Sorting of Ligand-activated Epidermal Growth Factor Receptor to Lysosomes Requires Its Actin-binding Domain*

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Ligand-induced down-regulation of the epidermal growth factor receptor (EGFR) comprises activation of two sequential transport steps. The first involves endocytic uptake by clathrin-coated vesicles, the second transfer of endocytosed EGFR from endosomes to lysosomes. Here we demonstrate that the second transport step requires a domain of the EGFR that encompasses residues 985–996 and was previously found to interact with actin. Deletion of domain 989–994 (Δ989–994 EGFR) did not interfere with EGFR uptake but completely abrogated its degradation. In contrast, both uptake and degradation were affected for K721A EGFR, a kinase-deficient EGFR mutant. To measure intracellular EGFR sorting, we developed a novel cell fractionation assay toward which cells were co-transfected for chicken hepatic lectin, a receptor for agialoglycoproteins. These cells were incubated with agialofetuin-coupled colloidal gold, which was targeted to lysosomes after receptor-mediated endocytosis. Compartmental within the lysosomal pathway gained buoyant density because of the presence of colloidal gold and could be isolated from cell homogenates by ultracentrifugation through a high-density sucrose cushion. In contrast to endocytosed wild type EGFR, both Δ989–994 EGFR and K721A EGFR were largely not retrieved in gold-containing endocytic compartments. These results are supported with morphological data. We conclude that sorting of endocytosed EGFR into the degradation pathway requires both its kinase activity and actin-binding domain.

Receptor tyrosine kinases elicit a diversity of cellular responses, including proliferation, apoptosis, differentiation, and migration. The epidermal growth factor receptor (EGFR),1 also named ErbB-1 or HER, is a member of the EGFR family of receptor tyrosine kinases that can, depending on the cell type and ligand, induce several of these distinct responses (1).

EGFR responses are initiated by the binding of epidermal growth factor (EGF) and other ligands that activate its intrinsic tyrosine kinase activity. EGFR activation stimulates, in addition to several intracellular signaling cascades, its clearance from the cell surface by clathrin-mediated endocytosis. After endocytic uptake, activated EGFR first enters sorting endosomes from where it is sorted to lysosomes for proteolytic degradation. Prior to its transfer to lysosomes, however, endocytosed EGFR continues to signal, and some EGFR substrates are in fact thought to associate primarily with endosomes (2–4). The combined sorting processes, recruitment into clathrin-coated pits at the plasma membrane and sorting from endosomes to lysosomes, result in effective down-regulation of activated EGFR. EGFR down-regulation is critically important for proper tissue development as illustrated by uncontrolled growth and metastasis of cancer cells that fail to down-regulate EGFR.

The molecular mechanism that regulates endocytic uptake of the EGFR is complex and still ill defined. Ligand-stimulated endocytosis requires endocytic codes within its cytoplasmic domain that are thought to be cryptic in unstimulated EGFR and exposed only upon ligand-induced EGFR autophosphorylation (5). For example, activated EGFR binds directly and stoichiometrically to the clathrin adaptor protein complex AP-2. This interaction, however, does not seem to be essential for rapid endocytosis (6–8). In addition to AP-2, src homology 2 and phosphotyrosine-binding domain-containing proteins, such as c-Cbl, Grb2, and Shc, have been proposed to participate in EGFR endocytosis (9–13). Finally, ubiquitination of the EGFR has been implicated in its endocytosis (14). After endocytic uptake and incorporation into sorting endosomes, EGFR/EGF complexes are recruited to vesicles that bud into the lumen of endosomes. Endosomes that have accumulated many such internal vesicles are referred to as multivesicular bodies (MVB). Incorporation of endocytosed EGFR into the vesicles within MVB is crucial to prevent recycling to the plasma membrane and is, like uptake, dependent on EGFR kinase activity (15–17). Sorting of EGFR into MVB also requires ubiquitination of its cytoplasmic domain (18). Ubiquitination is performed by c-Cbl, an enzyme that associates with autophosphorylated tyrosine residue 1045 of the EGFR. In addition to its ubiquitination site, multiple other domains at its carboxyl terminus, including a dileucine motif at position 679–680 (19), residues 1022–1063 and 1063–1123 (15) and 945–991 (20) are important determinants for intracellular EGFR sorting. Within the latter domain, a tyrosine-leucine motif (954–958) (21) interacts with SNX-1, a cytoplasmic and membrane-associated protein that participates in EGFR sorting to lysosomes (22) through its association with ESCRT I (endosomal sorting complex required for transport I) component Hrs (23). Distal from this domain,
residues 985–996, and more specifically residues 989–994, have been demonstrated to determine an F-actin binding site (24–26). The actin-binding domain has homology with actin binding sites of many other actin-binding proteins and is conserved in the EGFR family members ErbB2, v-erbB, and Xmrk. EGFR binding rapidly increases the fraction of cytoskeleton-associated EGFR (27). We anticipated that this association might drive EGFR down-regulation. In the current report we demonstrate that the actin-binding domain indeed plays a crucial role in sorting endocytosed EGFR to lysosomes.

**EXPERIMENTAL PROCEDURES**

**Generation and Maintenance of Cells**—Stable NIH-3T3 cell lines expressing either human wild type EGFR, K721A EGFR, or Δ989–994 EGFR are described elsewhere (25, 28). They were cultured in DMEM containing 10% fetal calf serum, penicillin/streptomycin, and 600 μg/ml Geneticin (G418; Invitrogen). The expression vector pCHL233 containing a cDNA clone for chicken hepatic lectin (CHL) (20) was generously provided by Dr. K. Drickamer (Columbia University, New York). An EcoRI fragment containing the cDNA for CHL was cloned into a single EcoRI site downstream of the SV40 origin of replication of the amplifiable expression vector pFRSV (30). Cells expressing EGFR or EGFR mutants were transfected with this construct and selected by gradually increasing the concentration of methotrexate (Pharmacia) to 50 μM. Stable clones that express 1.2–1.4 × 10^5 CHL and 1.3–1.7 × 10^5 (mutant) EGFR/cell, as determined by Scatchard analysis of surface-bound ^125I-agiallofetuin (AGOR) and ^125I-EGF, were selected and maintained in culture medium containing both 600 μg/ml Geneticin and 50 μM methotrexate. These clones homogeneously expressed CHL and (mutant) EGFR as determined by immunofluorescence microscopy and were used for all experiments, unless stated otherwise.

**Preparation and Labeling of Ligands**—Sialic acid was removed from fetuin (Sigma) and orosomucoid (Sigma) by incubating at 50 °C for 60 min in 50 mM HSO₄. Subsequently, galactose was removed from asialofetuin and orosomucosid by periodate oxidation followed by sodium borohydride reduction and mild acid hydrolysis (31), yielding agiallofetuin (AGF) and AGOR. AGOR, Fe³⁺-saturated transferrin (TI), and EGF (Invitrogen) were labeled with ^125I using iodo-beads (Pierce) according to standard procedures and separated from free ^125I on Sephadex PD 10 columns (Amersham Biosciences).

Colloidal gold (5 nm) was prepared principally as described (32). The gold sol was boiled for 1 h after the addition of 0.1% H₂O₂. The sol was then cooled down to room temperature, the pH set at 10–11 using 0.1 mM NaOH, and saturated with AGF. The pH was then lowered to 7.4 using 0.1 N HCl, after which BSA was added to 1 mg/ml. The sol was collected and washed with 0.1% H₂O₂ with 10 mM Hepes/NaOH, pH 7.4, 1 mg/ml BSA by ultracentrifugation. For uptake studies AGF-gold was diluted to a final optical density of 10 at 520 nm.

**EGF-induced Uptake and Degradation of EGFR**—Cells were seeded on 6-well plates and cultured for 1 day in medium containing 10% fetal calf serum, for a second day in medium containing 0.5% fetal calf serum, and then used for experiments. To measure EGFR uptake, cells were incubated in bicarbonate-lacking DMEM supplemented with 20 mM Hepes/NaOH, pH 7.5, 0.5% fetal calf serum, and then used for experiments. To measure EGFR degradation, cells were incubated in bicarbonate-lacking DMEM supplemented with 20 mM Hepes/NaOH, pH 7.5, 0.5% fetal calf serum, and then used for experiments. When indicated, the cells were washed and chased for 30 min in the absence of ligands. Endocytosis was stopped by washing the cells with phosphate-buffered saline at 4 °C. The cells were fixed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.8, for 30 min at 20 °C. Free aldehyde groups were quenched with 50 mM NH₄Cl, and the cells were permeabilized and immunolabeled in phosphate-buffered saline containing 1 mg/ml saponin and 20 mg/ml BSA. In stably transfected cells EGFR was labeled with Car5 (see above), transferrin receptor (TIR) with the mouse monoclonal antibody H88.4 (Zymed Laboratories Inc.), and LAMP-1 with rabbit polyclonal antibodies directed against its cytoplasmic domain (generously provided by Dr. C. Hopkins, University College, London, UK). Primary antibodies were detected with fluorescein isothiocyanate or Cy5-conjugated goat anti-rabbit IgG or Cy3-conjugated goat anti-mouse IgG (all from Jackson). Confocal sections of labeled cells were made using a Leica confocal laser scanning microscope (CLSM).

**Sorting Assay**—Cells were cultured on 6-cm gelatin-coated culture dishes, washed with prewarmed DMEM, 20 mM Hepes/NaOH, pH 7.5, 1% BSA, and incubated at 37 °C in the presence or absence of 5 nm AGF-gold (optical density of 10 at 520 nm; see above), 100 ng/ml ^125I-EGF or 100 ng/ml EGF, and, when indicated, 2 μg/ml ^125I-Tf or 2 μg/ml ^125I-AGOR. After endocytosis, the cells were washed four times at 0 °C with DMEM, 20 mM Hepes/NaOH, pH 7.5, 1% BSA to remove unbound ligands. Subsequently, surface-bound ligands were removed at 0 °C by washing the cells for 5 min in a low pH buffer (20 mM sodium citrate buffer, pH 3.5, supplemented with 130 mM NaCl, 2 mM CaCl₂, 1 mg/ml BSA, and 50 μM desferal) followed by 10 min in DMEM, 20 mM

![Figure 1. EGFR degradation and uptake](http://www.jbc.org/content/11563/3/11563/F1.large.jpg)

**FIG. 1.** EGFR degradation and uptake. A, cells expressing either wild type EGFR (●), Δ989–994 EGFR (■), or K721A EGFR (▲) were incubated at 37 °C for the indicated time in the presence of EGF. The cells were then lysed, and intact EGFR was detected by Western blotting. The signals were quantified and expressed relative to values for each cell line obtained at t = 0. Values are averages of two independent experiments ± S.D. B, cells expressing either wild type EGFR (●), Δ989–994 EGFR (■), or K721A EGFR (▲) were incubated at 37 °C for the indicated time in the presence of ^125I-EGF and washed rapidly to remove non-associated label. Plasma membrane-associated ^125I-EGF was dissociated and collected in a low pH buffer. Remaining intracellular ^125I-EGF was expressed as a percentage of total cell-associated label (plasma membrane + intracellular). Values are averages of two independent experiments ± S.D.
Hepes/NaOH, pH 7.5, 1% BSA, 50 μM desferal. The cells were then washed with and scraped in 1 ml of homogenization buffer (0.25 M sucrose, 10 mM Hepes/NaOH, pH 7.4, 2 mM EDTA, 0.05% NaN₃, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 g/ml leupeptin, 0.5 g/ml pepstatin A). Scraped cells were homogenized by passing them 20 times through a 23-gauge needle mounted on a syringe. Nuclei were removed from the homogenates by centrifugation at 3000 × g for 1 min in an Eppendorf centrifuge. Nuclear pellets contained less than 10% of internalized 125I-labeled ligands. 400-μl samples of the postnuclear supernatants were loaded on top of 3 ml of 0.4 or 1.3 M sucrose in 2 mM EDTA, 10 mM Hepes/NaOH, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A. The samples were centrifuged for 60 min at 50,000 rpm in a SW60 rotor using a Beckman ultracentrifuge. The supernatants were removed and the pellets analyzed for the presence of 125I using a γ-counter. The pellet below the 0.4-M sucrose cushion contained all endocytic compartments, whereas the pellet be-
low the 1.3-M sucrose cushion contained only those endosomal and lysosomal vacuoles that were loaded with AGF-gold.

RESULTS

EGFR Degradation Requires Kinase Activity and Domain 989–994—NIH 3T3 subclone 2.2 cells express undetectable levels of endogenous mouse receptors. Stable cell lines transfected for either wild type or mutant human EGFR were used to determine the contribution of the kinase (K721A EGFR) and actin-binding domain (Δ989–994 EGFR) to EGFR trafficking. K721A EGFR was used as a control for which ligand-induced trafficking had already been demonstrated to be defective. Scatchard analysis indicated similar binding constants and receptor numbers (1.3–1.7 × 10⁷/cell) for wild type and mutant receptors (data not shown). To study EGFR-induced degradation of the EGFR, cells were incubated at 37 °C in medium containing EGF, lysed, and analyzed for the presence of intact EGFR by Western blotting. The amount of wild type EGFR was reduced in the presence of EGF with a t½ of ~60 min (Fig. 1A). In contrast, both Δ989–994 EGFR and K721A EGFR were markedly resistant to EGF-induced degradation.

EGFR Endocytosis Requires Kinase Activity but Not Domain 989–994—Lack of degradation could result either from inefficient uptake or deficient transfer of endocytosed receptors to lysosomes. To discriminate between these possibilities we first measured EGFR uptake. Cells were incubated at 37 °C in medium containing 125I-EGF after which cell surface-associated 125I-EGF and internalized 125I-EGF were determined (Fig. 1B). Wild type EGFR endocytosed with a t½ of ~5 min. The initial rate of uptake for K721A EGFR was ~30% compared with wild type EGFR, consistent with a requirement for EGFR kinase activity. In contrast, the initial rate of uptake for Δ989–994 EGFR was comparable with wild type EGFR. After 10–30 min, however, the relative amount of intracellular 125I-EGF was slightly lower for Δ989–994 EGFR as compared with wild type EGFR-expressing cells, probably as a consequence of increased recycling for Δ989–994 EGFR (see below). Combined, the data in Fig. 1, A and B suggest that although Δ989–994 EGFR was endocytosed normally, its transfer from endosomes to lysosomes may be abrogated. For K721A EGFR, degradation was affected more severely than uptake, consistent with a role for EGFR kinase activity in both uptake and sorting from endosomes to lysosomes.

Sorting of Endocytosed EGFR Requires Both Kinase Activity and Domain 989–994 As Determined by CLSM—To study intracellular EGFR trafficking, the subcellular distribution of endocytosed EGFR was first analyzed by CLSM. As a marker for the lysosomal pathway we used Cy3-conjugated agialooro-somucoid (Cy3-AGOR). This ligand was efficiently endocytosed by CHL, a receptor that was stably co-transfected into EGFR-expressing cells. After receptor-mediated uptake, glycoproteins containing terminal N-acetylgalcosamine, such as AGOR, dissociate from CHL because of the low pH in endosomes and are then transferred to lysosomes (29). TIR was used as a marker for the recycling pathway. Cells were incubated for 30 min at 37 °C with EGF in the presence or absence of Cy3-AGOR, fixed, and then immunolabeled for EGFR only or for both EGFR and TIR. Endocytosed wild type EGFR co-distributed with Cy3-AGOR rather than with TIR-containing compartments (Fig. 2), indicating transport to late endosomes and lysosomes. In contrast, both Δ989–994 EGFR and K721A EGFR co-distributed predominantly with TIR rather than with Cy3-AGOR (Fig. 3), demonstrating entry into the recycling pathway.

To further resolve the itinerary of Δ989–994 EGFR and demonstrate that its behavior is not affected by co-expression of CHL and is not a peculiarity of the stable cell lines used above, cells transiently transfected for wild type EGFR or Δ989–994 EGFR were used. These cells were allowed to endocytose Alexa488-EGF and Alexa546-Tf for 30 min and then chased for another 30 min at 37 °C in the absence of ligands. After fixation the cells were immunolabeled with Cy5 for LAMP-1 (LAMP, panels A and B, middle top), washed, and chased for another 30 min at 37 °C. After fixation, the cells were immunolabeled for LAMP-1 with Cy5 (LAMP, panels A and B, middle bottom). Dual color combinations of triple-labeled cells are in the right column. The Cy5 label for LAMP-1 was artificially colored red for better comparison. The images are representative for all transfected cells. EGF/wild type EGFR localized predominantly with LAMP-1-labeled late endosomes/lysosomes, whereas EGF/Δ989–994 EGFR was largely confined to Tf-containing recycling endosomes.

EGFR were used. These cells were allowed to endocytose Alexa488-EGF and Alexa546-Tf for 30 min and then chased for another 30 min at 37 °C in the absence of ligands. After fixation the cells were immunolabeled with Cy5 for LAMP-1, a membrane protein marker of late endosomes and lysosomes (Fig. 4). In wild type EGFR-expressing cells, EGF was pulse-chased predominantly into LAMP-1-containing compartments (Fig. 4A), whereas in Δ989–994 EGFR-expressing cells EGFR was largely confined to Tf-containing recycling endosomes (Fig. 4B). Again, these data illustrate that endocytosed Δ989–994 EGFR is sorted into the recycling pathway rather than into the degradation pathway.

A Novel Method to Isolate Compartments within the Degradation Pathway from the Plasma Membrane and Recycling Endosomes—To investigate intracellular EGFR trafficking further, we developed a method to isolate sorting endosomes and lysosomes from recycling endosomes. Again, we used stable NIH 3T3 cell lines that co-express (mutant) EGFR and CHL. In this case CHL was employed to endocytose AGF coupled to 5 nm gold particles. Following endocytic uptake, AGF-gold dissociated from CHL in endosomes and was targeted to lysosomes (Fig. 5A). Vacular sorting endosomes and lysosomes...
contained up to hundreds of endocytosed AGF-gold particles as demonstrated by whole-mount electron microscopy (Fig. 5B) (34). In biochemical assays we used trace amounts of $^{125}$I-AGOR, which was, like AGF-gold, endocytosed by CHL and targeted to lysosomes. After endocytic uptake, the cells were washed at 4 °C to remove unbound ligands, followed by a treatment at pH 3.5 to strip AGF-gold and $^{125}$I-labeled ligands from the plasma membrane. Alternatively, surface-bound AGF-gold and $^{125}$I-AGOR could be removed with EDTA (data not shown), confirming the specificity of their interaction with CHL (29).

After cell homogenization and removal of nuclei by centrifugation, equal samples from each postnuclear supernatant were loaded on top of 0.4- and 1.3-M sucrose cushions and ultracentrifuged. All membranes pelleted through the 0.4-M sucrose cushion (total membrane fraction). In contrast, only those compartments loaded with AGF-gold were collected below the 1.3-M sucrose cushion due to their gain in buoyant density (Fig. 5C).

To validate this technique, CHL-expressing cells were incubated for 120 min at 37 °C, during which $^{125}$I-AGOR was present only during the final 15 min and AGF-gold was either absent or present during the final 15, 30, 60, or 120 min (Fig. 6A). In the absence of AGF-gold (0 min) only $\sim$10% of vacuole-associated $^{125}$I-AGOR was recovered at the bottom of the 1.3-M sucrose cushion. In contrast, $\sim$60% was recovered when AGF-gold was present concomitantly (15 min) with $^{125}$I-AGOR. The efficiency of this procedure did not increase when the cells were additionally loaded with AGF-gold prior to the 15-min pulse with $^{125}$I-AGOR (30–120 min) or at higher AGF-gold concentrations (not shown). Efficient endocytosis of $^{125}$I-AGOR as well as the AGF-gold-induced density shift of endocytic compartments relied on the expression of CHL (not shown). We next studied whether different compartments encountered within the lysosomal pathway (e.g. sorting endosomes, late endosomes, and lysosomes) were collected with equal efficiencies.

Cells were incubated with AGF-gold for 60 min, of which $^{125}$I-AGOR was present during the last 5–60 min (Fig. 6B). As expected, $^{125}$I-AGOR accumulated linearly with time, indicating progressive loading of the degradation pathway. Again, $\sim$60% of $^{125}$I-AGOR could be collected, irrespective of the loading time, indicating similar efficiencies for the isolation of “early” versus “late” compartments within the lysosomal pathway. The plasma membrane was stripped from AGF-gold prior to cell homogenization and thus not expected to pellet through 1.3 M sucrose. To verify this directly, AGF-gold-loaded and surface-stripped cells were biotinylated prior to cell homogenization to label plasma membrane proteins. The pellets at the bottom of 0.4- or 1.3-M sucrose cushions were extracted using TX114, and biotinylated plasma membrane proteins were detected by Western blotting (Fig. 6C). Membranes collected under the 0.4-M sucrose cushion contained many biotinylated plasma membrane proteins, whereas those pelleting through 1.3 M sucrose contained none, indicating the absence of plasma membrane in the latter fraction. In contrast to endocytosed $^{125}$I-AGOR, only $\sim$20% of the late-endosomal/lysosomal marker LAMP-1 was collected under the 1.3-M sucrose cushion after 60 min of loading with AGF-gold, indicating that only a limited

FIG. 5. Subcellular fractionation using colloidal gold. A, schematic drawing showing endocytosis of AGF-gold by CHL and EGF by EGFR. Endocytosed CHL/AGF-gold is uncoupled in sorting endosomes after which CHL is recycled to the plasma membrane via recycling endosomes and AGF-gold targeted into the degradation pathway. The EGF-EGFR complex is stable after uptake, sorted in MVB to the MVB internal vesicles and transported together with AGF-gold further into the degradation pathway. B, cells expressing CHL and EGFR were allowed to endocytose AGF-gold for 30 min and then processed for whole-mount electron microscopy. Two large vacuolar structures (asterisks) are shown, representing sorting endosomes or MVB, each containing hundreds of 5-nm colloidal gold particles. Bar, 200 nm. C, scheme for subcellular fractionation. Postnuclear supernatants (PNS) of cells that were incubated either in the presence (+ Au) or absence (− Au) of AGF-gold were split, loaded on 0.4- and 1.3-M sucrose cushions, and ultracentrifuged. All membranes migrated through 0.4 M sucrose, whereas only AGF-gold-loaded endosomes and lysosomes pelleted through 1.3 M sucrose.

Fig. 5. Subcellular fractionation using colloidal gold. A, schematic drawing showing endocytosis of AGF-gold by CHL and EGF by EGFR. Endocytosed CHL/AGF-gold is uncoupled in sorting endosomes after which CHL is recycled to the plasma membrane via recycling endosomes and AGF-gold targeted into the degradation pathway. The EGF-EGFR complex is stable after uptake, sorted in MVB to the MVB internal vesicles and transported together with AGF-gold further into the degradation pathway. B, cells expressing CHL and EGFR were allowed to endocytose AGF-gold for 30 min and then processed for whole-mount electron microscopy. Two large vacuolar structures (asterisks) are shown, representing sorting endosomes or MVB, each containing hundreds of 5-nm colloidal gold particles. Bar, 200 nm. C, scheme for subcellular fractionation. Postnuclear supernatants (PNS) of cells that were incubated either in the presence (+ Au) or absence (− Au) of AGF-gold were split, loaded on 0.4- and 1.3-M sucrose cushions, and ultracentrifuged. All membranes migrated through 0.4 M sucrose, whereas only AGF-gold-loaded endosomes and lysosomes pelleted through 1.3 M sucrose.
fraction of these compartments was reached by the endocytosed markers, consistent with the CLSM data in Fig. 4. In conclusion, this technique can be used to measure sorting of AGF-gold from other concomitantly endocytosed markers but not to assess the subcellular distribution of endogenous proteins.

Sorting of Endocytosed EGFR Requires Both Kinase Activity and Domain 989–994 as Determined by Subcellular Fractionation—The subcellular fractionation assay was then used to measure intracellular sorting of endocytosed 125I-EGF (Fig. 7). 125I-AGOR and 125I-Tf were used in parallel experiments together with non-labeled EGF to label the lysosomal pathway or recycling pathway, respectively. Cells expressing CHL together with wild type EGFR, Δ989–994 EGFR, or K721A EGFR were incubated for 30 min with the indicated ligands. In the presence of AGF-gold, ~70% of 125I-AGOR from all three cell lines pelleted through 1.3 M sucrose, indicating its presence in sorting endosomes/lysosomes. When AGF-gold was omitted, only ~10% was recovered from all cell lines. Only ~30% of endocytosed 125I-Tf pelleted through 1.3 M sucrose in the presence of AGF-gold, indicating that the majority had been sorted to recycling endosomes, away from the AGF-gold-containing lysosomal pathway. 125I-EGF that was endocytosed by wild type EGFR-expressing cells behaved similar to 125I-AGOR rather than to 125I-Tf, consistent with sorting of the 125I-EGF-EGFR complex to lysosomes. In contrast, both Δ989–994 EGFR- and K721A EGFR-expressing cells sorted the majority of endocytosed 125I-EGF, like 125I-Tf, away from AGF-gold-containing compartments. These results are consistent with the morphological data and indicate that both EGFR kinase activity and an intact actin-binding domain are required for EGFR sorting to lysosomes.

DISCUSSION

EGFR Kinase Activity Is Required for Sorting at the Plasma Membrane and in Endosomes—In this report we demonstrate that EGF-induced uptake of EGFR relies on an active EGFR kinase domain but not on its actin-binding domain, whereas
sorting of endocytosed EGFR to lysosomes requires both domains. The dependence on EGFR kinase activity is consistent with other studies that demonstrate ligand-induced uptake by clathrin-coated vesicles (1, 5, 35). The involvement of kinase activity in postendocytic trafficking of the EGFR has also been documented (15–17). Others reported, however, that EGFR kinase activity was not necessary for intracellular sorting of endocytosed EGFR to lysosomes (36–38). This discrepancy could be explained by the observation that relatively high amounts of EGFR may saturate the endosomal sorting machinery (15, 37). The data in the present study are consistent with the idea that EGFR kinase activity is required for efficient sorting of activated endocytosed EGFR into the lysosomal pathway. First, kinase-deficient EGFR was not degraded in the presence of EGF (Fig. 1A), whereas endocytotic uptake was reduced only 3-fold as compared with wild type EGFR (Fig. 1B). Combined, these effects can be explained only if insufficient transport from endosomes to lysosomes. Second, CLSM experiments demonstrated that endocytosed wild type EGFR resides within the lysosomal pathway, whereas kinase-negative EGFR predominately localized to Tfr-containing recycling endosomes (Figs. 2–4). Finally, we confirmed these distinct intracellular itineraries in cell fractionation experiments (Fig. 7). Felder et al. (16) demonstrated that divergence in trafficking between wild type and kinase deficient EGFR occurs at MVB. Wild type receptors are efficiently transferred to the internal vesicles of the MVB, whereas K721A EGFR is retained at the peripheral membrane. The molecular machinery that drives sorting of wild type EGFR at MVB is incompletely resolved but may involve the recruitment of essential phosphotyrosine-binding proteins, such as c-Cbl, and EGFR ubiquitination (18). In addition, autophosphorylation-induced conformational changes of the EGFR may allow association of cytoplasmic sorting molecules, such as SNX-1 (22) and possibly actin (see below).

**EGFR Sorting in Endosomes Requires Its Actin-binding Domain**—Unlike wild type EGFR, ligand-activated endocytosed Δ989–994 EGFR was not transferred to lysosomes, as can be concluded from three independent lines of evidence. First, Δ989–994 EGFR was not degraded in the presence of EGF despite efficient uptake (Fig. 1). Second, CLSM revealed that endocytosed Δ989–994 EGFR distributed with recycling TfrT rather than with lysosomal markers (Figs. 3 and 4). Finally, sorting from the degradation pathway was also demonstrated by the cell fractionation assay (Fig. 7). EGFR is an actin-binding protein, as revealed by *in vitro* and *in vivo* interactions with F-actin (24, 26). A domain encompassing residues 989–994 is responsible for this interaction because it was not observed for Δ985–996 or Δ989–994 EGFR (25, 26). Furthermore, a synthetic peptide homologous to domain 984–996 interacted with actin in *vitro*, and excess of this peptide as well as peptide-specific antibodies interfered with EGFR binding to actin (24).

Although our present study demonstrates that domain 989–994 is required for proper EGFR sorting, it cannot be concluded that association with actin is required. After all, this domain may serve functions in addition to actin binding. Interference with actin depolymerization cannot be used to demonstrate a requirement of actin-EGFR association in EGFR transport because many generic steps in vesicular transport, including endocytosis, and endosome and lysosome function (39) rely on the requirement of actin-EGFR association in EGFR transport be-

The molecular machinery behind the function of the actin-binding domain in intracellular EGFR sorting remains unclear. Possibly, the association of EGFR to actin is required to prevent it from being incorporated into the recycling pathway or to drive its transfer into domains at the limiting membrane of endosomes destined for incorporation into the inwardly budding vesicles of MVB. The latter possibility would be consistent with the presence of actin in exosomes (43), vesicles that are secreted by cells as a consequence of fusion of MVB with the plasma membrane. It should be noted, however, that, although the actin binding property of the EGFR may be critical for its sorting in MVB, we cannot exclude that an additional property of domain 989–994 rather than its actin binding capacity is essential for proper EGFR sorting.

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