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Lysosomal Targeting of Epidermal Growth Factor Receptors via a Kinase-dependent Pathway Is Mediated by the Receptor Carboxyl-terminal Residues 1022–1123*

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Binding of epidermal growth factor (EGF) to its receptor induces rapid internalization and degradation of both ligand and receptor via the lysosomal pathway. To study the mechanism of intracellular sorting of EGF-EGF receptor complexes to lysosomes, NIH 3T3 cells transfected with wild-type and mutant EGF receptors were employed. The kinetics of ^{125}I-EGF trafficking was analyzed using low concentrations of the ligand to avoid saturation of the specific sorting system. The relative size of the pool of internalized ^{125}I-EGF-receptor complexes that were capable of recycling decreased as receptors traversed the endosomal system. The rate of ^{125}I-EGF sequestration from the recycling pathway correlated with the rate of ^{125}I-EGF transition from early to late endosomes as measured by Percoll gradient fractionation. Deletion of the last 63 amino acids of the EGF receptor cytoplasmic tail did not inhibit the process of sequestration and targeting to the late endosomes and lysosomes. Truncation of the 123 residues, however, resulted in impaired lysosomal targeting and increased recycling of EGF. Receptor mutant in which 165 residues were deleted displayed maximal ability to recycle and a minimal extent of sorting to the late endosomes. The data suggest that two regions of the EGF receptor molecule, residues 1022–1063 and to a lesser extent residues 1063–1123, contribute in the regulation of routing of EGF receptors to the degradation pathway. The kinase-negative receptor mutant recycled EGF more intensively compared with the wild-type receptor, and the transport of this mutant to late endosomes was inhibited. These results support the view that the receptor kinase activity is important for ligand-induced sorting of EGF receptors to the pathway of lysosomal degradation.

Binding of epidermal growth factor (EGF)[1] to its receptor results in acceleration of endocytosis and down-regulation of receptors (1–3). Two regulatory steps distinguish endocytosis of EGF receptors and other signaling receptors from the endocytic trafficking of receptors that function as nutrient carrier, for example, transferrin receptors. First, the efficient internalization of signaling receptors via clathrin-coated pits requires activation by the ligand binding. Second, a significant pool of internalized growth factor receptors escape recycling and are sorted to lysosome-degradation pathway (reviewed in Refs. 4 and 5).

Whereas molecular signals involved in coated pit endocytosis and the main elements of endocytic apparatus are largely known, the mechanism by which internalized EGF-activated receptors are routed to the lysosomal pathway is less well understood. In earlier studies it was proposed that the receptor activation by EGF prevents receptor recycling from endosomes (2, 6, 7). Later, it was found that EGF does not dissociate from the receptor in early endosomes (9–12), and that the significant pool of internalized EGF-receptor complexes are rapidly recycled (13–17). Analysis of post-endocytic trafficking of EGF-receptor complexes in comparison with that of transferrin receptor suggested that the differences in the ability of these receptors to recycle are only quantitative (15).

Morphological studies demonstrated sequential appearance of labeled EGF and its receptors in "early" peripheral endosomal compartments and "late" perinuclear/juxtanuclear multivesicular endosomes (MVE) (2, 8, 9, 18, 19). During the passage through endosomal compartments, EGF and its receptors tend to accumulate in the vesicular parts of endosomes, particularly in the internal membrane structures of MVE (9, 19, 20). The exclusion of EGF-receptor complexes from tubular extensions of endosomes is thought to result in the sequestration of these complexes from the recycling pathway (9, 19, 20).

It is believed but not proven that recycling is a default pathway of receptors from endosomes, whereas lysosomal targeting requires interaction of receptor sequences with the specific elements of endosomal sorting machinery. Sequences responsible for the EGF-induced lysosomal targeting of EGF receptors have not been precisely mapped. It was previously proposed that residues 945–958 are important for degradation of EGF receptors (21). This domain was sufficient to support rapid degradation of the receptor lacking the entire core kinase domain regardless of the presence of EGF (21). However, studies of ^{125}I-EGF trafficking suggested that the region of the EGF receptor distal to residue 1022 is critical for the retention in

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[1] The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; PNS, postnuclear supernatant; TES, triethanolamine buffer; MVE, multivesicular endosomes; WT, wild-type; KN, kinase-negative mutant.

endosomes and degradation of ^{125}I-EGF (16).

There is still disagreement on whether tyrosine kinase activity of the EGF receptor plays a role in lysosomal targeting. Ultrastructural studies of the EGF receptor endocytosis demonstrated the importance of kinase activity for the receptor sorting to a non-recycling compartment, *i.e.* internal structures of MVE (20). Kinetics studies by Wiley and co-workers (15, 17), however, revealed no role for kinase in the receptor transport to late endosomes and degradation. Another study of EGF recycling at different levels of receptor occupancy showed that the regulatory role of receptor tyrosine kinase can be revealed at low EGF concentrations when lysosome-targeting apparatus is not saturated (16).

In this study we have analyzed the role of kinase activity and carboxyl-terminal regions of the EGF receptor in the sorting of EGF-receptor complexes to the recycling or degradation pathways in cells exposed to low concentrations of EGF. The data indicate that inactivation of kinase abolishes lysosomal targeting of EGF receptors. The regions of the carboxyl terminus between residues 1022 and 1063 and, to a lesser extent, residues 1063 and 1123 are important for the sequestration of EGF-receptor complexes from the recycling compartments.

EXPERIMENTAL PROCEDURES

Materials—Mouse receptor-grade and human recombinant EGF were obtained from Collaborative Research Inc. Mouse receptor-grade ^{125}I-EGF was prepared as described elsewhere (1). The specific activity of preparations varied from 1×10^5 to 2×10^5 cpm/ng. Chemicals were from Sigma, Fisher, and Pharmacia Biotech, Inc.

EGF Receptor Mutants and Cell Culture—Human EGF receptor mutants were obtained using site-directed mutagenesis of human EGF receptor to mutate Lys-721 to alanine (KN, kinase-negative mutant) or to insert a premature stop codon for different carboxyl terminus truncation mutants, as described previously (22–25). The receptor mutant constructs are depicted in Fig. 1. Mouse NIH3T3 cells, which contain about 5,000 endogenous EGF receptors/cell, were used for transfections. Transfections and G418 selection were performed as described previously (22–25). As determined by quantitative ^{125}I-EGF binding, cells expressing wild-type receptors (clone pCO 11) and deletion mutants Dc63 and Dc123 had $1.0–1.5 \times 10^5$ EGF receptors/cell. KN receptor and the deletion mutant Dc165 were expressed at the level of 0.7×10^5 and $2.5–3.0 \times 10^5$/cell, respectively. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes, 10% newborn calf serum with antibiotics and glutamine.

Kinetics of Recycling and Degradation of ^{125}I-EGF—Cells on 35-mm dishes were washed with binding medium (DMEM, 20 mM HEPES, 0.1% bovine serum albumin) and incubated with 1 ng/ml ^{125}I-EGF at 4 °C for 1 h. The cells were then washed with cold DMEM and allowed to internalize receptors for 5–60 min at 37 °C, followed by one rinse with cold DMEM. ^{125}I-EGF that had not been internalized during the 37 °C incubation was removed from the cell surface by a 2.5-min acid wash (0.2 M sodium acetate, 0.5 M NaCl, pH 4.5) as described (13, 14). At this point the cells are referred to as "^{125}I-EGF-loaded cells."

Cells were then incubated with 100 ng/ml unlabeled EGF at 4 °C for 1 h to saturate surface receptors. Trafficking of ^{125}I-EGF-receptor complexes in the loaded cells was initiated by incubating the cells at 37 °C for 0–60 min in binding medium with unlabeled EGF (100 ng/ml). Excess of unlabeled EGF in the medium and at the surface minimized re-binding and re-internalization of recycled ^{125}I-EGF. At the end of the chase incubation, the medium was collected to determine the amount of intact and degraded ^{125}I-EGF, and cells were subjected to a harsh acid wash (pH 2.8) to determine the amount of surface-bound ^{125}I-EGF. Finally, cells were solubilized in 1 N NaOH to determine the amount of intracellular ^{125}I-EGF.

The intact ^{125}I-EGF in the chase medium was separated from low molecular weight products of ^{125}I-EGF degradation by precipitation with the mixture of trichloroacetic acid and phosphotungstic acid as described previously (14). Briefly, trichloroacetic acid/phosphotungstic acid were added to the medium to a final concentration 3%/0.3%, and the mixture was incubated for 30 min at 4 °C and centrifuged to bring down protein precipitates. Supernatants and precipitates, solubilized in 1 N NaOH, were used to determine the amount of degraded and intact ^{125}I-EGF, respectively.

The amount of recycled ^{125}I-EGF was calculated by summing the radioactivity appeared on the cell surface and in the medium (trichloroacetic acid-precipitable) during chase incubation, and expressed as fraction of the total radioactivity present in the cell and medium. The amount of degraded ^{125}I-EGF was calculated as percent of trichloroacetic acid-soluble radioactivity of the total radioactivity present in the cell and medium. Trichloroacetic acid precipitation analysis of intracellular radioactivity revealed only negligible amounts of degraded ^{125}I-EGF.

Percoll Gradient Fractionation—Cell were grown on 100-mm dishes and used when confluent. Before experiments the cells were rinsed two times in binding medium and incubated in binding medium for 30 min at 37 °C. The cells were endosome-loaded with ^{125}I-EGF (1–2 ng/ml) for 5–60 min as described above for recycling experiments, and the surface ^{125}I-EGF was removed by mild acidic wash. Acid-treated cells were then washed twice with DMEM, one time with cold phosphate-buffered saline, and processed for Percoll gradient fractionation as described previously (26, 27). Briefly, the cells were scrapped from the plate in 1 ml of TES buffer (10 mM triethanolamine, 1 mM EDTA, 0.25 M sucrose, pH 7.2) with rubber policeman, pipetted 40 times with a 1-ml Gilson Pippetman, and adjusted to 2 ml with TES and centrifuged at 900 rpm for 10 min using Beckman rotor GH-3.8. The postnuclear supernatant (PNS) was removed, 2 ml of TES was added to the pellet, and pipetting and centrifugation were repeated as with initial homogenate. The two PNS were collected and combined with the stock suspension of Percoll (9 parts of Percoll and 1 part of 2.5 M sucrose) so that the final concentration of Percoll was 17%. 12-ml gradients were formed by centrifugation at $50{,}000 \times g$ for 25 min in a rotor Ti65 (Beckman). Density of gradients was monitored by Density Marker Beads (Pharmacia). Gradients were fractionated from the bottom into 20 fractions, and the radioactivity of fractions was measured.

The ratios of radioactivities in heavy (late endosomes plus lysosomes) and light fraction peaks (early endosomes) ($R_{H/L}$) were calculated using GRADIENT software generously provided by N. Aksenov (Institute of Cytology of Academy of Sciences, St. Petersburg, Russia). Total radioactivity of PNS layered on gradient was calculated by summing radioactivity of all 20 fractions. To avoid differences between experiments arising from the different degrees of cell disruption, the radioactivity in the pellet (unbroken cells) was taken into account in all calculations assuming that the distribution of ^{125}I-EGF in unbroken cells is the same as in PNS. Radioactivity of each fraction was corrected for nonspecific (free non-membrane bound) radioactivity by subtracting the constant component (minimal radioactivity per fraction among all fractions of a given gradient). This component was probably due to ^{125}I-EGF released from the receptor during homogenization and centrifugation and was typically not more than 10–20% of the maximal value of radioactivity/fraction. The corrected radioactivities of the fractions were expressed as percent of the total cell-associated and medium radioactivity. Such normalization allowed us to compare results obtained with cell lines expressing different levels of EGF receptors. The value of $R_{H/L}$ was estimated for each gradient by dividing the sum of radioactivity in the fractions in the first peak to that of the second peak. The border of peaks was considered to be the fraction with minimal radioactivity (typically fractions 6–8) that was included in the first peak.

The 17% gradient is not linear at densities higher then 1.050 g/ml. The linearity in a density range of 1.05–1.10 g/ml can be achieved, however, by using a higher (≈28%) concentration of Percoll. Therefore, to separate lysosomes (p = 1.07–1.11 g/ml) and late endosomes (p = 1.048–1.060), the first 7 fractions obtained from 17% Percoll gradient (peak of heavy endosomes and lysosomes) were collected, the volume was adjusted to 12 ml with 34% Percoll solution, and the samples were centrifuged as described for a 17% gradient. The ratio of ^{125}I-EGF in a heavy peak (lysosomes) to that in a lighter peak (late endosomes) of a 28% gradient was calculated as described above for a 17% gradient. Positions of organelles in the 17% gradient were identified by marker enzymes 5′-nucleotidase (plasma membrane), NADPH-cytochrome *c* reductase (endoplasmic reticulum), UDP-galactosyltransferase (Golgi apparatus), acid phosphatase, and *N*-acetyl-β-D-glucosaminidase (lysosomes) as described previously (26, 27). The specific activity of three lysosomal marker enzymes, acid phosphatase, *N*-acetyl-β-D-glucosaminidase, and cathepsin D was 2–7-fold higher in the lysosome compared with late endosome peaks of the 28% gradient.

RESULTS

Recycling of ^{125}I-EGF-Receptor Complexes at Different Points of the Endocytic Pathway—In our previous studies, rapid recycling of internalized ^{125}I-EGF-receptor complexes in A-431 and NIH 3T3 cells has been demonstrated under conditions of subsaturation or saturation of receptors with ^{125}I-EGF (13, 14).

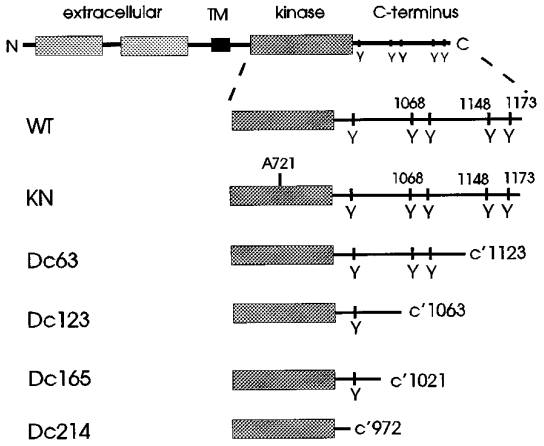

FIG. 1. **Schematic representation of the wild-type and mutant EGF receptors.** Wild-type EGF receptor (*WT*) drawn with the extracellular, transmembrane (*TM*) and intracellular domain (*kinase* and *C-terminus*). Major tyrosine phosphorylation sites (residues 992, 1068, 1086, 1148, and 1173) are indicated for the wild-type receptor. Deletion mutants have carboxyl-terminal truncations of 63 (*Dc63*), 123 (*Dc123*), 165 (*Dc165*), and 214 amino acid residues (*Dc214*). In kinase-negative mutant (*KN*), lysine 721 is mutated to alanine.

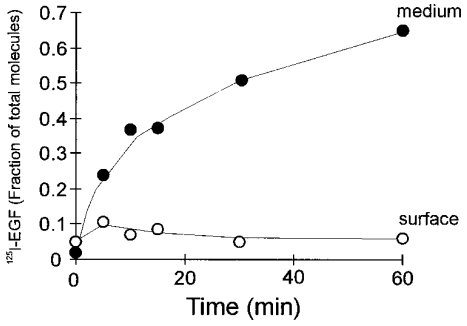

FIG. 2. **Recycling of ^{125}I-EGF in WT cells.** NIH 3T3 cells expressing wild-type receptors were incubated with 1 ng/ml ^{125}I-EGF at 4 °C for 1 h, then for 5 min at 37 °C, and ^{125}I-EGF that had not been internalized was removed by the acid wash. ^{125}I-EGF-loaded cells were incubated with unlabeled EGF for 1 h at 4 °C, and then incubated with unlabeled EGF at 37 °C for indicated periods of time. At the end of chase incubation, the amount of medium intact and degraded ^{125}I-EGF, surface and intracellular radioactivity was determined as described under "Experimental Procedures." The sum of intact medium and surface ^{125}I-EGF was expressed as fraction of the total radioactivity in the cell and medium at each time point of the chase incubation. The increase in this fraction compared with *time 0* corresponds to recycled ^{125}I-EGF.

Hence, we examined the dynamics of ^{125}I-EGF recycling in NIH 3T3 cells expressing wild-type EGF receptors (WT) in the presence of low concentrations of ^{125}I-EGF, so that the internalization system is not saturated. Cells were incubated with 1 ng/ml ^{125}I-EGF at 4 °C and allowed to internalize ^{125}I-EGF-receptor complexes for 5 min at 37 °C. ^{125}I-EGF that had not been internalized during 37 °C incubation was removed from the cell surface by mild acid wash as described under "Experimental Procedures." In^{125}I-EGF-loaded cells more than 90% of the total cell-associated ^{125}I-EGF was acid-resistant, *e.g.* intracellular (Fig. 2, *time 0*).

Loaded cells were then incubated with 100 ng/ml unlabeled EGF at 4 °C for 1 h to occupy surface receptors, and trafficking of ^{125}I-EGF-receptor complexes was initiated by incubating the cells at 37 °C for 0–60 min with unlabeled EGF. Excess of unlabeled EGF in the medium and at the surface prevented re-binding and re-internalization of recycled ^{125}I-EGF. During a 37 °C chase incubation, ^{125}I-EGF reappeared on the cell surface and in the medium (Fig. 2). Typically, the amount of recycled ^{125}I-EGF reached maximum (~60–65% of total loaded pool) after 1 h of 37 °C incubation (Fig. 2). Therefore, 60–65% of ^{125}I-EGF-receptor complexes, which had been internalized during 5 min, can return to the surface at least one time. In contrast, less than 1% of endosome-loaded ^{125}I-EGF degraded after 1 h of incubation as determined by trichloroacetic acid precipitation technique. Apparently, ^{125}I-EGF-receptor complexes are recycled faster than sorted to the degradation pathway. In addition, endosomes are rapidly saturated during the chase incubation with unlabeled EGF, which could reduce the rate of ^{125}I-EGF degradation (16).

Does the population of ligand-receptor complexes capable of recycling change as the receptors traverse the endosomal system? To address this question, the cells were allowed to internalize ^{125}I-EGF for 5, 15, 30, or 60 min at 37 °C, and the percent of ^{125}I-EGF capable of recycling relative to the total internalized ^{125}I-EGF was measured by incubating the cells with the excess unlabeled EGF at 4 °C and then at 37 °C for 1 h as described in Fig. 2. As shown in Fig. 3, the pool of recyclable ^{125}I-EGF decreased with the increase of the time of internalization. When ^{125}I-EGF was internalized for 1 h, only 14–16% of the endosomal pool escaped endosomes as intact ligand during the following recycling assay (1-h chase incubation with unlabeled EGF). These experiments demonstrated gradual sequestration of the receptors from the recycling pathway with prolonged internalization. As stated before, ^{125}I-EGF that was retained from recycling was degraded with the slow rate. Trichloroacetic acid precipitation of the chase medium revealed that 19.9 ± 4.7% (S.E.) of the total ^{125}I-EGF in 1-h loaded cells was degraded after a 1-h chase incubation with unlabeled EGF.

Deletion of the Receptor Carboxyl Terminus and Mutational Inactivation of the Kinase Affect Sequestration of EGF from Recycling—Experiments with cells expressing wild-type receptors revealed sequestration of ^{125}I-EGF from the recycling pathway during endocytosis. To identify the region of the receptor important for this process, the extent of recycling was measured in cells expressing EGF receptor deletion mutants presented in Fig. 1. All mutant receptors tested were capable of intensive ^{125}I-EGF recycling (>60%) after short times of internalization (5 min) (Fig. 3). However, after internalization of ^{125}I-EGF for 15 min or longer periods of time, the extent of ^{125}I-EGF recycling decreased with different rate in cells expressing WT and mutant receptors. The Dc63 mutant recycled ^{125}I-EGF with slightly lower efficiency than WT receptor (Fig. 3). In contrast, truncation of additional 60 residues (Dc123 mutant) resulted in elevated recycling as compared with WT and Dc63 mutant. Dc165 mutant displayed a maximum ability to recycle compare with other mutants at all stages of endocytosis. A larger truncation of the carboxyl terminus in Dc214 mutant did not lead to further acceleration of recycling. Therefore, a region of the EGF receptor responsible for the retention of EGF-receptor complexes from recycling is located between residues 1022–1123. Furthermore, the extent of recycling of the receptor mutant with mutationally inactivated kinase (KN) was within the range of that observed for Dc123 and Dc165 receptors. Therefore, kinase activity of the receptor appears to be important for re-routing of EGF-receptor complexes from the recycling to the degradation pathway.

A maximum of 20% of ^{125}I-EGF, internalized during a 1-h incubation, was degraded after a subsequent 1-h chase incubation with unlabeled EGF in WT (19.9 ± 4.7%), KN (19.1 ± 4.0%), and Dc123 cells (18.2 ± 4.1%), whereas the degradation was slower in cells expressing Dc165 receptor (11.4 ± 0.4%) after a 1-h chase incubation. The degradation of ^{125}I-EGF was 2-fold slower in cells expressing Dc63 receptor (10.1 ± 2.9%) than in WT cells. We currently do not have the explanation for the reduced rate of EGF proteolysis in Dc63 cells, although an

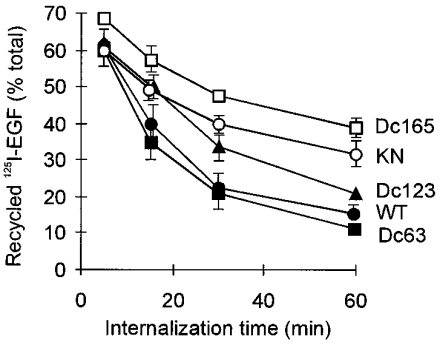

FIG. 3. **The pool of ^{125}I-EGF-receptor complexes capable of recycling depends on the time of endocytosis.** Cells expressing WT and mutant EGF receptors were allowed to internalize 1 ng/ml ^{125}I-EGF for 5, 15, 30, or 60 min, and the remaining surface ^{125}I-EGF was removed by the mild acid wash as described in Fig. 2. The ^{125}I-EGF-loaded cells were then incubated with unlabeled EGF at 4 °C, followed by 1 h at 37 °C to measure the fraction of ^{125}I-EGF capable of recycling. The recycled ^{125}I-EGF was calculated as the increase in the fractions of intact medium and surface ^{125}I-EGF during a 1-h chase incubation.

increased accumulation of ^{125}I-EGF in lysosomes of these cells is described below. In control experiments, the degradation of platelet-derived growth factor-BB was identical in WT and Dc63 cells (data not shown). Importantly, analysis of the pulse-chase recycling experiments (Figs. 2 and 3) suggests that the fastest process, recycling of preloaded ^{125}I-EGF, is the major parameter that determines the efficiency of intracellular sorting. In this type of experiment, the endosomal pool of ^{125}I-EGF-receptor complexes rapidly splits into recycled and endosome-retained pools. The latter pool appears to be degraded with a slow rate independently of the receptor kinase activity and carboxyl terminus.

Transition of ^{125}I-EGF from Early to Late Endosomes—The experiments presented in Figs. 2 and 3 demonstrated that the partition of internalized ^{125}I-EGF-receptor complexes in recycling is dependent on the duration of endocytosis and is regulated by the receptor kinase and carboxyl-terminal sequences. We further examined whether the ability of the EGF-receptor complexes to recycle correlates with the rate of their transition from the early to the late endosomal compartments. Centrifugation of the cellular membranes on 17% Percoll gradients separates early and late endosomes/lysosomes, which peak in the light (1.035–1.042 g/ml) and heavy fractions of the gradient (1.048–1.070 g/ml), respectively (26–28). In previous experiments, we observed the accumulation of labeled transferrin, a marker of early endosomes, exclusively in the low density fractions (29) as well as the block of EGF transition to the high density fractions at 18 °C (data not shown). These observations strongly support the notion that the peak of radioactivity in the heavy fractions of the gradient contains late endosomes and lysosomes.

^{125}I-EGF (1–2 ng/ml) was internalized for 15–60 min after prebinding of the ligand at 4 °C as described in recycling experiments (Fig. 3). After removal of the surface ^{125}I-EGF by an acidic buffer wash, the ^{125}I-EGF-loaded cells were homogenized and subjected to gradient fractionation. As seen in Fig. 4, most of ^{125}I-EGF was associated with light endosomal fractions after 15 min of continuous internalization at 37 °C in cells expressing WT and mutant receptors. Although plasma membrane and early endosome fractions sediment at a very similar density, the radioactivity in the light peak was considered as internalized ^{125}I-EGF because the surface ^{125}I-EGF was removed by the acid wash prior to cell homogenization. In all cell lines, the ^{125}I-EGF-receptor complexes in the early endosome fractions could be chemically cross-linked with the efficiency similar to that for the plasma membrane complexes (data not shown). This observation supports the view that EGF does not significantly dissociate from the receptor at the early stages of endocytosis (9–12).

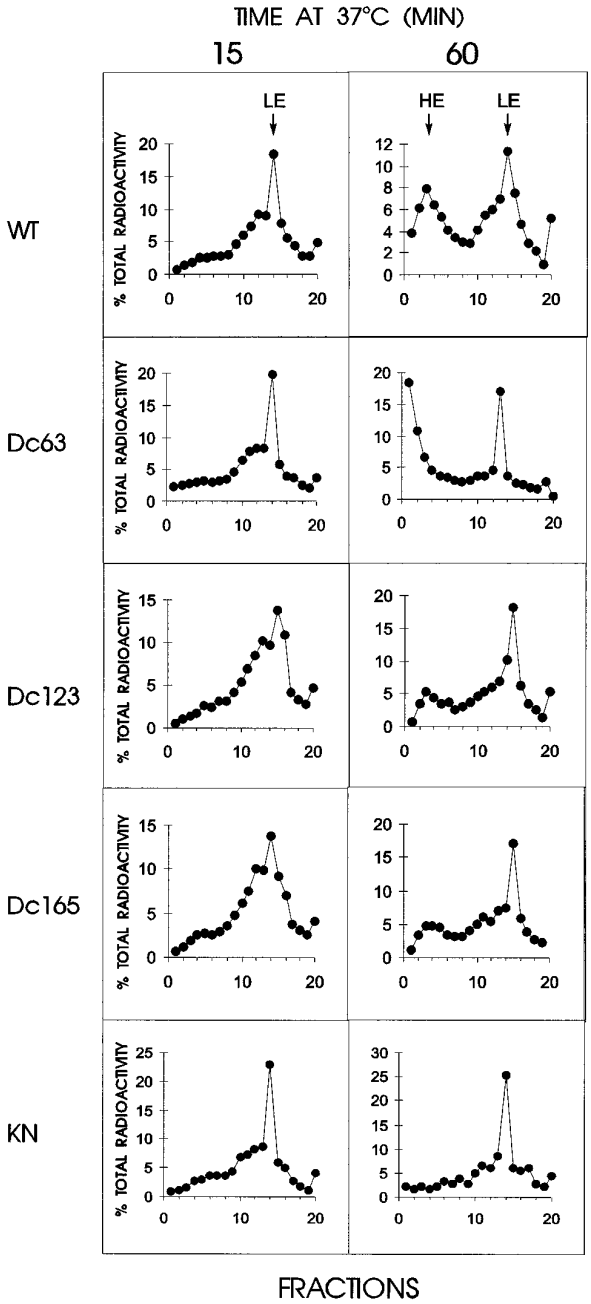

FIG. 4. **Transition of ^{125}I-EGF from early to late endosomes.** Cells expressing WT and mutant EGF receptors were incubated with 2 ng/ml ^{125}I-EGF at 4 °C and then for 15 or 60 min at 37 °C without EGF. The surface ^{125}I-EGF was then removed by the mild acid wash. The cells were homogenized and subjected to centrifugation on 17% Percoll. The radioactivity of the fractions was measured and expressed as percentage of the total radioactivity present in the cell and medium as described under "Experimental Procedures." *Arrows* indicate the position of the light (*LE*) and heavy endosome fractions (*HE*).

With prolonged periods of endocytosis (30–60 min) in WT and Dc63 cells, the significant amount of the ligand accumulated in heavy fractions that comprise late endosomes and lysosomes (Fig. 4). In contrast, very little ^{125}I-EGF was detected in heavy fractions after 1 h of continuous endocytosis in cells expressing Dc165 and KN receptor. The redistribution of the label from the early to late endosomal fractions was also significantly delayed in Dc123 compared with WT cells.

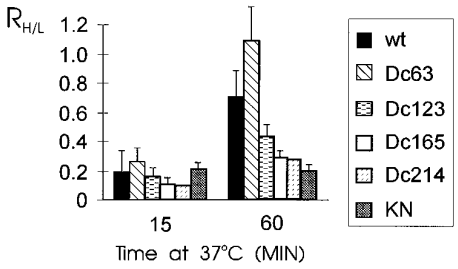

FIG. 5. **The ratio of ^{125}I-EGF in heavy and light peaks of the gradient ($R_{H/L}$) in WT and mutant EGF receptor cells.** The values of $R_{H/L}$ were calculated from the fraction distribution data obtained by Percoll subcellular fractionation of cells that were allowed to internalized ^{125}I-EGF for 15 and 60 min at 37 °C as described under "Experimental Procedures." $R_{H/L}$ represents mean value from 3–5 experiments performed as in Fig. 4.

The amount of ^{125}I-EGF in light and heavy peaks was estimated from gradients obtained in several experiments performed as in Fig. 4. The quantitation of the ratio of radioactivity in heavy and light fractions, $R_{H/L}$ (Fig. 5), revealed that the extent of EGF recycling (Fig. 3) inversely correlated with this ratio. The effective targeting of ^{125}I-EGF to late compartments in WT and Dc63 cells resulted in rapid sequestration of the receptor complexes from the recycling pathway. Therefore, the elevated recycling of the Dc123, KN and Dc165 mutants could be due to inefficient transport of EGF-receptor complexes to late endosomes. All together results of kinetics and Percoll fractionation experiments suggest that the domain between residues 1023–1123 is important for targeting of EGF receptors to kinase-dependent lysosomal pathway.

As seen in Fig. 5, the $R_{H/L}$ is higher in Dc63 than in WT receptor cells. The accumulation of ^{125}I-EGF in heavy fractions of the gradient in Dc63 cells was accompanied with the slower rate of ^{125}I-EGF degradation in these cells compared with WT cells. Therefore, we examined whether the degradation of ^{125}I-EGF in Dc63 receptor cells was impaired at the late stages of endocytosis. The distribution of ^{125}I-EGF between late endosomes and lysosomes in WT and Dc63 was compared by centrifugation of heavy membrane fractions obtained from the first 17% Percoll gradient on a 28% Percoll gradient (Fig. 6). In both cell lines, WT and Dc63, there was an accumulation of radioactivity in heavy fractions of the secondary gradient that correspond to the mature lysosomes in NIH 3T3 cells. The ratio of lysosomes and late endosomes was much higher in Dc63 cells (1.57 ± 0.55) than in WT cells (0.65 ± 0.20). Moreover, the peak of ^{125}I-EGF in heavy lysosome fractions in Dc63 could be detected as early as after 30 min of internalization. Therefore, both the rapid sorting from early compartments and the inefficient proteolysis of ^{125}I-EGF to low molecular weight products appear to result in accumulation of ^{125}I-EGF in lysosomes in these cells. However, for the main purpose of this study, *i.e.* the identification of the receptor sorting domain, it is important that the transition from early to late endosomes is not inhibited by the truncation of 63 amino acids of the receptor tail.

DISCUSSION

In this paper we demonstrate that the sorting to the late endosomal compartment and the sequestration of EGF from the recycling pathway is regulated by both the carboxyl-terminal domain and the tyrosine kinase activity of the EGF receptor. These observations, therefore, reveal the similarity in the regulation of the two key steps of EGF-induced endocytosis: internalization and lysosomal targeting of the receptor. The importance of receptor activation by EGF, as well as the presence of receptor regulatory regions distal to the core kinase domain in endocytosis, is generally acknowledged, although

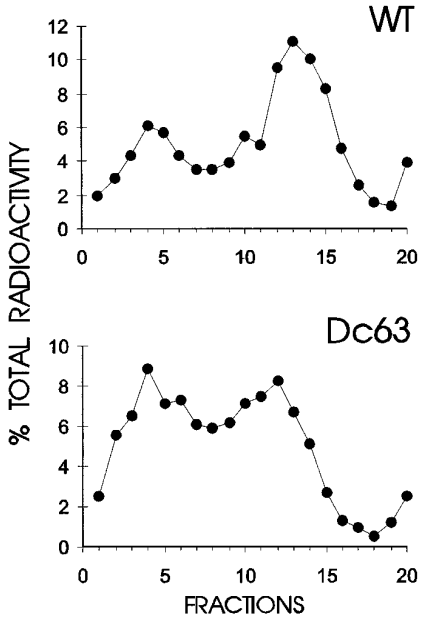

FIG. 6. **Accumulation of ^{125}I-EGF in lysosomes of WT and Dc63 cells.** The fractions of radioactivity corresponding to a heavy peak of the 17% Percoll gradients were combined and subjected to centrifugation on 28% Percoll to resolve late endosomes (peak in *fractions 11–14*) and lysosomes (peak in *fractions 2–4*).

not necessarily well understood at more than a superficial level. One source of confusion is the limited capacity or saturability of the endocytic pathways of EGF-bound receptors. Because of the saturation of internalization (30, 31) and intracellular sorting steps (16), different mechanisms regulate these processes when low or high numbers of receptors are activated by EGF. For instance, the role of EGF receptor kinase activity in internalization could be appreciated only at low levels of receptor occupancy by EGF (31–33). Therefore, studies of the specific mechanisms of ligand-dependent lysosomal targeting required analysis of endocytosis parameters under physiological conditions of low levels of receptor occupancy.

Hence we have used low concentrations of EGF to stimulate cells expressing moderate levels of receptors. Under these conditions, the ability of ligand-receptor complexes to recycle is higher in cells expressing KN receptor than in cells with WT receptor (Fig. 3). Previous studies of recycling of EGF-receptor complexes in B82 cells suggested that the rate of EGF recycling and transition to late compartments was not dependent on kinase activity of the receptor (15, 17). However, in latter reports, the conditions appeared to favor the saturation of the sorting system. Correspondingly, in our experiments there was no difference in the extent (Fig. 3) and the rate of the recycling (data not shown) of EGF in WT and KN cells that internalized EGF for 1–5 min at 37 °C. In contrast, when the extent of recycling was measured in cells loaded with low concentration of EGF (0.17–0.34 nM) for 15–60 min (Fig. 3) or for 2 h to a steady-state EGF distribution (16), the enhanced recycling was observed in cells expressing KN receptor mutant. The kinetics experiments are supported by the data of the subcellular fractionation analysis, which demonstrated a very slow accumulation of ^{125}I-EGF in late endosomes of KN cells (Figs. 4 and 5).

So far it is not known whether the lysosomal sorting system of other growth factor receptors is saturable. However, it has been shown that kinase activity is important for the rapid degradation of the PDGF β receptor and the c-*fms* receptor (34, 35). In both these studies the levels of receptor expression were low, and the receptor half-lives were significantly shorter than

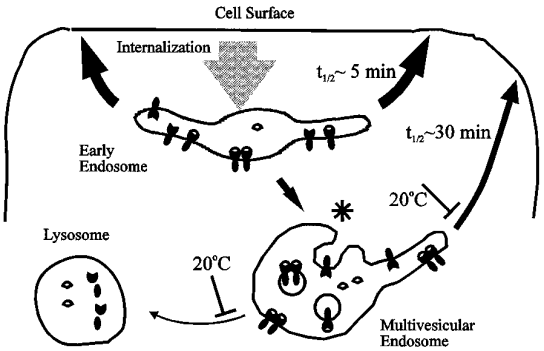

FIG. 7. **Pathways of intracellular sorting of EGF-receptor complexes.** In early endosomes, a large pool of EGF-receptor complexes are rapidly recycled. During each round of internalization, 35–40% of receptor complexes are recruited to MVE. EGF-receptor complexes are also recycled from MVE, however, each cycle is significantly longer than that from early endosomes. Receptor complexes that are incapable of recycling remain in MVE, mostly within internal membrane structures. MVE fuse with lysosomes directly, or, alternatively, receptors are transported to lysosomes through late endosomes (prelysosomal compartment). Both recycling and transport to lysosomes of the receptors are blocked at low temperature (20 °C). *Asterisk* indicates the involvement of kinase activity and carboxyl terminus of the receptor in the receptor sorting in MVE. The width of the *arrows* approximately corresponds to the specific rates of the processes.

that of EGF receptors (14, 24), which might indicate that the endocytic system was not saturated.

In addition to enzymatic activity of the EGF receptor, the carboxyl-terminal sequences distal to the core kinase domain of the receptor are critical for the proper trafficking of the receptor (21, 32). During preparation of this manuscript, Kurten and co-workers (36) reported that the region 945–958 of the EGF receptor contains endosome-retention signal and is involved in receptor interaction with the novel sorting protein. Based on experiments with mutants lacking the entire kinase domain, it has been proposed that this region does not function in the unoccupied EGF receptor due to conformational restrains by the kinase domain (21, 36). It is not clear, however, whether sequence 945–958 mediates EGF-dependent lysosomal sorting of the full-length receptor. On the other hand, studies of EGF recycling at steady-state conditions reveal that receptor mutant truncated at residue 1022 is recycled with higher efficiency than WT receptor (16). This difference could be observed only if small number of EGF-occupied receptors is internalized. Our studies indicate that the domain between residues 1022 and 1123 is required for the rapid sorting of EGF-receptor complexes to late endosomal compartments to prevent their effective recycling from early endosomes. Within this 100-amino acid region, sequence 1022–1063 appears to contribute more significantly in sorting than residues 1063–1123. The enhanced recycling of Dc123 mutant compared with WT receptors can be in part due to the slow internalization and, therefore, delayed transition of the Dc123 mutant through the early endosomes (24, 32). Since internalization of Dc165 receptor is identical to that of WT receptor (32),[2] the effect of truncation of residues 1022–1063 on the extent of recycling and transition of EGF to late endosomes should be strictly attributed to the defect in the intracellular sorting parameters.

The mechanism by which COOH-terminal sequences and kinase activity regulate lysosomal targeting of the growth factor receptors is still unclear. It is possible that tyrosine phosphorylation of one of endosomal proteins is necessary for the lysosomal targeting. Alternatively, receptor autophosphorylation may lead to the recruitment of proteins that mediate lysosomal targeting, or may yield the conformational changes within the receptor itself, allowing it to interact with sorting proteins. The latter view is supported by the observation that the lysosomal targeting of the KN mutant is not reduced to a minimal level displayed by the Dc165 receptor (Fig. 3). Whereas KN receptor lacks intrinsic kinase activity, some EGF-induced tyrosine phosphorylation of this mutant, presumably due to its association with another tyrosine kinase or to the endogenous mouse receptors, was detected in several studies (24, 37, 38). One example of a signaling molecule that is associated with autophosphorylation sites of the receptors is phosphotidylinositol 3-kinase, which is implicated in early-late endosome traffic (39). The role of this protein in lysosomal targeting of EGF receptors remains to be determined.

Finally, in the working model of intracellular sorting of EGF-receptor complexes presented in Fig. 7, we attempt to combine results of this report with as yet contradictory data on the role of EGF activation of its receptor in the sorting process. We suggest that rapid exchange between plasma membrane and early endosomes occurs immediately after endocytosis. This cycling of ligand-receptor complexes resembles retroendocytosis of insulin (40) and rapid recycling of asialoglycoprotein and transferrin (28, 41). The rapid recycling of EGF receptors ($t_{1/2} \leq 5$ min) is not regulated by kinase activity and carboxyl-terminal sequences of the receptor. The second pathway of recycling of EGF-receptor complexes is slower ($t_{1/2} \sim 30$ min), occurs in MVE, and is highly temperature-sensitive (13, 19, 20, 29). The sorting in MVE to the recycling pathway or nonrecycling compartments (internal structures of endosomes) can be regulated by the receptor kinase activity (20) and, possibly, carboxyl-terminal domain of the receptor (Ref. 16; this report). EGF-receptor complexes can recycle from MVE back to the plasma membrane directly or through a specialized late recycling compartment (42), a pathway also taken by transferrin receptor (29, 42). MVE can fuse with lysosomes (43), or, alternatively, EGF can be delivered to lysosomes via prelysosomal compartments (44), that probably correspond to the heavy endosomes in Percoll fractionation. Whereas EGF does not dissociate from the receptor in early endosomes and multivesicular endosomes (8–11), rapid release of EGF and degradation of the receptor is taking place in the prelysosomal compartments and lysosomes. Mapping the sorting domains of the receptor should make it possible to identify the proteins that interact with these domains in MVE and to assess the role of such interactions in the sorting process.

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Lysosomal Targeting of Epidermal Growth Factor Receptors via a Kinase-dependent Pathway Is Mediated by the Receptor Carboxyl-terminal Residues 1022-1123

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