The epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase family. Ligand (epidermal growth factor or EGF) binding to the EGFR results in the coordinated activation and integration of biochemical signaling events to mediate cell growth, migration, and differentiation. One mechanism the cell utilizes to orchestrate these events is ligand-mediated endocytosis through the canonical clathrin-mediated endocytic pathway. Identification of proteins that regulate the intracellular movement of the EGF-EGFR complex is an important first step in dissecting how specificity of EGFR signaling is conferred. We examined the role of the small molecular weight guanine nucleotide-binding protein (G-protein) rab7 as a regulator of the distal stages of the endocytic pathway. Through the transient expression of activating and inactivating mutants of rab7 in HeLa cells, we have determined that rab7 activity directly correlates with the rate of radiolabeled EGF and EGFR degradation. Furthermore, when inhibitory mutants of rab7 are expressed, the internalized EGF-EGFR complex accumulates in high-density endosomes that are characteristic of the late endocytic pathway. Thus, we conclude that rab7 regulates the endocytic trafficking of the EGF-EGFR complex by regulating its lysosomal degradation.

rab7 Activity Affects Epidermal Growth Factor:Epidermal Growth Factor Receptor Degradation by Regulating Endocytic Trafficking from the Late Endosome*

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The epidermal growth factor receptor (EGFR) is a ubiquitously expressed cell surface receptor tyrosine kinase that functions in the growth and development of differentiated cells (1). Under normal physiological conditions, the coordinated secretion of ligand (epidermal growth factor or EGF) and subsequent receptor stimulation function to maintain cell growth in a regulated manner to maintain the proper cell physiology.

In the resting state, the EGFR exists as a monomeric, single membrane spanning protein. Binding of its ligand to the extracellular domain of the receptor induces dimerization of the receptor and stimulation of its intrinsic kinase domain. This kinase transphosphorylates a number of carboxyl-terminal tyrosine residues in the cytoplasmic portion of the receptor that then serve as docking sites for the Src homology 2 domains of downstream signaling effectors (2, 3). These activated effectors, alone or in concert with other effectors, induce a variety of outcomes such as cell proliferation, migration, and differentiation (1, 4). Many cancers are characterized by the overexpression of the EGFR and unregulated EGFR signaling. As such, the EGFR has been a useful chemotherapeutic target in a number of cancers (5).

In addition to the signaling events initiated by ligand binding, EGFR stimulation also triggers the internalization of the ligand-receptor complex via the canonical clathrin-mediated endocytic pathway (6). Through a series of budding and fusion reactions, the ligand and receptor traffic through clathrin-coated vesicles, early and late endosomes, and lysosomes. There are at least three known functions of the endocytic pathway. First, it regulates the duration of the EGF-EGFR complex by causing the dissociation of the two proteins in acidic compartments. Second, EGFR levels are controlled through lysosomal degradation after ligand stimulation. Third, it places the activated EGFR in the proper cellular locale to allow it to signal to the appropriate downstream effectors (6–9). Endocytic trafficking of the EGFR is necessary for the proper temporal and spatial regulation of receptor signaling leading to normal cell physiology.

Thus far, the primary focus in the literature has been on the most proximal steps of endocytosis. Mutation and/or knockdown of proteins in recruitment to or the function of clathrin-coated pits such as dynamin, clathrin, EPS15, Grb2, and β-adaptin have been shown to alter the rate of ligand-stimulated endocytosis (10–13). The biochemical and physiological consequences of altering the rate of internalization of the EGFR have been well established (6). This article explores EGFR endocytic trafficking at more distal stages along the pathway. Identification of proteins that regulate these transitions will be useful for generating tools to examine the functional significance of various compartments in the regulation of EGFR signaling.

Rab proteins are a family of small molecular weight guanine nucleotide-binding proteins (G-proteins) that regulate vesicular budding and fusion reactions by oscillating between active, GTP and inactive, GDP bound forms (14). Over 60 different Rab proteins have been identified; each is unique in cellular and subcellular localization as well as the vesicles that they fuse (15). Many G-proteins have been implicated in EGFR endocytic trafficking. There is literature indicating that dynamin coordinates EGFR endocytosis (13), rab5 has a role in mediating entry of the receptor into the early endosome (16–18), and rab11 facilitates EGFR recycling to the plasma membrane (19).

rab7 is a 23-kDa protein that is localized to late endosomes (20). There is a defined role for rab7 in regulating the later stages of the endocytic pathway for a number of proteins. Expression of inhibitory forms of rab7 have been implicated in slowing endocytic trafficking of proteins such as low density lipoprotein, vesicular stomatitis virus, mannose 6-phosphate receptor, and angiotensin II type 1A receptor (21–24). However, it is unclear whether rab7 coordinates endocytic trafficking of all proteins or if rab7 regulates the movement of select cargoes. Specifically, it is unclear if functional rab7 is needed for normal EGF-EGFR endocytic trafficking.
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Second, it is unclear in which compartment cargo accumulates if rab7-mediated trafficking were disrupted. Currently, the literature remains controversial as to whether rab7 coordinates movement between the early and late endosome or whether it regulates trafficking between the late endosome and lysosome (22, 23, 25, 26). To understand the physiologic significance of endocytic trafficking, a better characterization of the compartments affected by the expression of mutant forms of regulatory proteins is needed.

In this report, dominant negative and constitutively active mutants of rab7 are transiently overexpressed in HeLa cells to study the role of this GTPase in EGFR trafficking. We find that changes in rab7 activity do not alter either the steady-state distribution of the EGFR or ligand-mediated entry of the receptor into the cell. However, inhibition and activation of rab7 activity differentially modulate the kinetics of radiolabeled EGF degradation. Similarly, expression of dominant negative rab7 slows the rate of EGFR degradation. Separation of endocytic vesicles from cells expressing dominant negative rab7 on an isotonic Percoll gradient reveals that cells expressing dominant negative rab7 accumulate internalized radiolabeled EGF in more dense endosomes. From these findings, we conclude that functional rab7 is required for the degradation of the EGF-EGFR complex by the lysosome.

MATERIALS AND METHODS

Cell Culture—tTA-HeLa cells (HeLa cells stably transfected with the tetracycline transactivator (tTA)) were a gift of Dr. Sandra Schmid (The Scripps Research Institute, La Jolla, CA). Cells were maintained in Dulbecco’s minimal essential media (DMEM) of 5% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine (27). Cell lines were maintained at 37 °C in 5% CO2.

Generation and Usage of Adenoviruses—This study uses canine rab7 constructs that are 99% homologous to the human rab7 protein (GenBank®). Constitutively active rab7, denoted rab7(Q67L) refers to rab7 with a glutamine to leucine mutation at amino acid 67 and has been documented extensively elsewhere (22, 23, 25). This mutation, in the GTPase domain of the protein, renders the GTPase inactive and maintains the protein predominantly in the GTP-bound state. Dominant negative rab7 has a point mutation of asparagine to isoleucine at amino acid 125, and is referred to as rab7(N125I); it cannot bind guanine nucleotide (22, 23).

Adenoviruses were generated and maintained as described previously (16, 17). Adenoviruses were cesium chloride purified and concentrated to minimize any potential effects of cytosol or media from the HEK293 cells in which they were amplified. Adenoviruses were subjected to sequence analysis at the Oklahoma Medical Research Foundation DNA Sequencing facility.

For adenoviral expression experiments, subconfluent dishes of tTA-HeLa cells were infected at a multiplicity of infection of 20 plaque forming units/cell for 2 h in serum-free DMEM at 37 °C in 5% CO2, followed by the indicated length of incubation in growth media. Mock infected cells were incubated with virus in the presence of 1 μg/ml tetracycline during infection and recovery. For all experiments, recombinant protein expression from adenoviruses was verified by immunoblotting and/or indirect immunofluorescence with a dish of cells treated in parallel.

Cell Lysate Preparation and Immunoblotting—Cell lysates were generated by washing the cells twice with phosphate-buffered saline (PBS) and solubilizing cells in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM phenylmethylsulfonyl fluoride) on ice. Proteins were solubilized by rotating the lysis buffer/cell mixture end over end for 10 min at 4 °C; insoluble material was removed by microcentrifugation for 10 min at 4 °C. The protein concentration of the solubilized protein was assessed by BCA assay (Pierce), and samples were diluted in SDS sample buffer. Equivalent amounts of protein were separated by the indicated percentage using SDS-PAGE, transferred to

FIGURE 1. Functional expression of tetracycline-regulatable adenoviruses encoding for HA-rab7(N125I) and HA-rab7(Q67L). A, tTA-HeLa cells were infected at a multiplicity of infection of 20 plaque forming units/cell with either nothing or adenovirus encoding for either HA-rab7(Q67L) or HA-rab7(N125I) in the absence or presence of 1 μg/ml tetracycline as indicated. Fifty micrograms of collected cell lysates were resolved on a 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against rab7 or hemagglutinin (12CA5 antibody). Antibody binding was visualized with the appropriate horseradish peroxidase-conjugated secondary antibody and ECL. Shown is a representative blot from an experiment repeated three times.

FIGURE 2. Adenovirally expressed HA-rab7(Q67L) stably expresses for 72 h, whereas adenovirally expressed HA-rab7(N125I) is maximal within 24 h. A, tTA-HeLa cells were infected as described in the legend to Fig. 1. Cell lysates were harvested 24, 48, and 72 h after infection. Cell lysates (50 μg) were resolved by 12% SDS-PAGE and immunoblotted (IB) with an antibody against the HA epitope. After incubation with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody, proteins were visualized by ECL. Shown is a representative blot from an experiment repeated three times.
nitrocellulose, detected using monoclonal antibodies against the hemagglutinin (HA) epitope (12CA5) (Roche), LAMP-1 (BD Transduction Laboratories, San Jose, CA), EEA1 (BD Transduction Laboratories), or a rabbit polyclonal antibody against EGFR (Santa Cruz Biotechnology, Santa Cruz, CA), rab7 (Santa Cruz Biotechnology) followed by probing with a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody. Detected proteins were visualized by enhanced chemiluminescence (ECL) and either film or the UV Products Imaging system.

Relative intensity of the immunoreactive protein bands was determined by scanning multiple film exposures of the same immunoblot from the same experiment to ensure the signal was in the linear range. Densitometric analysis was performed using Image J software (NIH).

Lysotracker and Indirect Immunofluorescence—To visualize the presence of acidic lysosomes, infected cells were incubated with 50 nM LysoTracker DND-99 (Molecular Probes, Eugene, OR) in serum-free DMEM for 30 min at 37 °C. Lysotracker DND-99 is a fluorescent dye that accumulates in acidic compartments, but the fluorescent intensity does not change in response to pH.

Cells were fixed in a 4% p-formaldehyde:PBS2− (PBS, 0.5 mM MgCl2, 0.5 mM CaCl2) solution at room temperature for 5 min and on ice for 15 min. Excess formaldehyde was removed with three 5-min washes in PBS2−. Cells were then permeabilized for 20 min in 0.1% saponin, 5% fetal bovine serum:PBS2−, and washed 3X 5 min using PBS2−. After washing, cells were incubated for 1 h with the indicated antibody. The EGF was detected with a mouse monoclonal antibody (α-EGFR Ab-5, Oncogene Research Products, La Jolla, CA). HA-tagged rab7 constructs were detected using the anti-HA 12CA5 mouse monoclonal antibody (Roche). Unbound primary antibody was removed with 3X 5-min washes in PBS2− and cells were incubated 1 h with an Alexa 488-conjugated goat anti-mouse secondary antibody (Molecular Probes). After six 10-min washes with PBS2−, coverslips were rinsed in Millipore water and mounted on a slide with Prolong Antifade (Molecular Probes). Confocal images were collected at the Warren Medical Research Institute Flow and Image Cytometry Laboratory (on the University of Oklahoma Health Sciences Center Campus) using a Leica TCS NT microscope and analyzed using the Leica TCS NT software (Wetzlar, Germany). Fluorescent images were collected using a Nikon Eclipse TE-2000-U microscope and processed using OpenLab Imaging Software. Pixel intensities of individual endosomes were quantified using Image J software (NIH).

125I-EGF Degradation and Secretion—Cells were incubated for 7.5 min with 125I-EGF (1 ng/ml) at 37 °C in binding buffer (DMEM, 20 mM HEPES, 0.1% bovine serum albumin, pH 7.3). Cells were washed four times in ice with ice-cold PBS to remove external, unbound 125I-EGF. Pre-warmed, 37 °C media was added to the cells and they were returned to 37 °C for the indicated periods of time. At each time point, the media was collected. The remaining cells were solubilized in 1% Nonidet P-40, 20 mM Tris, pH 7.4. Cell lysates were incubated with 10% trichloroacetic acid and 1% BSA as a carrier protein on ice for 1 h. Intact protein was detected using the anti-HA 12CA5 antibody (anti-HA epitope) and an Alexa 488 goat anti-mouse secondary antibody. Images were collected on a Nikon Eclipse TE2000-U microscope using a 60 objective. Size bar = 20 μm. C. Representative micrographs from experiments repeated at least three times. IB, immunoblot; DAPI, 4,6-diamidino-2-phenylindole.
separated from degraded by centrifugation for 15 min at 14,000 × g in an Eppendorf microcentrifuge at 4 °C. The radioactivity for all three fractions (secreted, intact, and degraded) was determined by calculating the amount of radioactivity in each fraction. Thirty-five samples were treated with 10 ng/ml EGF for the indicated amounts of time. Thirty-five were treated with 10 ng/ml EGF for the indicated time. Eighteen hours post-infection, cells uninfected control.

**RESULTS**

**Functional Expression of Dominant Negative and Constitutively Active Mutants of rab7**—To determine the effect of rab7 activity on EGFR endocytic trafficking, we generated tetracycline-regulatable adenoviruses that express amino-terminal HA-tagged mutants of rab7. HA-rab7(N125I) has a mutation of asparagine to isoleucine at amino acid 125, rendering the small molecular weight G-protein unable to bind guanine nucleotide. Overexpression of HA-rab7(N125I) has been shown to inhibit endogenous rab7 function (22, 23). A constitutively active form of rab7 was generated by substitution of glutamate with leucine at amino acid 67 (HA-rab7(Q67L)). This mutation inactivates the endogenous GTPase activity of the protein, causing the protein to stay in the GTP bound form (25).

Adenoviruses were expressed in HeLa cells because they contain a physiological number of EGFRs and the EGFR endocytic trafficking has been well characterized (11, 13, 16). The tetracycline regulation of the adenoviruses require the co-expression of the tTA and these studies utilized HeLa cells stably transfected with the tTA (tTA-HeLa) (27). The use of the tetracycline-regulatable adenoviral expression system in the tTA-HeLa cells has been an effective tool in the study of proteins involved in endocytic trafficking (16, 17, 31, 32). These adenoviruses are able to infect a high percentage of cells (>95%, see Fig. 3C) and protein expression can be attenuated by the addition of tetracycline. The transient nature of infection helps circumvent any deleterious effects that prolonged expression of the exogenous protein may have on the cell. Furthermore, because the infection in the presence of tetracycline inhibits protein expression, any unintended effects from the viral infection are readily distinguished.

Cell lysates were prepared from infected cells, resolved by 12% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using antibodies against either rab7 (Fig. 1A, upper panel) or the HA-epitope (12CA5, Fig. 1A, lower panel). We estimate that the level of exogenous rab7 expression is ~10–20-fold of endogenous rab7, based on densitometric scans of multiple gels. Adenovirally infected HA-rab7(N125I) migrates faster than HA-rab7(Q67L) on SDS-PAGE. Whereas the biochemical cause of this altered migration is not known, this observation is consistent with what has been reported in baby hamster kidney cells stably transfected with rab7(N125I) (23).
When cells are infected in the presence of tetracycline, mutant rab7 expression is completely abrogated. Under these infection conditions, greater than 95% of cells are infected (see Fig. 3C for representative infection). For all experiments, parallel dishes of cells were infected with the same amount of adenovirus (either HA-rab7(N125I) or HA-rab7(Q67L)) in the presence of 1 μg/ml tetracycline, to control for adenoviral effects and are noted as “mock” infected in subsequent figures.

To determine whether each rab7 mutant was functioning as previously reported, we examined the ability of the infected cells to form acidic vesicles in the presence of exogenous rab7. After infection with the indicated adenovirus or mock infection, cells were allowed to recover overnight. The next day, cells were incubated with LysoTracker DND-99 (Molecular Probes, Eugene, OR), a fluorescent, but pH-insensitive dye that selectively accumulates in acidic lysosomes. Cells were fixed, processed for indirect immunofluorescence using the 12CA5 antibody that selectively recognizes the HA-tagged exogenous rab7, and examined under a confocal microscope for the presence of LysoTracker-labeled acidic lysosomes (Fig. 1, red) and the relative distribution of the exogenous rab7. In Fig. 1B, acidic vesicles are observed under all three infection conditions (rab7(N125I), rab7(Q67L), and mock infected). However, the acidic vesicles seen in the rab7(Q67L) expressing cells were brighter and larger in size. These data are consistent with what others have reported using these rab7 mutants and are consistent with our expression system generating the expected consequences on acidic vesicles (21).

To determine the optimal time frame to examine the effect of rab7 activity on EGFR trafficking, we collected cell lysates from HeLa cells at 24-h intervals post-infection. From this, we found that 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against the HA epitope (12CA5). HeLa cells robustly expressed rab7(Q67L) at levels that did not significantly change over the 24–72-h time course examined (Fig. 2A). However, cells infected with HA-rab7(N125I) expressed the mutant protein at the 24-h time point, but the levels trailed off dramatically at later time points. Thus, our subsequent analysis of EGFR trafficking used cells that had been infected within 18–24 h.

**rab7 Mutants Do Not Affect EGFR Expression, Localization, or Ligand-stimulated Endocytosis**—Prior to examining the role of rab7 on EGFR endocytic trafficking, it was important to first determine whether the rab7 activity had any effect on the steady-state distribution and expression levels of the EGFR. The localization of the unliganded EGFR, like many proteins, is dynamic. In resting cells, the vast majority of EGFRs reside at the plasma membrane, however, there is a small percentage of receptors that continually shuttle to and from the plasma membrane (33). Past studies have shown that continual expression of activated mutant of the small molecular weight G-protein rab5 causes the redistribution of the EGFR into endocytic vesicles and subsequent degradation in the absence of ligand stimulation (17). To determine whether our rab7 mutants could elicit similar effects, we examined both the steady-state levels of total cellular EGFR and its cellular distribution.

Cell lysates from rab7-infected HeLa cells were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the presence of the EGFR. Expression of activated or inactive rab7 resulted in EGFR levels that were indistinguishable from controls (Fig. 3A).

To determine whether rab7 activity affects the cellular localization of the EGFR, parallel sets of infected cells were fixed and processed for indirect immunofluorescence using an antibody specific for the EGFR. Fluorescent micrographs of those cells indicate that the unstimulated EGFR does not localize to endosomes. Staining of the EGFR is diffuse with regions of intense staining localized to the periphery of the cell under all experimental conditions (Fig. 3B, -EGF).

To examine if the proximal steps of ligand-stimulated endocytic trafficking are disrupted by the activity of rab7, we examined the distribution of the EGFR after EGF treatment. A redistribution of cell surface EGFR to endosomes indicates that the endocytic process is still intact. Infected HeLa cells were incubated with EGF for 10 min at 37 °C. Cells were fixed, processed for indirect immunofluorescence using an antibody against the EGFR, and examined using a fluorescent microscope (Fig. 3B, +EGF). The fluorescent micrographs reveal that under all infection conditions, EGF treatment resulted in the accumulation of EGFRs in endosomes. These data are consistent with rab7 activity not inhibiting ligand-stimulated EGFR internalization.

For this experiment and all others, parallel dishes of cells were infected and subjected to indirect immunofluorescence using the
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Activated rab7 Accelerates Ligand Degradation and Inactive rab7 Slows Ligand Degradation—To determine the effect of rab7 activity on later stages of endocytic trafficking, we examined the rate of 125I-EGF degradation in HeLa cells expressing mutant rab7. Briefly, cells were loaded with 125I-EGF and then chased with radioligand-free media. Intact and degraded 125I-EGF were separated from one another by trichloroacetic acid precipitation of intact radioligand. A parallel set of uninfected cells were treated with the ionophore monensin, as a positive control for inhibition of radioligand degradation (29). Data are plotted as the percentage of total 125I-EGF that is intact at each time point assayed (Fig. 4A). Expression of HA-rab7(Q67L) resulted in an accelerated rate of 125I-EGF degradation at early time points and reached control levels by 30 min. Conversely, cells expressing HA-rab7(N125I) had the same initial kinetics of degradation as uninfected cells, but a slower rate of degradation at later time points (Fig. 4A). Whereas the inhibition on degradation is not as robust as that seen in the presence of monensin treatment, it is statistically significant compared with the rate of degradation in uninfected cells. These temporal differences in the amounts of 125I-EGF degradation are consistent with HA-rab7(Q67L) accelerating and HA-rab7(N125I) decelerating the rate of EGF-EGFR trafficking.

As a second approach to determine whether rab7(N125I) affects EGFR degradation, we examined EGFR accumulation in a single cell assay. Infected cells were treated with EGF for 30 min. Cells were fixed and the EGFR was probed with an anti-EGFR antibody. Shown in Fig. 5A, cells infected with rab7(N125I) exhibit an increase in the amount of detectable EGFR at the cell surface as determined by densitometry. When multiple experiments (n = 6) were quantified, there was a statistically significant decrease in the rate of EGFR degradation in cells expressing rab7(N125I) compared to controls and cells expressing rab7(Q67L).

Quantification of the endosomal fluorescent intensity reveals that rab7(N125I) expressing cells have a greater percentage of endosomes with higher fluorescent intensity as compared with rab7(Q67L)-infected and mock infected cells (Fig. 6B). Data from these experiments suggest that rab7(N125I) may cause a decrease in the rate of EGFR degradation.
Evidence that there was a decrease in EGFR degradation in the presence of rab7(N125I).

Rab7 Activity Affects the Density of Endosome In Which Internalized 125I-EGF Accumulate—To determine whether the rates of ligand and receptor degradation were because of changes in protein trafficking or to alterations in the activity of proteases, the density of compartments in which internalized radioligand from rab7 mutant-expressing cells were biochemically assessed. Post-nuclear supernatants from cells that had been pulse-labeled with 125I-EGF were separated over 17% Percoll gradients to resolve endosomes based on density (30). Gradients were fractionated into 10 drops (~350 µl) fractions and assayed for the presence of radioactivity. The percentage of total radioactivity in each fraction was plotted as a function of relative migration on the Percoll gradient (Fig. 7). Cells incubated with 125I-EGF for 15 min accumulate 125I-EGF in light endosomes (density > 1.035–1.040 g/ml) for all experimental conditions (data not shown). This is consistent with rab7 activity not affecting endocytosis of the EGFR or movement to the early endosome. Cells that were incubated with radioligand for 15 min followed by a 45-min chase in radioligand-free media had dramatically different distributions of 125I-EGF. Cells infected with rab7(N125I) had an accumulation of radioligand in high density endosomes (mean density ~1.080 g/ml) (Fig. 7B). No peak in 125I was seen in rab7(Q67L) or mock infected cells (Fig. 7, A and C). This endosomal density is slightly higher than the previously reported densities of the late endosome (density = 1.048–1.070 g/ml) (37–39).

Individual fractions were probed for the presence of endosomal marker proteins, namely LAMP-1 (lysosome) and EEA1 (early endosome) (40, 41). The peak in radioactivity seen with rab7(N125I) expression was slightly more buoyant than the fraction containing the bulk of LAMP1. Taken together, these data are consistent with the accumulation of radioligand in a late endosomal compartment.

**DISCUSSION**

Using a series of biochemical assays, we have shown a correlation between rab7 activity and trafficking of the EGF-EGFR complex. In our most sensitive assay, radioligand degradation, we observe a 28% increase in the rate of 125I-EGF degradation at short time points with HA-rab7(Q67L) expression (Fig. 4A, 15 min post-125I-EGF addition). When HA-rab7(N125I) was expressed, there was a 54% decrease in the rate of radioligand degradation, which was most striking at longer time points (60–90 min). The ability of an activating mutant of rab7 to accelerate the kinetics and a dominant negative mutant slowing the kinetics are consistent with rab7 being a key regulatory molecule in the degradation pathway. This notion was supported by the slowed EGFR degradation in cells expressing HA-rab7(N125I) (Figs. 5 and 6). The changes in degradation correspond temporally with when the EGF-EGFR complex would be trafficking into (15–30 min) and out of (30–60 min) the late endosome (42). These data are kinetically consistent with rab7 regulating trafficking out of the late endosome.

The analysis of 125I-EGF distribution over Percoll sedimentation gradients confirms a role for rab7 in regulating trafficking of the EGFR from the late endosome. Expression of HA-rab7(N125I) results in the accumulation of 125I-EGF in the higher density endosomes. The density of these fractions and the migration of lysosomal marker suggests that the defect was at the late endosome/lysosome interface.

There has been debate in the literature regarding which endocytic stages rab7 regulates. Data support a role for rab7 at the early endosome/late endosome interface as well as regulating the transition from late endosome to lysosome. Clearly our data support the latter model. However, we cannot exclude the possibility that rabs may vary based on cellular context or experimental methodology. Alternatively, interpretations may be altered based on the tools used to define various endocytic compartments.

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