Role of the Small GTPase RAB7 in the Late Endocytic Pathway*

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Rab7 is a small GTPase localized to the late endosomal compartment. Its function was investigated by overexpressing dominant negative or constitutively active mutants in BHK-21 cells. The effects of such overexpression on the internalization and/or degradation of different endocytic markers and on the morphology of the late endosomal compartment were analyzed. We observed a marked inhibition of the degradation of \(^{125}\)I-low density lipoproteins in cells transfected with the Rab7 dominant negative mutants while the rate of internalization was not affected. Moreover in these cells there was an accumulation of many small vesicles scattered throughout the cytoplasm. In contrast, overexpression of the activating mutants led to the appearance of atypically large endocytic structures and caused a dramatic change in the distribution of the cation-independent mannose 6-phosphate receptor. Our data indicate that the Rab7 protein in mammalian cells is present on a late endosomal compartment much larger than the compartment labeled by the cation-independent mannose 6-phosphate receptor. Rab7 also appears to play a fundamental role in controlling late endocytic membrane traffic.

Rab proteins are small GTPases localized at the cytoplasmic face of specific subcellular compartments in the endocytic and exocytic pathways. Several studies, using in vitro or in vivo assays, demonstrated a key role for these proteins in the regulation of intracellular vesicle trafficking (1, 2). Although the specific function of Rab proteins is not yet understood, the currently accepted model postulates that Rab proteins are restricted to the cation-independent mannose 6-phosphate receptor (CI-MPR) positive compartment.

In this study we have performed a detailed analysis of the Rab7 localization and function by transiently overexpressing several mutants in the BHK-21 cell line. We then investigated the changes induced by these proteins on the morphology of the late endosomal compartment and on the uptake and/or degradation of different endocytic markers, two of which (transferrin and LDL) are physiological ligands. Our results indicate that the Rab7 protein plays an important role in the organization of the late endosomal compartment and that its localization is not restricted to the cation-independent mannose 6-phosphate receptor (CI-MPR) positive compartment.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids Rab5, Rab5N133I, and the human transferrin receptor have been previously described (11, 12). Rab7 mutants were constructed by polymerase chain reaction-mediated mutagenesis (13). The oligonucleotides used in the first amplification for Rab7T22N, Rab7I41M, Rab7Q67L, Rab7N125I, and Rab7C were, respectively: 5'-TCGGAGTTGGTAAAGATCTCATGATGACACG-3', 5'-CACGAAGTCTGTCCCTCCTGGACTGTGTA-3', 5'-GGAAAGCGCTTCAGCAGCCGCTGTCGTC-3', 5'-CGTGTTTTCGAGGTCAATCTTGATTCCC-3', and 5'-CTGGAGTTGGTAAAGATCTCATGATGACACG-3'.

The abbreviations used are: wt, wild type; CI-MPR, cation-independent mannose 6-phosphate receptor; LDL, low density lipoproteins; LDI, lipoprotein-deficient serum; vT7, recombinant vaccinia T7 virus; PBS, phosphate-buffered saline.

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Cell Culture and Transient Transfection—All tissue culture reagents were from Life Technologies, Inc. BHK-21 cells were grown in Glasgow minimal essential medium supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin and grown in a 5% CO₂ incubator at 37 °C. BHK-21 cells were infected with the V77 recombinant vaccinia virus and transfected as described (11). The transfection reagent was either DOTAP obtained by Boehringer Mannheim or made by sonication dioleoyl-1-α-phosphatidylethanolamine and dimethylcholesterol ammnonium bromide as described (16). Cells were transfected for 4–6 h and then processed for immunofluorescence or biochemical assays.

Antibodies and Confocal Immunofluorescence Microscopy—The 9E10 anti-myc monoclonal and the CI-MPR polyclonal antibodies were kind gifts from Dr. S. Fuller and Dr. B. Hofflack, respectively. The anti-Rab7 antibody was prepared as described (4). Anti-LY antibodies were bought from Molecular Probes. Cells grown on 11-mm round glass coverslips were permeabilized and fixed as described previously (11). The secondary antibodies fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-conjugated anti-mouse or anti-rabbit were from Sigma or Amersham. Cells were viewed with the EMBL confocal microscope.

Western Blot and GTP Overlay—Cells were lysed in standard SDS sample buffer and extracts were electrophoresed on 12% SDS-polyacrylamide gels. For immunoblotting, separated proteins were transferred to nitrocellulose membrane. The filter was then blocked in 5% milk in PBS for 40 min at room temperature. Then primary mouse monoclonal 9E10 anti-myc antibody was added at the appropriate dilution and incubated for 2 h at room temperature. The filters were washed, incubated with a secondary anti-mouse horseradish peroxidase-conjugated antibody for 1 h at room temperature, and the bands were visualized using the enhanced chemiluminescence system (ECL, Amersham). For GTP overlay, after electrophoresis the gel was treated, transferred to nitrocellulose filter, and incubated with [α-32P]GTP as described (11). Estimation of Transferase Binding, Endocytosis, and Recycling—Human transferase (Sigma) was labeled to a specific activity of 105 cpm/mg using IODO-GEN (17). BHK-21 cells were transfected with the human transferase receptor plasmid alone or together with the different plasmids encoding Rab7 wt and mutant proteins and treated as described (18, 19). The amount of transferrin endocytosed, recycled, and membrane bound was measured by γ-radiation counting.

Estimation of Horseradish Peroxidase Internalization—BHK-21 cells were transfected for 4 h with the plasmids encoding wild-type or mutant Rab7 proteins and then allowed to internalize horseradish peroxidase (5 mg/ml) in serum-free Glasgow minimal essential medium for times ranging from 1 to 60 min. Cells were then transferred on ice, washed extensively with PBS, 0.1% bovine serum albumin, and lysed in 10 mM Hepes (pH 7.4), 0.2% Triton X-100. The horseradish peroxidase content in the postnuclear supernatant was estimated using o-dianisidine (20). The total amount of protein was determined by colorimetric methods (21). Internalization of Lucifer Yellow—Lucifer Yellow was diluted in Hank’s balanced salt solution at a concentration of 5–10 mg/ml. Cells grown on 11-mm round glass coverslip were allowed to internalize Lucifer Yellow for 5–10 min at 37 °C. The cells were then washed extensively with Hanks’ balanced salt solution and reincubated at 37 °C for various times in serum-deficient medium. After two washes with Hanks’ balanced salt solution and one wash with PBS the cells were permeabilized, fixed, and processed for immunofluorescence.

Preparation of LDL and Labeling with 125I—Human low density lipoprotein (LDL) (1.019 < d < 1.063 g/ml) was obtained by preparative ultracentrifugation (22) from serum of normolipidemic subjects to which 0.1% sodium azide, 1 mM EDTA disodium salt, and 0.17 TIU/ml of aprotinin were added, in order to prevent degradation of apoprotein. The h-LDL was then concentrated and further purified by double ultracentrifugation at density 1.063 g/ml. Lipoprotein-deficient serum (LDS) was prepared from the same serum samples used for LDL preparation by ultracentrifugation at density 1.210 g/ml. h-LDL and LDS were then extensively dialyzed for 24 h at 4 °C against 0.15 M NaCl, 0.24 mM disodium EDTA (pH 7.4). The protein content of LDL, determined as described (23), ranged between 8 and 10 mg/ml. LDL was labeled with 125I as described (24). The specific activity of [125I-LDL] ranged between 200 and 400 cpm/mg of protein; more than 98% 125I radioactivity was precipitable by trichloroacetic acid and less than 3% was extractable in chloroform-methanol. Radiolabeled LDL was always used within 2 weeks from preparation.

Estimation of 125I-LDL Internalization, Recycling, and Degradation—Cells were incubated for 24 h in medium complemented with human LDS before transfection. Transfected cells were allowed to internalize 125I-LDL that was added to the medium at a concentration of 20 μg/ml for 5 h and the amount of 125I-LDL surface bound, internalized, and degraded was estimated as described (25). Briefly, to determine the amount of 125I-LDL degraded, the medium was collected, treated with trichloroacetic acid, extracted with chloroform and hydroquinone, and iodine was precipitated with a mixture of cesium carbonate and cesium bromide as described (16). Cells were transfected for 4–6 h and then treated with 3 mg/ml Pronase in serum-free medium containing 10 mM HEPES (pH 7.5) for 1 h at 0 °C. The cells were recovered by centrifugation at 3000 rpm for 3 min. The radioactivity present in the Pronase-medium (125I-LDL surface bound), cell-associated (125I-LDL internalized), and the acid-soluble material from the incubation medium (125I-LDL degraded) was detected with a γ-counter.

RESULTS

Construction and Expression of Rab7 Mutants in BHK-21 Cells—Using polymerase chain reaction-mediated mutagenesis (13) we introduced point mutations in the GTP binding or hydrolysis domain of the Rab7 cDNA to obtain dominant negative or constitutively active mutants. Similarly to homologous mutations in ras, the mutant Rab7T22N should have a reduced affinity for GTP while the Rab7N125I should have a reduced guanine nucleotide binding. Analogous Rab5 mutant proteins (Rab5S34N and Rab5N135I) behaved as dominant negative mutants in both in vitro and in vivo assays (11, 19, 26, 27). On the other hand, the Rab7T41M and Rab7Q67L mutants are predicted to have an impaired intrinsic (Rab7Q67L) or GAP-mediated (Rab7T41M) GTP hydrolysis and should preferentially bind to the GTP-bound form. The Rab7AC mutant has the last three amino acids deleted. This mutant lacks the cysteines required for membrane association and remains cytosolic (data not shown).

We expressed all the constructs in BHK-21 cells using the vaccinia recombinant V77 infection/transfection system (28). Fig. 1 shows a Western blot (A) and a GTP-binding blot (B) of

FIG. 1. Western blot and GTP overlay on BHK-21 cells expressing myc-tagged Rab7 wt and mutant proteins. A, Western blot analysis on lysates of BHK-21 cells infected with V77 and transfected with pGEMI encoding Rab7, Rab7T22N, Rab7T41M, Rab7Q67L, Rab7N125I, Rab7AC, or nontransfected (NT). The analysis was performed using monoclonal anti-myc antibodies as detailed under “Experimental Procedures.” B, GTP overlay on the same lysates. [32P]GTP binding was performed as described under “Experimental Procedures.” 125I-LDL binding and internalization, after incubation with 125I-LDL, cells were washed 6 times with PBS, 0.1% bovine serum albumin, once with PBS, and then treated with 3 mg/ml Pronase in serum-free medium containing 10 mM HEPES (pH 7.5) for 1 h at 0 °C. The cells were recovered by centrifugation at 3000 rpm for 3 min. The radioactivity present in the Pronase-medium (125I-LDL surface bound), cell-associated (125I-LDL internalized), and the acid-soluble material from the incubation medium (125I-LDL degraded) was detected with a γ-counter.
BHK-21 cells infected with the vT7 virus and transfected with the wt or mutant Rab7 myc-tagged plasmids. As a control we used cells that were infected with the vT7 virus but nontransfected. Western blot analysis was performed with a monoclonal anti-myc antibody. The myc-tagged Rab7 wt and mutant proteins were expressed at comparable levels (Fig. 1A) and more than 15-fold over the endogenous levels (data not shown). This high expression was reached because of the presence of a perfect Kozak consensus sequence in front of the myc epitope (14). As expected, the Rab7 wt and the Rab7I41M, Rab7Q67L and Rab7D mutant proteins bind efficiently GTP whereas the Rab7T22N or Rab7N125I proteins do not bind the nucleotide (Fig. 1B).

Immunofluorescence Analysis of Cells Expressing Rab7 wt or Mutant Proteins—The Rab7 protein was previously localized to the late endosomes in BHK-21, Madin-Darby canine kidney, and NRK cells (4). We analyzed by double immunofluorescence the effect of the overexpression of the Rab7 wt and mutant proteins on the late endosomal compartment in BHK-21 cells. We used the monoclonal anti-myc antibody to detect the myc-tagged overexpressed proteins and a polyclonal anti-CI-MPR antibody to identify the late endosomal compartment. The CI-MPR is localized to early endosomes, late endosomes, and trans-Golgi network but the bulk of the protein is in the late endosomal compartment (29). Overexpression of the myc-tagged Rab7 wt protein results in the staining of a vesicular compartment mainly confined to the perinuclear area (Fig. 2E), similar to the localization of the endogenous protein (4). Surprisingly, there was only partial co-localization between the myc-tagged Rab7 wt protein and the CI-MPR in BHK-21 cells (Fig. 2, A and E). The two proteins were in the same perinuclear area but the wt Rab7 staining was more widely distributed in the cell compared to the CI-MPR staining. This would suggest that the CI-MPR is present only in restricted regions of the late endosomal compartment or that Rab7 is not confined to late endosomes. Expression of the two dominant negative mutants, Rab7T22N (Fig. 2F) or Rab7N125I (Fig. 2G), in BHK-21 cells resulted in the labeling of small vesicles scattered throughout the cytoplasm and also present under the plasma membrane. In contrast, the expression of the two activating mutants, Rab7I41M (data not shown) or Rab7Q67L (Fig. 2, H, M, and N), led to the formation of larger vesicles again not restricted to the perinuclear area. The distribution of the CI-MPR also changed dramatically upon overexpression of Rab7Q67L. Normally, the CI-MPR staining was mainly restricted to perinuclear structures such as in cells transfected with Rab7 wt, Rab7T22N, and Rab7N125I (Fig. 2, A-C). When the Rab7Q67L was expressed the CI-MPR staining was no longer confined to these perinuclear structures (Fig. 2D). Other cells transfected with Rab7Q67L are shown in Fig. 2, I, J, M,
and N. Arrows indicate nontransfected cells in which the distribution of the CI-MPR was unaffected. Expression of the Rab7D mutant resulted in a cytosolic localization of this protein and had no effect on the distribution of the CI-MPR (data not shown).

The Rab7 Compartment Is Reached by Lucifer Yellow—Lucifer Yellow is a fluid phase endocytic marker that labels early endosomes in the first 5–10 min of internalization and, after longer incubation times, late endosomes and lysosomes. We investigated whether this marker was able to stain the Rab7 compartment. Cells transfected with the different constructs were first allowed to internalize Lucifer Yellow for 5 min to primarily label the early endosomal compartment. After extensive washing the cells were reincubated at 37 °C for various times to allow accumulation of the marker in late endosomes and lysosomes. 5 min of internalization were sufficient to detect the Lucifer Yellow marker in small vesicles scattered throughout the cell. No co-localization with the Rab7 wt protein, that was mainly restricted to the perinuclear area (Fig. 2, K and O) or with any other Rab7 mutant protein (data not shown), was observed. After 30 min of chase Lucifer Yellow was mainly present in the perinuclear region and found to co-localize with the Rab7 wt protein (Fig. 2, L and P). A similar co-localization was found when the different Rab7 mutant proteins were expressed (data not shown).

Transferrin and Horseradish Peroxidase Endocytosis Are Not Altered in BHK-21 Cells Overexpressing Rab7 wt and Mutant Proteins—To analyze the effect of the overexpression of the Rab7 mutant proteins on the first steps of endocytosis we measured the internalization of transferrin and horseradish peroxidase.

Transferrin binds iron at the cell surface, is clustered into coated pits, and is internalized by coated vesicles. It is then transported to the early endosomes where it looses the iron and is recycled back to the membrane together with its receptor. Overexpression of Rab5 doubles the rate of internalization of transferrin whereas expression of the dominant negative mutant Rab5N133I inhibits endocytosis of this marker, as described previously (11, 18, 19). As expected the initial rate of internalization of 125I-transferrin was altered in cells expressing Rab5 or Rab5N133I but no significant change was detectable upon overexpression of Rab7 wt or any of the Rab7 mutants. After 5 min there was a 70% increase in the amount of internalized transferrin in cells overexpressing the Rab5 protein and a 30% decrease in cells expressing the Rab5N133I mutant protein (Fig. 3A). Overexpression of all the Rab7 constructs also did not affect the recycling of 125I-transferrin to the plasma membrane (data not shown).

Horseradish peroxidase is currently used as a marker of fluid phase endocytosis. Overexpression of Rab5 and Rab5N133I mutant changes the amount of horseradish peroxidase internalized in the cells (11). Overexpression of Rab5 wt doubles the amount of horseradish peroxidase accumulated in the cells after 60 min of internalization, while the expression of the Rab5N133I mutant causes a 30% decrease (Fig. 3B). No significant changes were detected after overexpression of the Rab7 wt or mutant proteins at this (Fig. 3B) and at earlier or later time points (data not shown).

LDL Degradation Is Severely Affected by the Expression of Dominant Negative Rab7 Mutants—To study the effect of Rab7 on the endocytic pathway we examined the internalization and degradation of 125I-LDL in BHK-21 cells overexpressing the Rab7 wt and mutant proteins. LDL binds a specific receptor on the surface of cultured fibroblast with high affinity. LDL is internalized and degraded, so that its cholesterol component is made available for cellular membrane synthesis while the receptor is recycled to the plasma membrane. Only a very small amount of LDL is recycled to the plasma membrane.

Before transfection, BHK-21 cells were incubated for 24 h in medium complemented with human LDS. After transfection the cells were allowed to internalize 125I-LDL added to the medium at a concentration of 20 μg/ml for 5 h and the amount of 125I-LDL surface bound, internalized, and degraded was estimated as described under “Experimental Procedures.” Fig. 4 shows a typical experiment of 125I-LDL internalization (Fig. 4A) and degradation (Fig. 4B). The accumulation of 125I-LDL in BHK-21 cells showed a 50% increase in cells overexpressing Rab5 (Fig. 4A). This is consistent with the contention that the Rab5 protein is able to accelerate the kinetics of endocytosis (11). An increase of approximately 50% in the intracellular accumulation of 125I-LDL was also observed in cells expressing Rab7T22N and Rab7N125I, compared to control cells (Fig. 4A). However, this effect was accompanied by a strong inhibition (more than 60%) of the degradation of LDL (Fig. 4B). Inhibition of 125I-LDL degradation was also observed in cells transfected with the Rab5N133I mutant protein but in this case the amount of 125I-LDL internalized was approximately 30% less than in control cells (Fig. 4A). This is consistent with previous findings that dominant negative mutants of Rab5 inhibit endocytosis (11, 18, 19).

No effect on 125I-LDL intracellular accumulation or degradation was detected upon overexpression of the Rab7AC mutant compared to control cells. This is consistent with the hypothesis that this mutant is not post-translationally modified, is not able to attach to the membrane, and therefore is not functional.

To demonstrate that Rab7 did not interfere with the first steps of endocytosis we measured the initial internalization rate of 125I-LDL in cells expressing the Rab7 and Rab5 wt and mutant proteins (data not shown). After transfection cells were allowed to bind 125I-LDL for 2 h at 4 °C and the excess of unbound ligand was removed by washing. In all transfected cells the number of surface 125I-LDL-binding sites was similar and 90% of 125I-LDL was specifically bound. To measure the rate of uptake the cells were then shifted at 37 °C for different periods of time ranging from 30 s to 60 min and the fraction of internalized 125I-LDL was determined. After 5 min control cells had internalized 70% of prebound 125I-LDL while cells expressing the dominant negative mutants of Rab5 had internalized only 40%. Conversely, the internalization rate of 125I-LDL was accelerated in cells expressing the Rab5 wt protein since in these cells about 70% of the prebound 125I-LDL was internalized within 2.5 min. These data are consistent with previous studies showing that Rab5 regulates the kinetics of receptor-mediated endocytosis (11, 19). No effect was detected on the initial internalization rate upon overexpression of Rab7 wt or any mutant protein (data not shown).

These data demonstrate that Rab5 and Rab7 have distinct roles in regulating the endocytic pathway. While Rab5 controls the early steps and is able to modify the kinetics of endocytosis, Rab7 does not influence the initial steps of the pathway but has a fundamental role in regulating transport to late endocytic compartments.

DISCUSSION

In this paper we have studied the role of Rab7 in the endocytic pathway. We transiently expressed in BHK-21 cells several mutants of the protein with impaired ability to bind or hydrolyze GTP and we monitored their effect on the morphology of the late endosomal compartment and on the internalization and/or degradation of different endocytic markers.

We demonstrated that Rab7 plays an important role in late
steps of endocytosis since the expression of the two dominant negative mutant proteins causes a marked inhibition of the degradation of 125I-LDL while the initial internalization rate remains unchanged. Moreover, no effect of the expression of any Rab7 mutant protein was detected in the internalization and/or recycling of transferrin or horseradish peroxidase. These findings show that Rab7 does not function in the early endocytic pathway and does not influence the kinetics of internalization. The early steps of endocytosis are instead regulated by the Rab5 proteins (18). Rab7 therefore functions in a later step compared to Rab5 and influences the late endosome organization. Consistent with this conclusion, expression of different mutants impaired in their ability to bind or hydrolyze GTP causes big changes in the morphology of the compartment as judged by the immunofluorescence distribution of CI-MPR and the Rab7 mutant proteins.

The Rab7 protein has been previously localized to late endosomes in NRK cells by co-localization with the CI-MPR by electron microscopy studies (4). However, surprisingly, the protein seems to be present mainly in a CI-MPR negative compartment in BHK-21 cells. This compartment is labeled in 30 min by Lucifer Yellow, a fluid phase marker of the endocytic pathway, suggesting that the CI-MPR labels only restricted areas of the late endosomal compartment (Fig. 2). Remarkably, we also noticed a drastic change in the distribution of the CI-MPR upon expression of the Rab7Q67L constitutively active
mutant. These data disagree with a previous report in which the distribution of the CI-MPR in a stable cell line expressing the Rab7Q67L did not show any detectable change (9). The organization of the endosomal compartment seems to be quite different between BHK-21 cells, used in this study, and the inducible HeLa cell lines used by Chavrier and colleagues (9). However, we have transiently expressed the Rab7Q67L mutant also in HeLa cells and could detect a similar effect even if less pronounced (data not shown). One possible explanation for this discrepancy is that in our experiments the use of the vT7 infection/transfection expression system causes a higher level of expression of the mutant protein (more than 15-fold over the endogenous) in a very short time (4 h). In the stable cell lines used by Chavrier and colleagues (9) lower levels of expression (4–5-fold over the endogenous) were achieved after a very long time (3 days of induction). During this time cells may have

Fig. 4. Effects of expression of Rab7 wt and the various mutant on 
$^{125}$I-LDL internalization and degradation. Cells were grown for 24 h in LDS, infected with the vT7 recombinant vaccinia virus, and transfected with the different constructs as indicated. After 4 h of transfection cells were washed extensively and incubated 5 h at 37 °C in the presence of 20 μg/ml $^{125}$I-LDL. The amount of $^{125}$I-LDL internalized (A) or degraded (B) is plotted as percentage of control cells. The data represent the average of five independent experiments with error bars.
adapted to the presence of the Rab7Q67L protein and might have been able to overcome the redistribution of the CI-MPR.

The expression of the constitutively active Rab7Q67L protein causes the formation of large endocytic structures while expression of the two dominant negative mutants (Rab7T22N and Rab7N125I) gives a very fine and punctate staining. These effects resemble those produced by Rab5 on early endosomes. Expression of the Rab5Q79L activating mutant induces the formation of giant early endosomes while expression of the Rab7 protein plays a fundamental role in the endocytic pathway at the level of late endosomes. This hypothesis is confirmed by the fact that \(^{125}\)I-LDL degradation is inhibited in cells expressing the Rab7T22N and Rab7N125I mutant proteins. In these cells a delayed delivery to the late endocytic compartment due to inhibition of fusion would prevent efficient degradation of \(^{125}\)I-LDL and would allow its accumulation in the cells (Fig. 4).

Our data do not discriminate whether the Rab7 protein controls early to late endosomes or late endosomes to lysosomes transport or both. The development of in vitro assays to study these two different steps will be required to solve this issue. However, our results indicate that the Rab7 protein plays a fundamental role in the endocytic pathway at the level of late endosomes, probably controlling fusion. This is demonstrated by the morphological changes of the compartment upon overexpression of the Rab7 wt and mutant proteins and by the strong effect on LDL degradation upon expression of the dominant negative mutants.

Further characterization of this late endosomal compartment by morphological and biochemical assays together with the identification of interacting components of Rab7 will be required to pinpoint the site of action of this protein and gain complete understanding of its role in the late endocytic pathway.