Receptor and Membrane Recycling Can Occur with Unaltered Efficiency Despite Dramatic Rab5(Q79L)-induced Changes in Endosome Geometry*

Received for publication, November 15, 2000, and in revised form, December 11, 2000 Published, JBC Papers in Press, January 2, 2001, DOI 10.1074/jbc.M010387200

Brian P. Ceresa‡, Marius Lotscher§, and Sandra L. Schmid¶

From the Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Endocytic vesicles deliver their content of membrane proteins, lipids, and luminal content to the early or sorting endosomal compartment consisting of tubular and vacuolar portions. Many receptor-ligand complexes dissociate in the mildly acidic environment of the early endosome (1). It has been proposed (1, 2) that endosomal morphology and resulting geometric considerations play a major role in controlling sorting efficiency in the early endosome. In this model, membrane proteins destined for recycling accumulate in long tubular extensions of the early endosome, which have a high surface to volume ratio. Fluid phase content including released ligands is deposited in the vacuolar portions of the early endosome, which, being spherical, approach a minimum surface to volume ratio. These vacuolar portions dissociate from tubular regions to carry their contents to late endosomes and/or lysosomes (3).

Rab5 is a small molecular weight GTPase associated with the plasma membrane and early/sorting endosomes. Rab5 controls homotypic early endosome fusion and thus functions in the formation of early endosomes (4–6). A point mutation in the GTPase domain (glutamine to leucine; denoted as Rab5(Q79L)) reduces Rab5 GTPase activity and results in a mutant Rab5 with an increased propensity to be in the active, GTP-bound state (7, 8). Expression of this constitutively active form of Rab5 enhances homotypic endosome fusion leading to the formation of enlarged early endosomes. It also has been reported that Rab5(Q79L) overexpression increases the rate of transferrin receptor uptake and decreases the rate of transferrin receptor recycling (8) although the mechanism for these effects remains obscure.

Rab5 is preferentially associated with the vacuolar portions of the early endosome (9), and Rab5(Q79L) overexpression leads to the formation of large spherical endosomes as visualized in semithick sections by electron microscopy (10). To test whether the Rab5(Q79L) effects on transferrin receptor endocytosis and recycling can be correlated with these dramatic changes in endosomal size and geometry, we utilized a tetracycline-regulatable adenoviral expression system that allows us to temporally control Rab5(Q79L) expression levels. The early endosomal compartment was dramatically enlarged in adenovirally infected HeLa cells overexpressing Rab5(Q79L). However, the presence of these enlarged endosomes did not alter the kinetics of endocytic membrane trafficking of either cell surface receptors or bulk membrane lipids. These unexpected results argue that geometric considerations may contribute to a lesser extent than previously assumed in determining the sorting and recycling efficiencies of the early endosomal compartment.

MATERIALS AND METHODS

Cell Culture—tTA-HeLa cells were cultured in DMEM1 supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (growth medium). Wild type and mutant (canine) Rab5α constructs (a gift of M. Zerial) were tagged with the hemagglutinin epitope on the amino terminus and subcloned into pUHD expression vectors (11). HA-Rab5(WT) and HA-Rab5(Q79L)-expressing cells were generated by cotransfecting the tTA-HeLa cells with cDNA that encodes HA-Rab5(WT) or HA-Rab5(Q79L). Rab5(Q79L) reduces Rab5 GTPase activity and results in a mutant Rab5 with an increased propensity to be in the active, GTP-bound state (7, 8). Expression of this constitutively active form of Rab5 enhances homotypic endosome fusion leading to the formation of enlarged early endosomes. It also has been reported that Rab5(Q79L) overexpression increases the rate of transferrin receptor uptake and decreases the rate of transferrin receptor recycling (8) although the mechanism for these effects remains obscure.

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1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; PBS, phosphate-buffered saline; WT, wild type; HRP, horseradish peroxidase; Tf, transferrin; TfnR, transferrin receptor; B-XX-Tfn, biotinylated Tf; C6-NBD-SM, N-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino) hexanoyl)-sphingosylphosphocholine.
washed out the existing tetracycline with two PBS (phosphate-buffered saline, pH 7.4) washes and incubating the cells in growth medium without tetracycline for 48 h.

**Alexa-Transferin Labeling and Immunofluorescence**—Cells, grown on coverslips to ~70% confluence, were washed twice with room temperature PBS and then incubated with 50 μg/ml Alexa-Transferin (Molecular Probes) in PBS *++* (PBS, 1 mM MgCl2, 1 mM CaCl2, and 0.2% bovine serum albumin) for the indicated times at 37 °C. Coverslips were moved to 4 °C, washed twice with ice-cold PBS *++* and then three times with ice-cold citrate buffer, and re-equilibrated with two additional ice-cold PBS *++* washes (12). The coverslips were fixed in a 4% formaldehyde-PBS *++* (PBS, 1 mM MgCl2 and 1 mM CaCl2) solution at room temperature for 5 min and on ice for an additional 15 min. Excess formaldehyde was removed with 3 × 5-min washes in PBS *++*. Cells were permeabilized in 0.1% saponin/5% goat serum/PBS *++* for 15 min. After 3 × 5-min PBS *++* washes, the coverslips were incubated with primary antibody for 1 h. Antibodies used (source in parentheses) were mouse monoclonal anti-HA tag 12CA5 (fan Wilson, The Scripps Research Institute) and mouse monoclonal anti-Rab5 (Transduction Laboratories). Unbound primary antibody was removed with 3 × 5-min PBS *++* washes, and the coverslips were incubated with the appropriate secondary antibody (noted in figure legends). Coverslips were subjected to 6 × 5-min PBS *++* washes, rinsed in Millipore water, and mounted on a coverslip slide using Fluoromount G (EM Sciences).

**Western Blot Detection of HA-Rab5**—35-mm dishes of cells were washed twice in room temperature PBS and put on ice with PBS to cool to 4 °C for 5 min. Cells were then harvested in 500 μl of ice-cold solubilization buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM phenylmethylsulfonyl fluoride). Lysates were solubilized by gently rocking for 10 min at 4 °C and centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 10 min at 4 °C to pellet the insoluble material. Protein concentration was determined by Bio-Rad, and 100 μg of solubilized protein was run on 13% SDS-polyacrylamide gel electrophoresis mini-gel, transferred to nitrocellulose, and immunoblotted using monoclonal antibodies against Rab5 or 12CA5 as above. Proteins were detected using secondary goat anti-rabbit antibody conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence.

**Adenovirus Generation**—HA-Rab5(WT) or HA-Rab5(Q79L) was put under the control of a tetracycline-regulatable promoter in the pAdlov vector 3' to the V5 packaging site and 5' to the poly(A) site. Adenoviruses were generated as previously described (13). Prior to use in experiments, adenoviruses were plaque-purified to a single viral population and then amplified.

**Adeno-associated Virus Infection**—In experiments in which cells expressed Rab5 continuously for 18 h, TTA-HeLa cells at ~70% confluency were infected with adenovirus at an m.o.i. of 10 plaque-forming units/cell. Cells were infected with adenovirus in DMEM with or without 1 μg/ml tetracycline for 2 h at 37 °C. After infection, the viral medium was removed and replaced with growth medium with or without tetracycline.

**Lipid Recycling**—Lipid recycling was performed as described by Hao and Maxfield (17). Quantitation of C6-NBD-SM in medium or in cell lysates was performed using a PerkinElmer Life Sciences fluorimeter at an excitation wavelength of 465 nm and measuring the peak height between 518 and 558 nm. Data are plotted as the C6-NBD-SM that remains cell-associated at each time point.

**RESULTS AND DISCUSSION**

**Expression of HA-Rab5(Q79L) Induces Enlarged Endosomes in Stably Transformed Cells**—To begin to probe the mechanism of Rab5(Q79L) effects on TfnR endocytosis and recycling, we generated stably transformed cell lines expressing HA-tagged wild type Rab5 (HA-Rab5(WT)) and constitutively active HA-Rab5(Q79L) under the control of a tetracycline-responsive expression system. Using this system, stable cell lines can be generated while avoiding any deleterious effects that may result from continuously altering cellular membrane trafficking (11). Stable cell lines generated in this manner express either HA-Rab5(WT) or HA-Rab5(Q79L) in a tetracycline-regulated manner as determined by Western blotting using either the 12CA5 anti-HA antibody (not shown) or antibodies against Rab5, which reveal both the more slowly migrating recombinant HA-tagged protein and the endogenous Rab5 (Fig. 1A). In these stably transformed cells, recombinant HA-Rab5 is expressed at roughly equimolar levels compared with endogenous protein. Expression of Rab5(WT) at these levels did not affect endosome morphology (Fig. 1B, upper left), whereas expression of Rab5(Q79L) at these levels was sufficient to cause the expected morphological phenotype—enlarged endosomes (upper right). Expression of HA-Rab5(Q79L) was suppressed when cells were cultured in the presence of tetracycline (upper middle panel). In all cases, the endosomes were functional in that fluorescently labeled Tf was internalized and delivered to them (Fig. 1B, lower panels). Unexpectedly, examination of the single-round uptake and recycling kinetics of Tf revealed that despite formation of enlarged endosomes there was no perturbation of Tf uptake in cells induced to express either WT or mutant Rab5 compared with uninduced control cells (Fig. 1C). Similarly, there was no change in the steady state accumulation of Tfn receptors in this endosomal compartment. These data suggested that the enlarged endosome phenotype is not predictive of defects in endocytic membrane trafficking.

**Adeno-associated Virus-mediated, Tetracycline-regulated Expression of Rab5(Q79L)**—Given that a threshold level of Rab5(Q79L) expression may be required to cause changes in membrane trafficking (8), it remained possible that the lack of changes in TfR trafficking was due to low levels of exogenous protein expression. Consequently, we elected to employ an adenoviral expression system to obtain reproducibly and uniformly higher levels of overexpression. In addition, adenovirus allows for the rapid induction of high levels of protein, thus circumventing problems that may occur as a result of chronic exposure of a foreign protein to the cell. The adenoviral expression system was designed to retain the tetracycline regulation so that any potential adenovirus effects could be controlled by infection of cells in the presence of tetracycline.

When cultured under inducing conditions for 18 h in the absence of tetracycline, TTA-HeLa cells infected with recombinant adenoviruses encoding either WT or mutant Rab5 expressed 50–100-fold higher levels of the desired protein compared with endogenous Rab5 (Fig. 2A). Importantly, WT and mutant Rab5 expression was not detectable when infected cells were cultured in the presence of tetracycline. As expected at these high levels of overexpression, the characteristically enlarged endosomal morphology was readily apparent in cells expressing HA-Rab5(Q79L) (Fig. 2B). These enlarged endosomes remained accessible to internalized Tf as indicated by the colocalization of fluorescently labeled transferrin (Alexa-Transferin) and Rab5 containing vesicles stained with an antibody...
that recognizes the HA epitope (Fig. 2B). Despite the fact that transferrin receptors were trafficking through these dramatically enlarged endosomes when the kinetics of transferrin receptor trafficking was measured, there were no changes in the uptake of the transferrin receptor (Fig. 2C) compared with control uninfected cells or cells infected with HA-Rab5(WT).

Like the stably transfected Rab5 tTA-HeLa cell lines, adenovirus expression of wild type and mutant Rab5 did not alter the steady state accumulation of Tfn within the cell.

**Transferrin Endocytosis and Recycling Are Unaffected by HA-Rab5(Q79L) Overexpression—**Our findings are inconsistent with previous results showing effects of both Rab5(WT) and Rab5(Q79L) overexpression on endocytosis and recycling of TfnR (5, 8). One trivial explanation for these differences is that previous studies were performed on adherent cells following internalization of \(^{125}\)I-Tfn, whereas our assay follows B-XX-Tfn uptake in nonadherent cells. However, similar results were obtained when we assayed endocytosis and recycling of \(^{125}\)I-Tfn in adherent adenovirally infected HeLa cells using the methodology of others (Refs. 5, 8, and data not shown). A second methodological difference was that previous studies employed a protocol that ensured a rapid bolus of Rab5 overexpression (5, 8). In contrast, the persistent overexpression of Rab5(Q79L) in our system may enable induction of a compensatory mechanism(s) that restores transferrin receptor trafficking to normal steady state rates. Therefore, we adapted our expression system for rapid induction of a bolus of protein expression. For these experiments, cells were infected with a 30-fold higher

### Fig. 1. HA-Rab5(Q79L) expression in stably transformed tTA-HeLa cells causes enlarged endosomes but does not affect Tfn endocytosis or recycling.

A, tTA-HeLa cells stably expressing either HA-Rab5(WT) or HA-Rab5(Q79L) under control of a tetracycline (tet)-regulatable promoter were clonally selected as described under “Materials and Methods.” Cells were incubated in the absence (induced) or presence (uninduced) of 1 \(\mu\)g/ml tetracycline for 48 h. A, immunoblots of cell lysates probed with antibodies against Rab5 showing expression of endogenous and HA-tagged Rab5.

B, transformed tTA-HeLa cells uninduced or induced to express either HA-Rab5(WT) or HA-Rab5(Q79L) as indicated were incubated with Alexa-Tfn (lower panels) for 20 min at 37 °C before fixation, permeabilized in 0.1% saponin, and processed for indirect immunofluorescence using the 12CA5 anti-HA antibody (upper panels), as described under “Materials and Methods.” C, single-round kinetics of uptake and recycling of prebound biotinylated Tfn in tTA-HeLa cells uninduced or induced to express either HA-Rab5(WT) or HA-Rab5(Q79L) for 48 h as described under “Materials and Methods.” Results are average ± S.D. of three independent experiments.

### Fig. 2. High levels of Rab5(Q79L) expression cause enlargement of endosomes without perturbing endocytic trafficking of transferrin.

A, tTA-HeLa cells infected with adenoviruses expressing either HA-Rab5(WT) or HA-Rab5(Q79L) under the control of a tetracycline-regulatable promoter were incubated for 18 h in the presence or absence of tetracycline as described under “Materials and Methods.” A, immunoblots of cell lysates probed with either anti-Rab5 antibodies (right panel) or anti-HA antibodies (left panel). B, immunofluorescence images of adenovirally infected tTA-HeLa cells expressing either HA-Rab5(WT) or HA-Rab5(Q79L) incubated with Alexa-Tfn and subjected to indirect immunofluorescence with anti-HA monoclonal antibody as described under “Materials and Methods.” C, single-round kinetics of uptake and recycling of prebound biotinylated Tfn in uninfected tTA-HeLa cells or in cells infected with either HA-Rab5(WT)-encoding adenoviruses or HA-Rab5(Q79L)-encoding adenoviruses assayed 18 h after infection as described under “Materials and Methods.” Inset shows expanded axis for early time points. Results are average ± S.E. of two independent experiments.
m.o.i. of adenovirus (see “Materials and Methods” for details), and Rab5 expression was controlled using cycloheximide. Briefly, tTA-HeLa cells were infected with adenovirus (m.o.i. of 300) for 2 h. Cells were then treated with 10 μg/ml cycloheximide for 3 h to accumulate mRNA in the absence of protein expression. The cycloheximide was washed from the cells, and protein was expressed for the indicated periods of time before experiments were performed. As shown in Fig. 3A, this protocol allows for tight control of protein synthesis while permitting a regulatable, high level of Rab5 expression. Within 1 h of HA-Rab5(Q79L) expression, exogenous HA-Rab5(Q79L) levels were estimated to be 50-fold over endogenous Rab5 (Fig. 3A). The level of HA-Rab5(Q79L) expression continued to increase with increased time of incubation in the absence of cycloheximide, plateauing at 6–12 h postinfection. A similar expression pattern was seen when cells were infected with HA-Rab5(WT) adenovirus (data not shown). After only an hour of protein synthesis, the enlarged endosomal phenotype could be detected (Fig. 3B). Increased duration of HA-Rab5(Q79L) synthesis caused a successive increase in the size of early endosomes, whereas increased HA-Rab5(WT) expression caused only minor increases in endosome size.

At each time point, we performed a kinetic analysis of transferrin uptake and recycling (Fig. 4). Consistent with our findings thus far, despite the dramatic changes in endosomal morphology seen at even the earliest time points of HA-Rab5(Q79L) expression (1 h), we were unable to detect changes in endocytosis or steady state accumulation of TfnR within the cell. Although endosome size continued to enlarge at 3 and 12 h of HA-Rab5(Q79L) expression, there was similarly no effect on the rates or efficiency of TfnR uptake and intracellular accumulation compared with uninfected cells or cells infected in the presence of 1 μg/ml tetracycline, which served as controls. In all cases, Tfn uptake was maximal at 5 min, and recycling occurred with a half-time of ~7–8 min, consistent with results of others (18–20).

Rates of Bulk Phase Endocytosis and Lipid Recycling Are Unaffected by Trafficking through Enlarged Endosomes—It has been proposed (1, 2) that sorting in the early endosome occurs, at least in part, by a default mechanism based on the geometry of the tubulovesicular early endosome. In this model, the high surface area of the tubular portions of the endosome facilitates recycling of membrane-associated components, perhaps through an iterative process (19, 21). Others have argued...
that more directed sorting mechanisms are required for the highly efficient endocytic trafficking of recycling receptors such as the TfnR (22). The appearance of coated buds containing TfnR on early endosomes (22) and the sorting motif-dependent inhibition of TfnR recycling by bafilomycin (23) support this latter hypothesis. Thus, our inability to detect an effect on the kinetics and efficiency of TfnR uptake and recycling in cells despite dramatic alterations in early endosome size and geometry may reflect the involvement of Rab5-independent, directed sorting events. Consistent with this possibility, one notable difference in our experiments compared with others is that in previous studies human TfnR vectors were introduced in parallel with the Rab5(WT) and Rab5(Q79L) constructs (8), whereas we are studying transport kinetics of endogenous receptors. Thus, it is possible that at higher levels of expression TfnR endocytic trafficking becomes more sensitive to alterations in endosomal morphology and/or Rab5 function than that of endogenous Tfn receptors. Importantly, we obtained similar results when examining endogenous TfnR endocytosis and recycling kinetics using adenoavirally infected HepG2 cells expressing Rab5(Q79L) (data not shown).

To determine whether the dramatic changes in early endosome morphology affect the bulk sorting properties of early endosomes as predicted by current models, we examined the kinetics of endocytosis of a bulk fluid phase marker and the kinetics of recycling of a bulk membrane lipid marker. To focus on the rates of volume endocytosis rather than the extent of volume accumulation, we analyzed the initial rate of fluid phase HRP uptake. As can be seen (Fig. 5, A–C), we were unable to detect differences in the rate of HRP endocytosis at either 1, 3, or 12 h after bolus induction of expression of WT or mutant Rab5 compared with either uninduced or uninfected controls. Previous studies on HRP uptake in Rab5-expressing cells (7) focused on later time points of uptake when changes in endosomal volume will be reflected by increased accumulation of HRP at steady state. Our results suggest that GTP hydrolysis by Rab5 is not rate-limiting for bulk or receptor-mediated endocytosis in HeLa cells.

We next measured the rates of bulk membrane recycling in cells overexpressing Rab5(Q79L), expecting that membrane lipids would accumulate in the enlarged vacuolar portions of the early endosome slowing their recycling. For these experiments we used C6-NBD-SM, a readily extractable, fluorescently labeled membrane lipid (17). Briefly, cells were labeled with C6-NBD-SM for 10 min at 37 °C to allow the C6-NBD-SM to traffic to the early endosomes. After extracting the plasma membrane C6-NBD-SM through a series of backwashes in a fatty acid-free bovine serum albumin solution, dissociable C6-NBD-SM was measured by the fluorescence in the medium. Unexpectedly, there was no appreciable difference in the rate or extent of C6-NBD-SM recycling from the endosome to the plasma membrane at any time point after induction of Rab5 WT or mutant overexpression (Fig. 5, D–F). Thus, in these cells, efficiency of recycling of either bulk membrane or TfnR was not affected by dramatic changes in endosome geometry.

Conclusions—We find that the early endosomal compartment significantly expands in cells overexpressing the constitutively active Rab5 mutant, Rab5(Q79L). This finding is consistent with previous work of others (8, 24) and with the model that Rab5 plays a critical role in early endosome biogenesis and morphogenesis by controlling the rate of endosome fusion events while in the Rab5/GTP-bound form (4, 5). Unexpectedly, and in contrast to previous reports (7, 8), in the presence of Rab5(Q79L) overexpression we observed no detectable acceleration in the kinetics of Tfn receptor or fluid phase uptake. Further, there were no changes in steady state, intracellular accumulation of the Tfn receptor or lipid recycling despite the appearance of these morphologically altered early endosomes. Our results were obtained at a variety of levels of Rab5(Q79L) overexpression, which caused varying degrees of change in endosome morphology and after even brief exposure to mutant Rab5 provided little opportunity for the induction of compensatory mechanisms.

Overexpression of dominant-negative Rab5 mutants (e.g. Rab5(N133I) or Rab5(S34N)) has been shown by several groups to inhibit TfnR and fluid phase endocytosis and endosome fusion (5, 8). These mutations block Rab5 function and can exert their inhibitory effects independently of downstream effectors. By contrast, the activating mutant studied here, Rab5(Q79L), will require interaction with downstream effectors to manifest its effects. Cell type and other variables may determine whether specific downstream effectors of Rab5 are limiting and therefore whether overexpression of Rab5(Q79L) will alter the kinetics of membrane trafficking along the endocytic recycling pathway. Thus, our results do not rule out a function for Rab5 in controlling membrane trafficking through the early/sorting endosome.

Regardless, the important findings from these studies are
2-fold. First, our results clearly establish that Rab5-induced changes in early endosomal morphology are not predictive of defects in endocytic membrane trafficking. Second, our results argue that geometric considerations may contribute to a lesser extent than previously assumed in determining the bulk sorting and recycling efficiencies of the early endosomal compartment. Morphometric measurements made of the tubular and vesicular portions of early endosomes in baby hamster kidney cells (25) show that 50–70% of total endosomal volume and 55–90% of total surface area are associated with the tubular portions of the endosome. Although there is considerable inherent error in these measurements (25), they also suggest that endosomal geometry would not be sufficient to account for the observed efficiency of sorting and recycling.

Acknowledgments—We thank Dr. Yoram Altschuler for assistance with recombinant adenovirus production, Dr. Shana Barbas for construction of HA-tagged Rab5, and Drs. Marino Zerial, Fred Maxfield, and members of the Schmid Laboratory for helpful discussion and/or critical reading of the manuscript.

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