were warmed to 37°C for various time intervals, and the amount of cell surface bound and internalized EGF were separately determined. For direct comparison of the rates of
Fig. 1 (continued)

internalization in the different cell lines, all values are presented as percent of total cell-associated [125I]-EGF radioactivity after 90-min incubation at 4°C (Fig. 2). When probed at saturating ligand concentration (>100 ng/ml), K721A showed only a small and transient decrease in surface ligand binding (data not shown), despite efficient ligand uptake. When probed at lower ligand concentration (10 ng/ml), the loss of the high affinity component of EGF binding led to a small, persistent decrease of ligand binding. Under similar conditions, wt and CD63 receptors almost completely disappeared from the cell surface. In cells coexpressing K721A and CD63, an intermediate level of receptor down-

Figure 2. Ligand internalization and surface receptor down-regulation. Cells expressing wt EGFR (HER), COOH-terminally truncated active receptor (CD63), kinase-negative receptor (K721A), and different clones coexpressing K721A and CD63 were incubated for 90 min on ice with 10 ng/ml [125I]-EGF, then warmed to 37°C for different time intervals. [125I]-EGF bound to cell surface receptors and internalized ligand were separated by an acid-wash procedure and quantitated separately. To be able to directly compare the internalization and downregulation curves for different cell lines, all values were scaled to total binding at (t = 0) = 100%. (A and B) Time course of ligand internalization (A) and surface receptor downregulation (B) for wt (solid lines), CD63 (dashed lines), and K721A (dotted lines). (C and D) Time course of ligand internalization (C) and surface receptor downregulation (D) for wt and K721A (solid lines) and for five different clones of K721A/CD63 cells (dotted lines).
Table I. EGF Receptor Downregulation by Pretreatment with Unlabeled EGF

| Mutant EGF receptors | k1    | n1   | k2    | n2   | Total receptor number | Percent downregulation |
|----------------------|-------|------|-------|------|-----------------------|------------------------|
| HER                  | 14    | 250,000 | 0.26  | 21,000 | 271,000               | 71                     |
| +EGF                 | 19    | 59,000  | 2.6   | 19,000 | 78,000                |                        |
| K721A                | 11    | 343,000 | 0.34  | 16,000 | 359,000               | 18                     |
| +EGF                 | 13    | 280,000 | 2.3   | 14,000 | 294,000               |                        |
| K721A/CD63 (1:1)    | 11    | 318,000 | 0.17  | 12,000 | 330,000               |                        |
| +EGF                 | 15    | 200,000 | 0.09  | 1,000  | 201,000               |                        |
| K721A/CD63 (1:4)    | 9.2   | 250,000 | 0.09  | 8,000  | 258,000               |                        |
| CD63                 | 11    | 540,000 | 0.53  | 65,000 | 605,000               |                        |
| +EGF                 | 5.1   | 85,000  | 0     | 0      | 85,000                |                        |
| CD63                 | 9.8   | 520,000 | 0.42  | 46,000 | 566,000               |                        |
| +EGF                 | 5.2   | 76,000  | 0     | 0      | 76,000                | 87                     |

Binding of $^{125}$I-EGF to cells pretreated with or without 50 ng/ml EGF for 2 h at 37°C was determined as described in Materials and Methods and in Fig. 3. The results were plotted in the form of Scatchard curves, which were best fitted to a model representing either one or two affinity binding sites to determine number ($n$) and apparent dissociation constants ($K \times 10^{-9}$ M) of the binding sites. The values in parentheses for the K721A/CD63 cells are the ratios of the level of expression of the two mutant receptors as determined by immunoblotting analyses.

Regulation was observed, depending on the ratio of expression of the two mutants in each of the cell lines tested (Fig. 2, B and D). To obtain a more quantitative estimate of the degree of receptor downregulation in the different cell lines, Scatchard plots of $^{125}$I-EGF binding to cells preincubated in the presence or absence of unlabeled EGF were compared. The degree of receptor downregulation observed in this experiment was consistent with the results of the internalization/downregulation experiment (Table I; Fig. 3). It is noteworthy that at room temperature, receptor internalization is not completely blocked. Yet, this does not influence the measurement of the number of binding sites, as this information is derived from extrapolation to infinite ligand concentration.

**Receptor Degradation**

Confluent cells were treated for various times with EGF, then lysed, immunoprecipitated, and analyzed by SDS-PAGE. After transfer to nitrocellulose, immunoblots were analyzed with either RK2 antipeptide antiserum, which recognizes wt, CD63 and K721A receptor, or with anti-C antipeptide antibodies, which recognize wt and K721A receptor but not CD63 mutant. The blots were probed with $^{125}$I-protein A to visualize EGF receptors by autoradiography. EGF was able to stimulate receptor degradation in cells expressing either wt receptor or CD63 mutant, but not in cells expressing K721A mutant (Fig. 4). However, in K721A/CD63 cells, loss of receptor protein was observed when probed with RK2 antiserum, which detects both mutant receptors, but not when probed with anti-C, which detects only the K721A mutant. Thus, the two mutant receptors responded differently to ligand stimulation, even when expressed in the same cell.

![Figure 3. Scatchard analysis of $^{125}$I-EGF binding to cells pretreated with EGF. Cells expressing wt EGFR (HER in A), COOH-terminally truncated receptor (CD63 in B), kinase-negative receptor (K721A in C), and cells coexpressing CD63 and K721A (D) were treated for 2 h at 37°C with or without 50 ng/ml EGF. Excess unlabeled ligand was removed and the cells incubated for 60 min at 22°C with different concentrations of $^{125}$I-EGF. Binding to the cells as a function of ligand concentration was represented in the form of Scatchard plots and numerically fitted by computer models for one- and two-affinity receptor binding to obtain receptor numbers and apparent dissociation constants (listed in Table I). Dotted lines indicate binding of $^{125}$I-EGF to cells, solid lines indicate binding to cells after down-regulation of EGFR.](image-url)
Figure 4. EGF-induced receptor degradation. Cells expressing wt EGFR (HER in a), kinase-negative receptor (K721A in b), COOH-terminally truncated active receptor (CD63 in c), and cells coexpressing K721A and CD63 (d) were treated for various time intervals up to 8 h at 37°C in absence or presence of 500 ng/ml EGF. After lysis of the cells, the EGF receptors were immunoprecipitated with RK2 and subjected to SDS-PAGE analysis. Blots were then reacted with either anti-C (A) or RK2 (B) antibodies followed by reaction with ~25I-protein A for visualization by autoradiography. Radioactive bands were excised for quantitation in a gamma counter. Band intensities were plotted against the incubation time with EGF (C). Solid symbols denote samples blotted with RK2, open symbols denote samples blotted with anti-C. Dashed lines indicate receptor levels in cells incubated with buffer alone.

Determination of Receptor Half-Life

Cells were pulse-labeled with [35S]methionine, then chased in the presence or absence of EGF. The immunoprecipitated receptors were quantitated as a function of time. As shown in Fig. 5, in the absence of EGF, wt and K721A receptors had a half-life of 12–15 h. In agreement with previous experiments (8) EGF decreased the half-life of wt receptor to ~1 h, and the half-life of K721A to 8–10 h. Cells expressing a single receptor species had a single life-time for the rate of receptor degradation. However, K721A/CD63 cells showed a biphasic degradation profile (Fig. 5). The curves consisted of a short-lived component with a half-life of ~1 h and a long-lived component with a half-life of 8–12 h. The relative contributions of the two components reflected the expression levels of the two receptor mutants in the different cell lines tested. Anti-C antibodies immunoprecipitated only the long-lived component while RK2 antibodies immunoprecipitated both long-lived and short-lived receptor populations. These results indicate that both CD63 and K721A retain their characteristic degradation times even when expressed in the same cells.
Discussion

We have generated transfected NIH-3T3 lacking endogenous EGFR which coexpress similar amounts of CD63 and K721A mutants and, as a control, NIH-3T3 cells expressing similar amounts of wt, CD63, or K721A alone. We have previously demonstrated that kinase activity is dispensable for expression of EGFR with normal ligand binding characteristics on the cell surface but indispensable for signal transduction, DNA synthesis, transformation, and normal receptor trafficking (8).

Covalent cross-linking experiments with cells expressing wt EGFR, CD63 mutant and K721A mutant alone or cells coexpressing both CD63 and K721A indicated that the kinase activity is not essential for receptor dimerization. Moreover, heterodimeric complexes of CD63 and K721A were formed in CD63/K721A cells in response to EGF. The extent of heterodimerization and the dimerization of K721A alone was enhanced by inhibition of endocytosis, probably reflecting the rapid divergence of the endocytic pathways of the active and kinase-deficient EGFR receptor mutants. We have also demonstrated that the addition of EGF to cells expressing CD63 and K721A led to cross-phosphorylation of K721A both in vitro and in living cells (12, 13). Like receptor dimerization, cross-phosphorylation was greatly increased at 4°C or in the presence of PAO which blocks receptor internalization. It appears that conditions that block receptor internalization enhance the interactions between receptors by preventing their separation in the course of their endocytic itinerary. The formation of heterodimers and cross-phosphorylation of K721A by the active receptor kinase could potentially modulate function and intracellular routing of the two receptor species.

Is autophosphorylation of EGFR involved in receptor routing? We have previously examined whether autophosphorylation plays a role in the regulation of intracellular trafficking by studying transfected cells that express mutant EGFR in which autophosphorylation sites were substituted by phenylalanine (Y1173F, Y1148F, Y1068F) or were removed by COOH-terminal truncations. For example, CD63 and CD126 mutants lack 2 and 4 autophosphorylation sites, respectively. Cell lines expressing these mutants were tested for ligand internalization, receptor downregulation, and ligand-induced receptor degradation (10, 11). We have shown that EGFR with altered autophosphorylation sites behaved like wt receptor with respect to receptor trafficking. Hence, autophosphorylation per se does not provide a "flag" to distinguish between receptors targeted to the lysosome and receptors destined to return to the cell surface for reutilization.

To further explore the factors that regulate receptor trafficking, we have generated transfected cells that express K721A mutants together with CD63. The CD63 mutant was used in this analysis because it behaves like wt receptor with respect to internalization, downregulation, and biological responsiveness but allowed an immunological distinction between receptor bearing an active kinase and kinase-defective mutant (K721A). In K721A/CD63 cells, the ratio of the two mutant receptors was determined by immunological means, using anti-C antibodies that recognize all three receptor types. Several clones, each expressing different amounts of both mutant receptors, were selected for further characterization. The K721A/CD63 cells bound ligand with similar affinity as cells expressing either wt or K721A alone and were responsive to EGF stimulation and DNA synthesis (12).

Although the efficiency of ligand internalization by transfected cells expressing wt, CD63, K721A, and K721A/CD63 did not differ significantly, surface receptor downregulation was less marked in K721A/CD63 cells as compared with CD63 cells or cells expressing wt receptors (Figs. 2 and 3). Depending on the ratio of the two receptor species, different lines of K721A/CD63 showed a behavior intermediate to that of CD63 or K721A when expressed alone. In these cell lines, EGF-induced receptor downregulation plateaued at higher levels of surface binding as compared with downregulation of wt or CD63 alone.

We followed the fate of the two receptor species expressed in the same cells with methods able to distinguish between these two mutants. Analysis of receptor degradation in response to EGF stimulation indicated that the two types of receptor retained their particular behavior even when coex-
pressed in the same cells. The presence of CD63 did not alter the half-life of the kinase-negative mutant, nor was the degradation rate of CD63 receptor slowed down by the presence of the K721A mutant receptor. Since coimmunoprecipitation, cross-phosphorylation (12), and covalent cross-linking experiments all indicated that the mutants are able to form mixed oligomers, it is unlikely that the two mutants receptors use different pathways of internalization. The two receptor types encounter each other at least when embedded in the plasma membrane. However, at some later stage in the endocytic pathway, the two receptor mutants clearly follow separate destinies. The mechanism that governs the itinerary of the two mutant receptors must operate at the molecular level as it distinguishes between receptor molecules according to their intrinsic kinase activities. The most plausible mechanism is that during endocytosis, the activated receptors phosphorylate a "sorting substrate(s)" which directs the activated receptors toward a degradation pathway. The kinase negative mutant that cannot phosphorylate the sorting substrate is delivered back to the cell surface by a constitutive recycling mechanism. In other words, the kinase activity prevents the return of EGFR to the cell surface by constitutive recycling mechanism, which is used by receptors lacking tyrosine kinase activity such as LDL receptor or transferrin receptor (5).

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