Rab7 is required for the normal progression of the autophagic pathway in mammalian cells

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Summary

Autophagy is a normal degradative pathway that involves the sequestration of cytoplasmic components and organelles in a vacuole called an autophagosome that finally fuses with the lysosome. Rab7 is a member of the Rab family involved in transport to late endosomes and in the biogenesis of the perinuclear lysosome compartment. To assess the role of Rab7 in autophagy we stably transfected CHO cells with wild-type pEGFP-Rab7, and the mutants T22N (GDP form) and Q67L (GTP form). Autophagy was induced by amino acid starvation and the autophagic vacuoles were labeled with monodansylcadaverine. By fluorescence microscopy we observed that Rab7wt and the active mutant Rab7Q67L were associated with ring-shaped vesicles labeled with monodansylcadaverine indicating that these Rab proteins associate with the membrane of autophagic vesicles. As expected, in cells transfected with the negative mutant Rab7T22N the protein was diffusely distributed in the cytosol. However, upon induction of autophagy by amino acid starvation or by rapamycin treatment this mutant clearly decorated the monodansylcadaverine-labeled vesicles. Furthermore, a marked increase in the size of the monodansylcadaverine-labeled vacuoles induced by starvation was observed by overexpression of the inactive mutant T22N. Similarly, there was an increase in the size of vesicles labeled with LC3, a protein that specifically localizes on the autophagosomal membrane. Taken together the results indicate that a functional Rab7 is important for the normal progression of autophagy.

Key words: Autophagy, Rab7, Autophagosome, Lysosome, LC3, Starvation

Introduction

Autophagy is the main pathway for sequestration and targeting of cytoplasmic components to the lytic compartment (for a review, see Klionsky and Emr, 2000). Morphological studies have indicated that autophagocytosis is used for bulk, nonselective transport of cytosol and even organelles to the lysosome (Dunn, 1990a). The autophagic process is initiated when an isolation membrane or phagophore, a cup-shaped membrane that probably originates from specialized regions of the ER, engulfs part of the cytoplasm (Dunn, 1990a). The resulting vacuole (i.e. autophagosome) is a double membrane structure that becomes acidic, loses the inner membrane and fuses with endocytic vesicles, to finally acquire hydrolytic enzymes by fusion with lysosomes. These autophagolysosomes are in charge of the degradation of the sequestered materials (Dunn, 1990a; Dunn, 1990b). Under starvation conditions autophagic degradation is enhanced (for a review, see Klionsky and Emr, 2000) in order to provide amino acids for essential metabolic pathways such us gluconeogenesis.

An overlap between endocytosis and autophagocytosis can be demonstrated in mammalian cells by the localization of endocytosed material in autophagic vacuoles (Gordon and Seglen, 1988; Gordon et al., 1992; Liou et al., 1997). Endocytic markers are not detected in autophagic vacuoles after a short uptake which filled the early endosome (Tooze et al., 1990; Punnonen et al., 1993; Munafo and Colombo, 2001). However, after 10 minutes endocytosis-internalized molecules can be detected in autophagic vacuoles. Therefore, the endocytic pathway converges with the autophagic pathway immediately after the early endosome stage (Tooze et al., 1990; Liou et al., 1997).

Rab GTPases constitute the largest family of small GTP-binding proteins with more that 60 members in the human genome (Martinez and Goud, 1998; Chavrier and Goud, 1999). Rab proteins and their cognate partners coordinate sequential steps of transport such as vesicle formation, vesicle motility and finally, tethering/docking to their target membranes (Waters and Pfeffer, 1999). Rab proteins localize in distinct intracellular compartments where they regulate specific transport events. Rab7 is a member of this large family that has been found in late endosomes (Chavrier et al., 1990; Feng et al., 1995; Press et al., 1998; Zuk and Elferink, 1999) and implicated in transport from early to late endocytic compartments (Feng et al., 1995; Press et al., 1998; Mohrmann and van der Sluijs, 1999; Ohashi et al., 1999). It has also been reported that Rab7 is present in a compartment connected to lysosomes (Meresse et al., 1995). More recently, it has been postulated that Rab7 controls the aggregation and fusion of late endosomes/lysosomes and is an essential protein for maintenance of the perinuclear lysosomal compartment (Bucci et al., 2000).

Therefore, given the convergence between the late

endocytic/lysosomal pathway and the autophagic pathway it was important to examine the role of Rab7 in autophagocytosis. This study was performed on stably transfected CHO cells that overexpress EGFP-Rab7 wt and mutants. The generated cell lines were used to test how the expression of these proteins influenced autophagocytosis. The usefulness of mutant Rab GTPases for producing a membrane trafficking block at a particular step has been demonstrated in several vesicular transport pathways. We have used both a GTPase-deficient mutant of Rab7 (Q67L) and a dominant negative mutant (T22N) to examine the localization and the consequences of overexpressing these proteins in the autophagic pathway. By combining morphological and biochemical analyses we show that Rab7 plays a role in the autophagic pathway. Furthermore, we present evidence that the Rab7 dominant negative mutant is targeted to the autophagosome vesicles by a signal transduction mechanism induced by amino acid deprivation.

Materials and Methods

Materials

Minimal essential medium (α-MEM) and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY, USA). All other reagents were from Sigma Chemical Co. (St Louis, MO, USA). The plasmid pEGFP-Rab7 wt and mutants were a generous gift from Bo van Deurs (University of Copenhagen, Copenhagen, Denmark). The pCI-neo Myc-LC3 plasmid was kindly provided by Drs Noboru Mizushima and Tamotsu Yoshimori (National Institute for Basic Biology, Okazaki, Japan). The rabbit polyclonal anti-cathepsin D antibody was a generous gift from Dr William Brown (Cornell University, Ithaca, NY, USA). The monodansyl cadaverine derivative, monodansylpentane (MDH), was kindly provided by Dr H. Elsässer (Marburg, Germany).

Cell Culture

Chinese hamster ovary (CHO) cells were grown to 80% confluence on coverslips in α-MEM supplemented with 10% fetal bovine serum (full nutrient medium) in 6-well plates at 37°C in an atmosphere of 95% air and 5% CO_2.

Generation of pEGFP-Rab7 stably transfected cells

CHO cells were transfected with the plasmids (1 μg/ml) with the LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the instructions supplied by the manufacturer. Stably transfected cells were generated by selection with 0.5 mg/ml geneticin. Cells were subsequently cloned and maintained with 0.1 mg/ml geneticin. The levels of overexpressed GFP proteins were determined by western blot analysis. For this purpose transfected cells were lysed in standard SDS sample buffer and 20 μg of proteins from total cell extracts were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% low fat milk in phosphate-buffered saline (PBS) for 1 hour at room temperature. The membrane was incubated for 2 hours with a mouse monoclonal anti-EGFP antibody (Roche) at 1:2000 dilution, washed and incubated with a secondary HRP-conjugated anti-mouse antibody (Amersham Biosciences Corp., Piscataway, NJ, USA) at a 1:4000 dilution. The bands were visualized with the ECL system (Amersham).

Transfection with myc-LC3

CHO cells stably overexpressing pEGFP-Rab7 wt or the T22N mutant were transiently transfected with the plasmid pCI-neo Myc-LC3 using the lipofectamine reagent (Gibco) according to the manufacturer's instructions. After 36 hours, colocalization studies were done by indirect immunofluorescence with the monoclonal anti-Myc epitope antibody 9E10 (BabCo).

Autophagy induction

Autophagy was induced by amino acid starvation. Cells were washed three times with PBS and incubated with 2 ml Earle's balanced salts solution (EBSS; Sigma) at 37°C for different lengths of time in the presence or the absence of the drugs. In some experiments cells were pre-treated with the inhibitors before induction of autophagy. Alternatively, autophagy was induced by treatment with rapamycin (50 ng/μl) for 2 hours in full nutrient medium (Noda and Ohsumi, 1998; Klionsky and Emr, 2000).

Measurement of autophagy

We have recently developed an assay that allows us to quantitate autophagy through the incorporation of the autofluorescent marker monodansylcadaverine (MDC) (Munafo and Colombo, 2001). Briefly, cells incubated in full nutrient medium or under starvation conditions, were labeled with 0.05 mM MDC in PBS at 37°C for 10 minutes. After incubation, cells were washed four times with PBS and collected in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured by fluorescence photometry (excitation wavelength: 380 nm, emission filter: 525 nm) in a Packard Fluorocount microplate reader. To normalize the measurements to the number of cells present in each well, a solution of ethidium bromide was added to a final concentration of 0.2 μM and the DNA fluorescence was measured (excitation wavelength: 530 nm, emission filter: 590 nm). The MDC incorporated was expressed as specific activity (arbitrary units).

Measurement of long-lived protein degradation

CHO cells overexpressing GFP-Rab7WT, GFP-Rab7Q67L and GFP-Rab7T22N or the vector alone, were plated at 7×10^5 cells/well in a 24-well plates. Cells were then labeled for 24 hours in medium containing [^{3}H]leucine (1 μCi/ml; Amersham Biosciences Argentina, Buenos Aires, Argentina). Cells were washed to remove unincorporated labeled leucine and then incubated for 1 hour in full medium containing unlabeled leucine to allow degradation of short-lived proteins. After three rinses with PBS, cells were incubated in either amino acid-free or full nutrient medium for 6 hours. At the end of the incubation both the trichloroacetic acid (TCA)-precipitable radioactivity of the cell monolayers and the TCA soluble radioactivity present in the medium were measured. Leucine release was calculated as a percentage of the radioactivity in the TCA-soluble supernatant of the total cell radioactivity (Tallóczy et al., 2002; Mizushima et al., 2001).

Cell fractionation

Stably transfected CHO cells overexpressing GFP-Rab7 T22N were incubated in control medium (full nutrient) or under starvation for 2 hours. Cells were trypsinized and centrifuged at 800 ***g*** for 5 minutes. The pellets were resuspended in homogenization buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes, KOH, pH 7.2) and passed 20 times through a 27-gauge needle connected to a syringe. Cell lysates were centrifuged at 1200 ***g*** for 10 minutes at 4°C and the post-nuclear supernatants were centrifuged at 100,000 ***g*** for 60 minutes at 4°C. The pellet containing the membranes was washed twice with 1 M NaCl and 20 μg of proteins (membranes or cytosol) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% low fat milk in PBS for 1 hour at room temperature. The nitrocellulose

membrane was incubated for 2 hours with a mouse monoclonal anti-EGFP antibody (Roche) at 1:2000 dilution, washed and incubated with a secondary HRP-conjugated anti-mouse antibody (Amersham Biosciences Corp., Piscataway, NJ, USA) at a 1:4000 dilution. The bands were visualized with the ECL system (Amersham, NJ).

Fluorescence microscopy

pEGFP-Rab7 transfected CHO cells were analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TE 300, Japan), equipped with a filter system for GFP (excitation filter: 450-490 nm, barrier filter: 515 nm). Images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using the MetaMorph Program, serie 4.5 (Universal Images Corporation). Confocal images were obtained with a Nikon C1 Confocal Microscope System and with the EZ-C1 program (Nikon, Japan). Images were processed using Adobe 7.0 (Adobe Systems).

Visualization of MDC-or MDH-labeled vacuoles

Autophagic vacuoles were labeled with monodansylcadaverine (MDC) by incubating cells grown on coverslips with 0.05 mM MDC in PBS at 37°C for 10 minutes (Biederbick et al., 1995; Munafo and Colombo, 2001). In some experiments cells were labeled with a MDC derivative, monodansylpentane (MDH; 0.1 mM in PBS) (Niemann et al., 2001). After incubation, cells were washed four times with PBS and immediately analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TE 300, Japan) equipped with a filter system (V-2A excitation filter: 380-420 nm, barrier filter: 450 nm). Images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using the program MetaMorph 4.5 (Universal Images Corporation).

Labeling of a late endosome/lysosomal compartment

Stably transfected CHO cells were allowed to internalize Rhodamine-dextran (0.5 mg/ml) for 20 minutes at 37°C by fluid phase endocytosis. The marker was chased for 20 minutes at 37°C to label the lysosomal compartment. Subsequently, cells were incubated for 2 hours in the medium without amino acids (EBSS) to induce autophagy and afterwards labeled with MDH as described above.

Indirect immunofluorescence

Cells were fixed with 2 ml of 3% paraformaldehyde solution in PBS for 15 minutes at room temperature. Cells were washed with PBS and blocked by incubating for 10 minutes with 50 mM NH_4Cl in PBS. Cells were permeabilized with 0.05% saponin in PBS containing 0.2% BSA, and then incubated with the mouse monoclonal anti-Myc epitope antibody 9E10 (BabCo) (dilution 1:50) or with a rabbit polyclonal anti-cathepsin D (dilution 1:50). Bound antibodies were subsequently detected by incubation with the corresponding Texas Red-conjugated secondary antibodies (dilution 1:125 and 1:200, respectively). Cells were mounted with Mowiol and analyzed by fluorescence microscopy using a 510-560 nm excitation filter and a 590 nm barrier filter.

Results

Autophagic vacuoles are decorated by EGFP-Rab7

The notion that the autophagic and endocytic pathways interconnect is widely accepted (Berg et al., 1998; Gordon et al., 1992). Since it has been shown that these pathways converge immediately after the early endosomal compartment we were interested in studying the role of Rab7, a marker for late endocytic structures (Chavrier et al., 1990; Feng et al., 1995; Press et al., 1998), in the autophagic process. For this purpose we generated stably transfected CHO cells that overexpress the chimeric proteins EGFP-Rab7 wt and the mutants Q67L and T22N. Autophagic vacuoles were labeled with the autofluorescent drug monodansylcadaverine (MDC), a specific autophagosomal marker (Biederbick et al., 1995; Munafo and Colombo, 2001). By fluorescence microscopy we observed that, in living cells transfected with pEGFP-Rab7 wt and the active mutant Rab7Q67L (Fig. 1A, d-i), the EGFP signal was associated with round vesicles labeled with MDC, indicating that Rab7 associates with the membrane of autophagic vesicles both in cells incubated under control (not shown) and starvation conditions. As expected, in cells transfected with the dominant negative mutant Rab7T22N the protein was mostly diffusely distributed in the cytosol, under full nutrient conditions (control, Fig. 1A, j-l). Strikingly, upon induction of autophagy by amino acid deprivation, the distribution of the Rab7-negative mutant markedly changed and the protein localized on the MDC-labeled vesicles (Fig. 1A, m-o). It is important to mention that this change in distribution was not observed with another dominant negative mutant, GFP-Rab5S34N (not shown), indicating the specificity of the association.

MDC has been described as a specific autophagic vacuole marker (Biederbick et al., 1995), but since this compound also acts as a lysosomotropic agent, MDC could eventually label some acidic compartments derived form the endocytic pathway. Therefore, we used monodansylpentane (MDH) that is a new derivative of MDC (Niemann et al., 2001). MDH staining of autophagic vacuoles is independent on the acidic pH but shows the same preferences for autophagic membrane lipids as MDC (Niemann et al., 2001). Furthermore, the authors have indicated that under in vivo conditions MDH is a more stringent marker for autophagic vacuoles than is MDC. As shown in Fig. 1B, MDH colocalized with both Rab7 wt and T22N-decorated vesicles in cells subjected to starvation, indicating the autophagic nature of these vesicles. The colocalization of the Rab7 wt- and T22N-decorated vesicles with the markers MDC and MDH was quantified. As shown in Table 1, under starvation condition, 48.2% of the Rab7 wt-labeled vesicles colocalized with MDC whereas the colocalization with MDH was 62.5%. In the case of the mutant Rab7 T22N the colocalization with MDC and MDH was 66.8 and 86.5%, respectively, while in cells overexpressing the GTPase-defective mutant the corresponding colocalizations were 49.28 and 54.57%.

In order to show that Rab7T22N indeed changes its distribution upon induction of starvation we performed a cell fractionation analysis. In cells overexpressing the mutant GFP-Rab7T22N and subjected to control or nutrient-deprived conditions, the membrane and cytosolic fractions were separated by differential centrifugation. The presence of Rab7 in both fractions was determined by western blotting using an antibody against GFP. Our results show that more Rab7 becomes membrane associated in cells subjected to starvation (Fig. 1C), indicating that effectively this Rab7 mutant is recruited to the membranes under conditions that stimulate autophagy.

Rab7 dominant negative mutant is targeted to autophagic vacuoles upon autophagy induction

Early autophagosomes are able to fuse with late endocytic

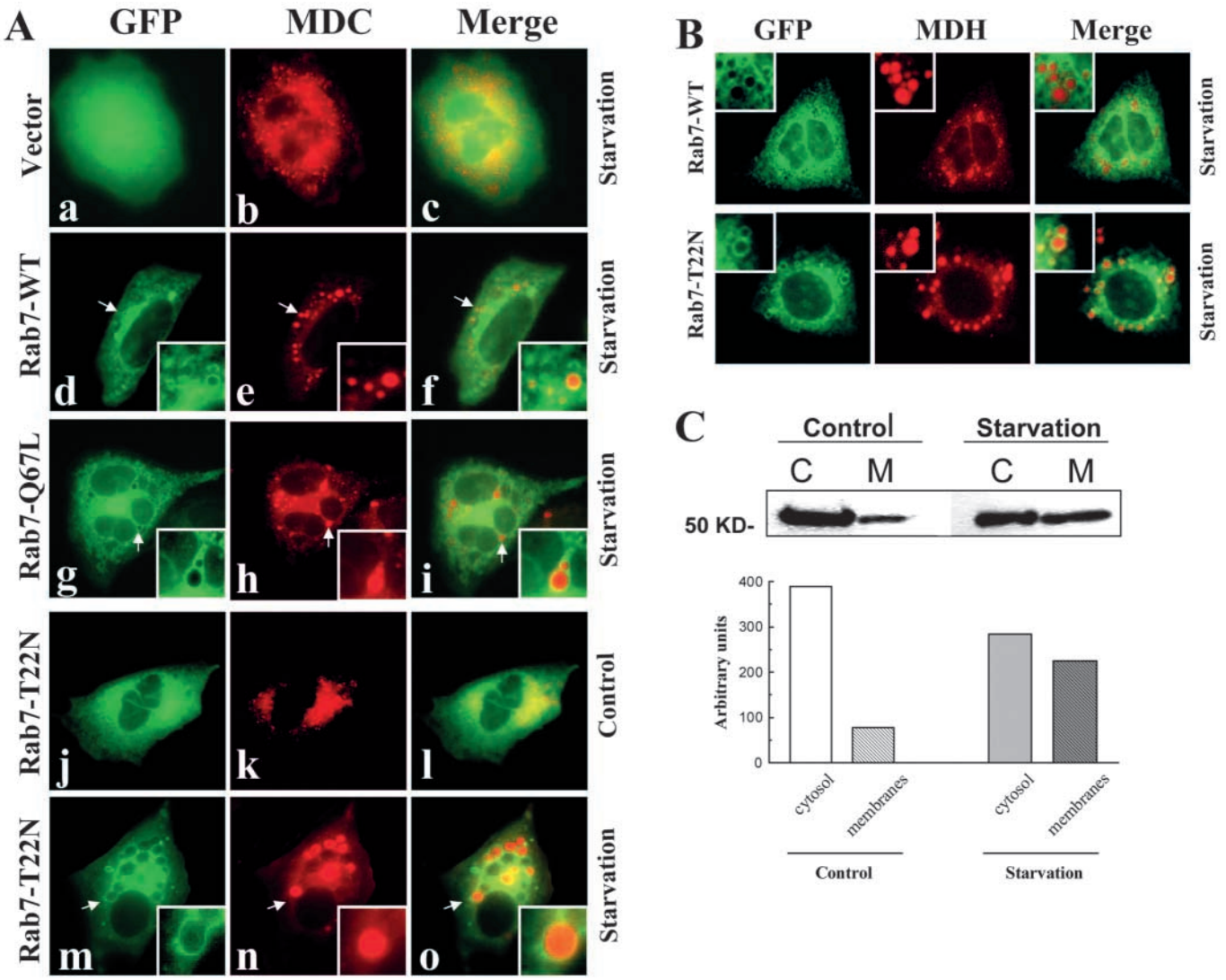

Fig. 1. EGFP-Rab7 colocalizes with monodansylcadaverine (MDC) and monodansyl pentane (MDH), markers for autophagic vesicles. CHO cells stably transfected with pEGFP-Rab7 wt, the mutants Q67L and T22N or with the vector alone (pEGFP) were incubated in full nutrient medium (j-k) or in EBSS medium (starved cells; a-i and m-o) at 37°C for 2 hours. Afterwards, cells were labeled with MDC (A) or MDH (B) and analyzed by fluorescence microscopy as described under Materials and Methods. Arrows indicate colocalization between Rab7 decorated vesicles and MDC. (C) Stably transfected CHO cells overexpressing GFP-Rab7T22N were incubated in control medium (full nutrient) or under starvation conditions for 2 hours. Protein extracts (membranes or cytosol) were subjected to electrophoresis on a 10% SDS-PAGE. The GFP protein was detected by western blotting (top panel) using specific antibodies. The bands were visualized with the ECL system (Amersham, NJ) and quantified (bottom panel) by densitometry. Figure represents one of three independent experiments.

structures to form an intermediate structure, called an amphisome (Gordon and Seglen, 1988; Gordon et al., 1992; Berg et al., 1998). Subsequently, the mature autophagosomes are transformed into autophagolysosomes by fusion with lysosomes. Since Rab7 participates in fusion events between late endocytic structures and also in the biogenesis of lysosomes, the presence of Rab7T22N on the autophagic vacuoles might be a consequence of a fusion event with endocytic vesicles or the protein may be directly targeted to the autophagosomal membrane. In order to distinguish between these two possibilities we have studied the localization of Rab7T22N in the presence of vinblastine, a microtubule disrupting agent, which impedes the influx of endocytosed material into the autophagic pathway, as well as fusion with the lysosomal compartment (Hoyvik et al., 1986; Gordon and Seglen, 1988; Gordon et al., 1992). As shown in Fig. 2 vinblastine did not alter the starvation-induced localization of

Table 1. Colocalization with autophagic and lysosomal markers

	Rab7-WT	Rab7-Q67L	Rab7-T22N
MDC (%)	48.2±5.31	49.28±7.87	66.86±3.24
MDH (%)	62.57±5.76	54.57±7.66	86.58±5.31
LC3 (%)	35.52±2.24	31.63±9.2	36.80±2.04
CathD (%)	51.87±3.15	65.23±8.36	23.87±2.03

Values are means±s.e.m. from three independent experiments. At least 100 Rab7-decorated vesicles were counted for each condition.

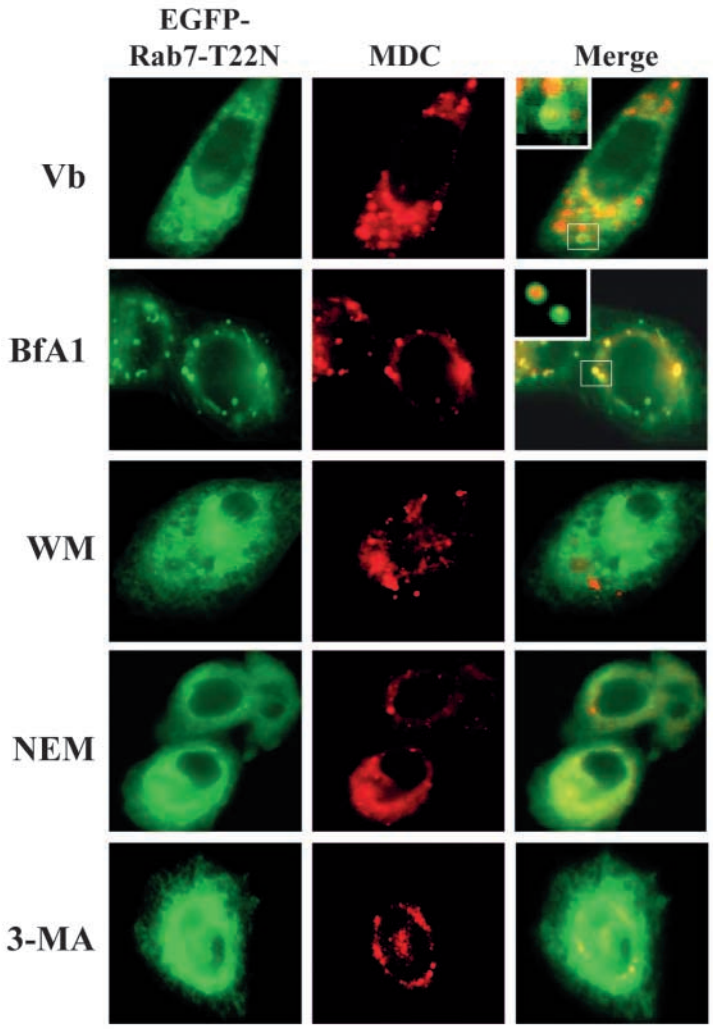

Fig. 2. Targeting of the mutant Rab7T22N to autophagic vacuoles is prevented by inhibitors of autophagosome formation. CHO cells expressing EGFP-Rab7T22N were incubated in EBSS medium (starved cells) at 37°C for 2 hours in the presence of 50 μM vinblastine (Vb), 0.1 μM bafilomycin A1 (BfA1), 200 nM wortmannin (WM) or with 30 mM 3-methyl adenine (3-MA). In this last case, cells were also preincubated with the drug for 2 hours before the starvation period. For the N-ethylmaleimide treatment (NEM) cells were pre-incubated for 15 minutes with 50 μM NEM before induction of starvation. Cells were washed and incubated for a further 2 hours in the starvation medium. Cells were subsequently labeled with MDC and immediately analyzed by fluorescence microscopy as described in Fig. 1.

the mutant Rab7T22N on autophagic vacuoles. Similar results were obtained with bafilomycin A1, an inhibitor of the vacuolar proton ATPase, which was reported to cause accumulation of autophagosomes under starvation conditions by inhibiting the fusion between autophagosomes and lysosomes (Yamamoto et al., 1998; Kabeya et al., 2000). Since bafilomycin A1 ensures the separation of autophagosomes and lysosomes, the presence of Rab7T22N on the autophagic membranes is unlikely to be due to the fusion with lysosomes. However, it is known that overexpression of the dominant negative mutant of Rab7, alters endocytic traffic. Therefore, it is likely that expression of this mutant might hamper the convergence between the endocytic and autophagic pathways (see results below). Taken together the results suggest that Rab7 does not seem to be acquired by the autophagic vacuoles via fusion events with vesicles from the endocytic pathway, rather it is likely that this Rab protein is targeted to the autophagosomal membranes by a signal transduction mechanism in response to amino acid deprivation.

Phosphatidylinositol 3-kinases (PI 3-kinases) participate in the regulation of autophagy (Blommaart et al., 1997; Simonsen et al., 2001). Wortmannin (WM) is a potent PI 3-kinase inhibitor that blocks the formation of autophagic vacuoles (Petiot et al., 2000; Munafo and Colombo, 2001). Therefore we tested the effect of WM on the change in distribution of Rab7T22N in response to amino acid withdrawal. As expected, WM inhibited the formation of autophagic vacuoles in response to starvation (Fig. 2). Moreover, WM prevented the starvation-induced targeting of Rab7T22N to the remaining autophagic vacuoles, indicating that a signal mechanism involving a PI 3-kinase participates in the targeting of this Rab7 mutant to the membranes of autophagosomes.

In a recent report we showed that an N-ethylmaleimide (NEM)-sensitive protein is required for the initial steps of the autophagic pathway. Treatment of cells with NEM blocked the formation of autophagic vacuoles (Munafo and Colombo, 2001). Similar to the results obtained with WM, NEM completely inhibited the localization of Rab7T22N on autophagic vacuoles labeled with MDC (Fig. 2). Similarly, treatment with 3-methyl adenine (3-MA), a classic inhibitor of autophagy, abrogated targeting of the Rab7 mutant. Therefore, our results indicate that targeting of the Rab7 dominant negative mutant is abolished by proven inhibitors of the autophagic pathway.

The TOR kinase pathway is involved in membrane recruitment of Rab7 T22N

Signal transduction cascades allow cells to sense the environmental conditions and to respond appropriately. In response to amino acid deprivation autophagy is upregulated to allow cell survival. TOR is a phosphatidylinositol kinase (PIK)-related kinase (Cutler et al., 1999; Cutler et al., 2001) that plays a central role in the amino-acid sensing mechanism (van Sluijters et al., 2000; Klionsky and Emr, 2000). Treatment with the macrolide antibiotic rapamycin inactivates m-Tor and induces autophagy in both yeast (Noda and Ohsumi, 1998) and mammals (Cutler et al., 1999). Rapamycin mimics the starvation response even under full nutrient conditions.

In order to test whether Rab7T22N is targeted to autophagosomal vacuoles via a Tor-kinase signal transduction mechanism, stably transfected CHO cells overexpressing Rab7T22N were treated with rapamycin and the autophagic vesicles were labeled with MDC. As expected, even in cells incubated under nutrient rich conditions numerous autophagic vacuoles are formed and the vesicles were clearly decorated by GPF-Rab7T22N (Fig. 3).

Taken together our results indicate that the cytosolic distribution of this Rab7 mutant markedly changes upon

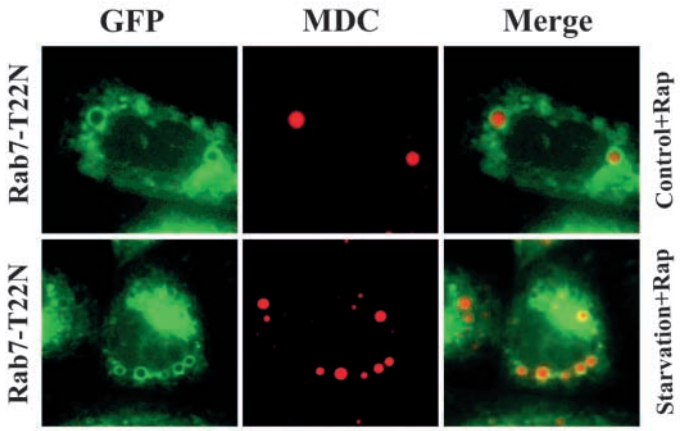

Fig. 3. Rapamycin induces the localization of Rab7T22N to autophagic vacuoles. CHO cells expressing EGFP-Rab7T22N were incubated at 37°C for 2 hours in α-MEM (control cells) or EBSS medium (starved cells) in the presence of 50 ng/μl rapamycin. Cells were briefly washed with PBS, labeled with MDC and immediately analyzed by fluorescence microscopy as described in Fig. 1.

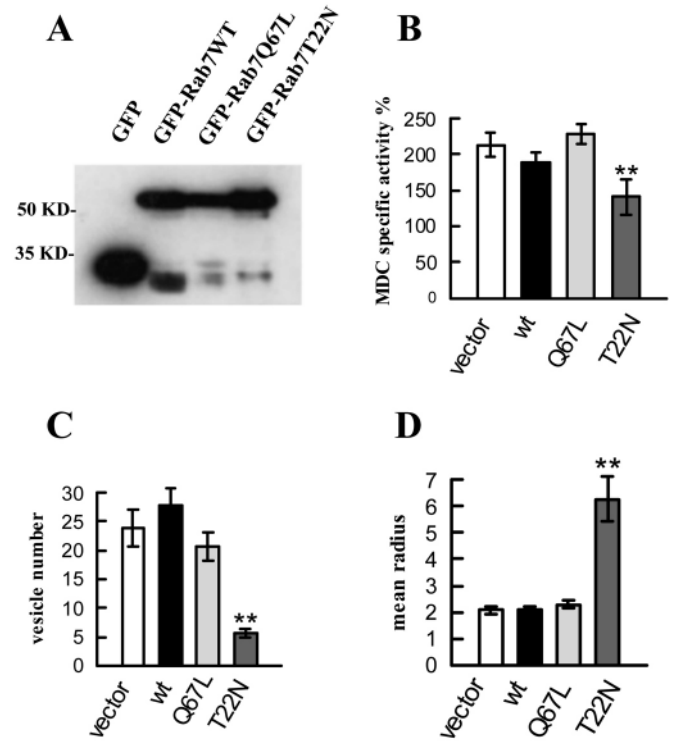

Fig. 4. Overexpression of Rab7T22N accumulates large autophagosomes. (A) The levels of overexpressed EGFP proteins were detected by western blot using an anti-EGFP antibody as indicated under Materials and Methods. (B) CHO cells expressing EGFP-Rab7 wt, the mutants Q67L or T22N, or the vector alone (EGFP) were incubated at 37°C for 2 hours in EBSS medium (starved cells). Following this incubation period, cells were incubated with 0.05 mM monodansylcadaverine (MDC) for 10 minutes at 37°C. Intracellular MDC was measured by fluorescence photometry as indicated under Materials and Methods. The data represent the mean ± s.e.m. of five independent experiments. (C,D) The number of MDC-labeled vacuoles per cell (C) and the size (D) were quantified by the Methamorph software, using the integrated morphometry analysis. Data represent the mean±s.e.m. (n=50 cells). $^{**}P<0.01$.

induction of autophagy either by amino acid deprivation or by treatment with rapamycin, which mimics the starvation response even under nutrient rich conditions. Furthermore, our results indicate that TOR is part of the signal transduction pathway that detects nutrients and regulates the translocation of Rab7 from the cytosol to the membranes of autophagic vesicles.

The dominant negative Rab7 causes the accumulation of larger autophagosomes

We have developed a morphological and biochemical assay to study autophagy in living cells based on the incorporation of the autofluorescent marker MDC (Munafó and Colombo, 2001). Therefore, to determine the impact of overexpressing Rab7 on the autophagic pathway, we measured by fluorometry the incorporation of MDC into cells transfected with EGFP-Rab7 wt or the mutants, incubated under nutrient-deprivation conditions. Fig. 4A, shows the levels of overexpression of the GFP proteins in the stably transfected cells. No major differences in the level of overexpression of GFP-Rab7 wt and GFP-Rab7T22N were observed, however, in cells overexpressing the dominant negative mutant T22N there was an inhibition in the amount of MDC accumulated (Fig. 4B). To further evaluate the role of Rab7 in the autophagic pathway we analyzed the amount and size of vesicles labeled with MDC by fluorescent microscopy. No significant differences were observed in cells expressing Rab7 wt or the mutant Q67L (Fig. 4C) compared with cells expressing the vector alone. However, in cells overexpressing the mutant Rab7T22N, the number of MDC-vesicles was significantly reduced. Interestingly, there was a marked increase in the size of the MDC vesicles, only in cells expressing Rab7T22N (Fig. 4D).

In order to confirm these observations we analyzed the distribution of LC3, a protein that specifically localizes on the membrane of autophagosomes (Kabeya et al., 2000; Mizushima et al., 2001). LC3 is the mammalian homologue of the yeast protein Aut7/Apg8, which is essential for autophagy in yeast (Klionsky and Ohsumi, 1999). LC3 was the first identified mammalian protein that localizes to the autophagosome membrane in response to amino acid withdrawal (Kabeya et al., 2000). Therefore, we first studied whether Rab7 colocalizes with this autophagic marker. For this purpose, stably transfected cells overexpressing either EGFP-Rab7 wt or the mutant T22N, were co-transfected with the plasmid myc-LC3. After 36 hours transfection, cells were starved for 2 hours and LC3 was detected by indirect immunofluorescence with an anti-myc antibody. Cells overexpressing myc-LC3 were clearly distinguished from untransfected cells (not shown). As shown in Fig. 5A (upper panels), in cells overexpressing Rab7 wt numerous vesicles were clearly labeled by LC3, indicating that Rab7 indeed localizes with autophagic vacuoles (arrows). As expected, in Rab7T22N-transfected cells, larger autophagic vacuoles labeled with LC3 were observed (Fig. 5A, lower panels). As shown in Table 1, LC3 colocalizes with 35.5 and 36.8% of the Rab7 wt and Rab7T22N labeled vesicles, respectively.

It is interesting to note that the large vesicles decorated by Rab7T22N accumulated LC3 inside the vacuoles (Fig. 5A, insets). This is consistent with a previous observation that processed LC3 is distributed both outside and inside isolated

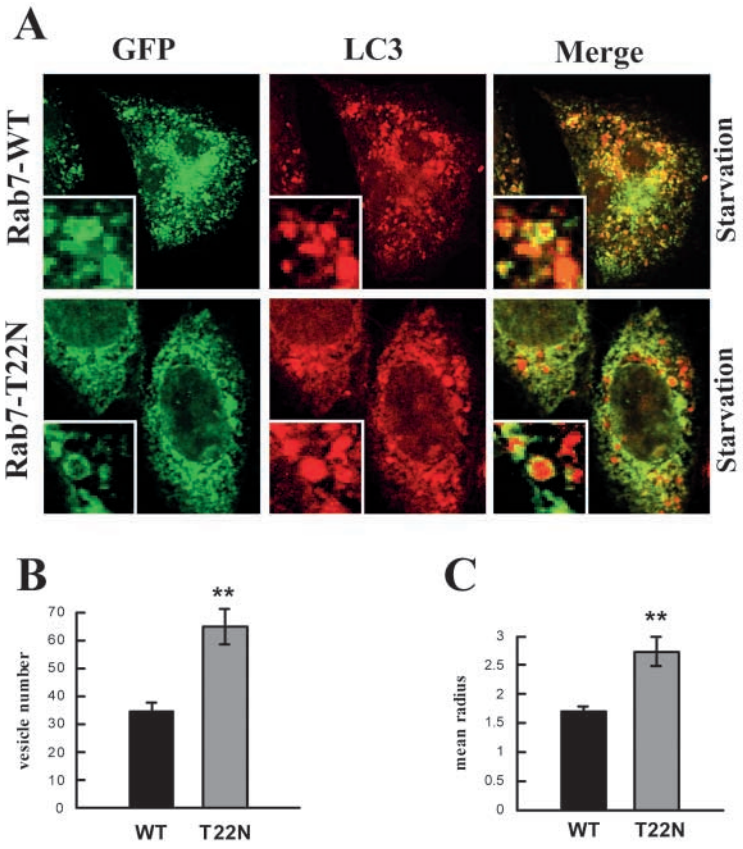

Fig. 5. The mutant Rab7T22N induces the accumulation of LC3-labeled autophagosomes. (A) CHO cells expressing EGFP-Rab7 wt or the mutant T22N were transiently transfected with the plasmid pCI-neo Myc-LC3. After 36 hours transfection, cells were subjected to indirect immunofluorescence as described under Materials and Methods and analyzed by confocal fluorescence microscopy. Briefly, cells were fixed, permeabilized and incubated with an antibody against the myc epitope. The corresponding Texas red-conjugated secondary antibody was used. Cells were mounted with Mowiol. (B,C) The number (B) and size (C) of LC3-labeled vacuoles per cell were quantified by the Methamorph software, using the integrated morphometry analysis. Data represent the mean±s.e.m. (n=25 cells). **P<0.01

autophagic vacuoles since over half of the protein is resistant to protease digestion (Kabeya et al., 2000; Mizushima et al., 2001). The number and size of vesicles labeled by LC3 was also quantified in both Rab7 wt- and T22N-expressing cells. Similar to the results observed with the autofluorescent marker MDC, the diameter of the autophagic vesicles labeled by LC3 markedly increased in cells expressing the mutant Rab7T22N (Fig. 5C). However, in contrast to the results obtained with MDC, the number of LC3 vacuoles increased significantly by overexpression of Rab7T22N (Fig. 5B) when compared with cells expressing the wild-type protein. We believe that the reason for this apparently contradictory result is that MDC and LC3 are probably labeling distinct subpopulations of autophagic vacuoles, although there is a subset of vesicles that has both markers. MDC preferentially labels mature autophagosomes and autolysosomes. In Rab7T22N-overexpressing cells we have observed that large MDC-labeled vesicles (probably mature autophagosomes) accumulate, but the total number is reduced, probably because autolysosomes are not formed. The MDC-labeled vesicles become enlarged, since, under starvation conditions, more material is incorporated and fusion with early endocytic compartments is not hampered. However, LC3 preferentially labels early stages in the autophagic pathway (i.e. immature autophagosomes). The number of LC3-positive vesicles increases because these cells are overexpressing LC3, and processing of LC3 is further enhanced under starvation, therefore a larger number of LC3-labeled vesicles are formed. Indeed, it has been shown that the number of autophagosomes formed corresponds to the amount of processed LC3 (Kabeya et al., 2000). We have also observed that overexpression of LC3 increases the basal level of autophagy (Munafó and Colombo, 2002). Therefore, it is not surprising that overexpressing LC3 increases the number of autophagic vesicles in Rab7T22N-transfected cells where the autophagic pathway is probably altered.

Taken together, our results suggest that overexpression of the dominant negative mutant of Rab7 causes the accumulation of large autophagosomal vacuoles probably by interrupting the normal progression of the autophagic pathway.

Rab7T22N overexpression impairs autolysosome formation

It has been shown that in the presence of vinblastine, a microtubule depolimerizing agent, known to block fusion between autophagosomes and lysosomes, large autophagic vacuoles accumulate (Marzella et al., 1982; Hoyvik et al., 1986; Munafó and Colombo, 2001). Similarly, we have observed that overexpression of Rab7 T22N causes the accumulation of autophagosomes. Therefore, to assess whether fusion between the autophagic vacuole and the lysosome might be hampered in cells overexpressing the mutant Rab7T22N, we first internalized Rhodamine-dextran by fluid phase endocytosis and chased for 20 minutes to label the late endosome/lysosomal compartment. Subsequently, cells were incubated in starvation medium for an additional period of 2 hours, and autophagic vacuoles were labeled with MDC (see Fig. 6A). As shown in Fig. 6B, in cells overexpressing EGFP-Rab7 wt colocalization between both markers was clearly observed, indicating that fusion between autophagosomes and late endosomes/lysosomes has occurred. In contrast, in cells overexpressing the mutant Rab7T22N practically no colocalization was observed, indicating that expression of this mutant protein hinders fusion of autophagosomes with lysosomes. To address whether a cargo internalized in an early endosomal compartment can reach the autophagosomes in cells overexpressing Rab7T22N the stably transfected cells were first subjected to starvation for 2 hours and then they were allowed to internalize the endocytic marker Rhodamine-dextran for 5 minutes. Subsequently, cells were labeled with MDC. Under these conditions we observed a partial colocalization of the endosomal marker in vacuoles labeled with MDC and decorated by Rab7, indicating that the autophagosomes are accessible to early endocytic cargo even in cells overexpressing the Rab7T22N mutant (data not shown).

To further determine that the formation of autolysosomes

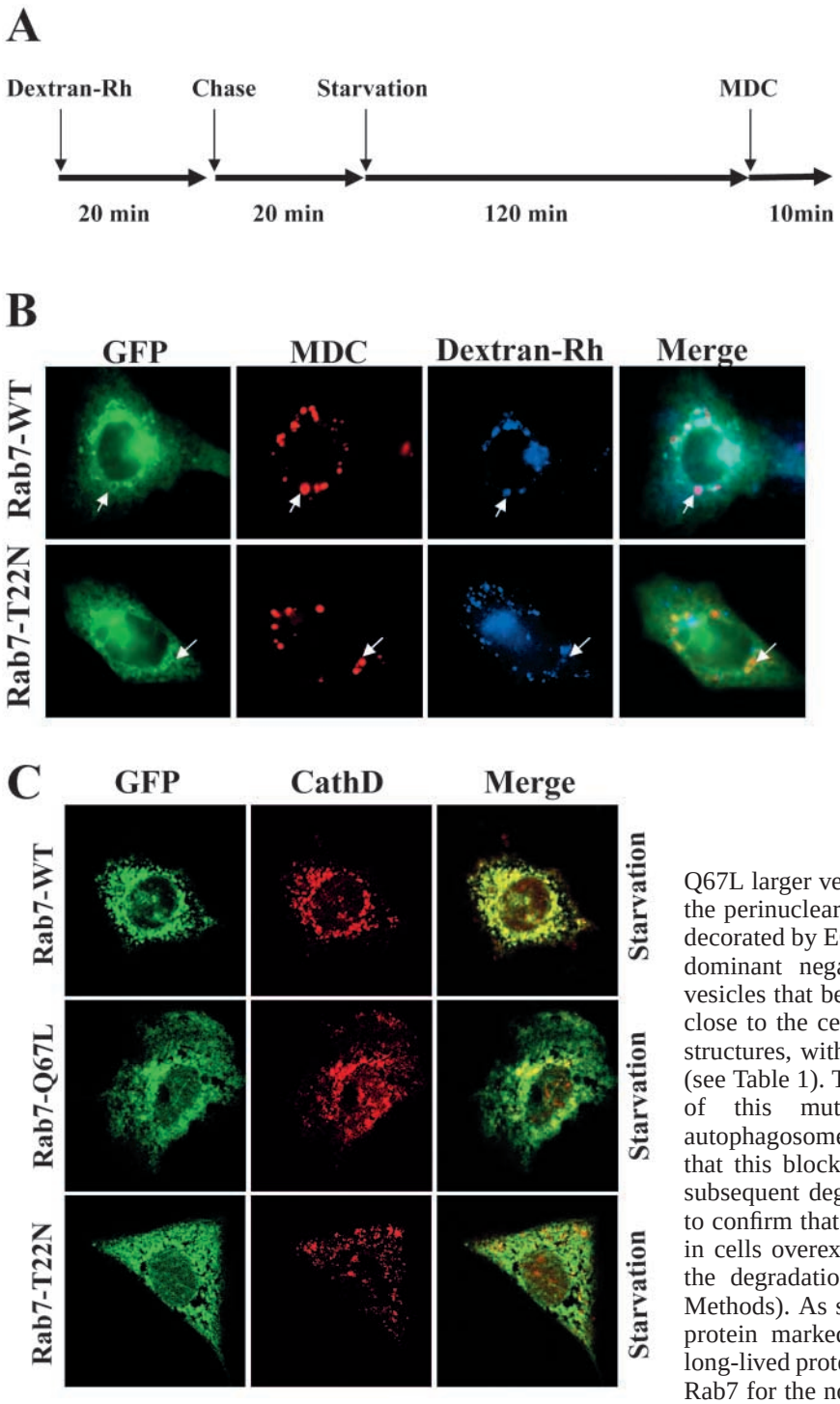

Fig. 6. Rab7 T22N hampers fusion between autophagosomes and late endosome/lysosomal compartment. **(**PA) Outline of the method used to label the late endocytic/lysosomal compartment with Rhodamine-dextran. (B) CHO cells overexpressing EGFP-Rab7 wt or T22N were allowed to internalize for 20 minutes at 37°C Rhodamine-dextran (0.5 mg/ml) by fluid phase endocytosis. The marker was chased for 20 minutes at 37°C to label the late endosome/lysosomal compartment. Subsequently, cells were placed for 2 hours in the starvation medium (EBSS) to induce autophagy and then were labeled with MDC as indicated in Fig. 1. Cells were immediately analyzed by fluorescence microscopy. In cells transfected with Rab7 wt arrows indicate Rab7 decorated vesicles that colocalized with both MDC and Rhodamine-dextran. In contrast, in cells overexpressing the mutant Rab7 T22N the MDC-labeled vesicles are not labeled with the endocytic probe (arrow). (C) CHO cells overexpressing EGFP-Rab7 wt or the mutants Q67L and T22N, were incubated under starvation conditions for 2 hours and afterwards subjected to indirect immunofluorescence to detect the lysosomal enzyme cathepsin D, as described under Materials and Methods. The corresponding Texas Red-conjugated secondary antibody was used. Cells were mounted and analyzed by confocal fluorescence microscopy.

was impeded we studied the distribution of the lysosomal enzyme cathepsin D (CthD), by indirect immunofluorescence, in cells incubated under starvation conditions. As shown in Fig. 6C, in cells overexpressing Rab7 wt, CthD had a typical vesicular pattern, with a marked perinuclear distribution. This is consistent with a previous observation that overexpression of Rab7 induces the aggregation of lysosomes in the perinuclear region (Bucci et al., 2000). As previously shown (Vitelli et al., 1997) by overexpressing the activating mutant Q67L larger vesicles are formed, but they are not restricted to the perinuclear area. Some of the CthD vesicles were clearly decorated by EGFP-Rab7 Q67L. In contrast, expression of the dominant negative mutant T22N resulted in labeling of vesicles that become dispersed throughout the cytoplasm and close to the cell periphery. Rab7T22N localized to vesicular structures, with a limited colocalization (23.87%) with CthD (see Table 1). This result clearly indicates that overexpression of this mutant protein hampers the transition of autophagosomes to autophagolysosomes and it is probable that this blockage disrupts the autophagic pathway and the subsequent degradation of the sequestered material. In order to confirm that digestion of sequestered material is hampered in cells overexpressing the mutant Rab7 T22N we analyzed the degradation of long-lived proteins (see Materials and Methods). As shown in Fig. 7, overexpression of this mutant protein markedly decreased the autophagic degradation of long-lived proteins, confirming the requirement of a functional Rab7 for the normal development of the autophagic pathway.

Discussion

Rab proteins are present in the cytosol in the GDP-bound conformation complexed to Rab-GDI (Rab GDP dissociation inhibitor). The complex Rab-GDI represents a pool of active Rab that delivers Rabs to the membranes of specific compartments. Upon membrane association, GDI is released via the interaction with a GDI displacement factor (Dirac-Svejstrup et al., 1997) and the Rab protein interacts with the exchange factor followed by GDP/GTP exchange. Therefore,

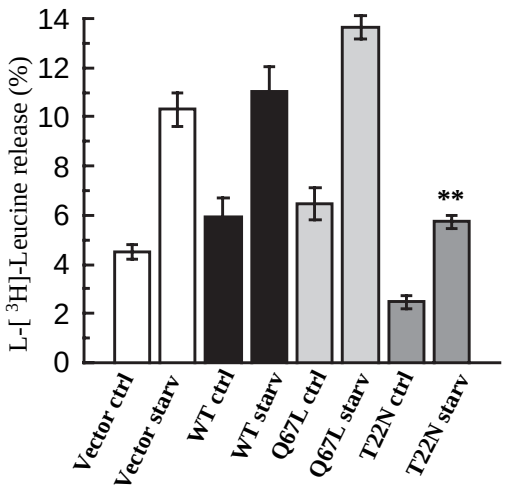

Fig. 7. Overexpression of Rab7 T22N suppresses the autophagic degradation of labeled long-lived proteins. CHO cells overexpressing EGFP-Rab7 wt, and the mutants Q67L or T22N, or the vector alone (EGFP) were labeled for 24 hours in medium containing 1 μCi/ml [^{3}H]leucine (see Materials and Methods). Both the trichloroacetic acid (TCA)-precipitable radioactivity of the cell monolayers and the TCA-soluble radioactivity present in the medium were measured. Leucine released (in the TCA-soluble supernatant) was calculated as a percentage of the total cell radioactivity. Results were expressed as the mean±s.e.m. **Significantly different from the vector under starvation conditions $P<0.001$ (compared with ANOVA-1 and Turkey-Kramer test).

prenylated Rab proteins first associate with membranes in their GDP-bound conformation and subsequently they bind GTP (Soldati et al., 1994; Ullrich et al., 1994). In this report we present evidence that upon induction of autophagy, either by amino acid starvation or by treatment with rapamycin, the dominant negative mutant of Rab7 was targeted to the membranes of autophagic MDC-labeled vacuoles. Our results strongly suggest that this protein was not present on autophagic vacuoles as a result of fusion events since neither vinblastine nor bafilomycin A1, agents known to block fusion with vesicles from the endocytic/lysosomal pathway, prevented Rab7 decorating autophagosomes. In contrast, targeting was hampered by treatment with inhibitors of early events in the autophagic pathway such as WM or NEM. These results clearly suggest that a signal transduction mechanism modulates the association of this protein with the autophagosomal membrane. It is probable that this mutant protein interacts with the exchange factor, but since it is unable to exchange GDP for GTP it remains associated with the membranes, although in an inactive conformation incapable of interacting with Rab effector proteins (Zerial and McBride, 2001). Targeting of the dominant negative form of Rab7 is also consistent with a previous observation that in yeast, the protein Vps39 directly binds to the GDP-bound form of Ypt7 (the Rab7 homologue in yeast) stimulating nucleotide exchange in Ypt7 (Wurmser et al., 2000).

The question is why is Rab7 targeted to the membranes of autophagic vacuoles. Compelling evidence indicate that Rab GTPases recruit tethering factors to establish firm bonds between the fusing membranes (for a review, see Zerial and McBride, 2001). A symmetrical requirement for a Rab protein in both fusion partners has been established in mammalian cells (Rubino et al., 2000) and in yeast (Haas et al.1995). Therefore, we propose that Rab7 is first recruited onto autophagosomal membranes to allow the subsequent fusion with late endocytic/lysosomal compartments. Indeed, we have observed that in cells overexpressing Rab7T22N large autophagosomes accumulate. Our results indicate that maturation to autophagolysosomes was hampered in these dominant-negative transfectants. Similarly, we have previously observed that vinblastine, an agent known to block autophagosome/lysosome fusion by altering microtubule polymerization, causes the accumulation of large autophagic vacuoles labeled with MDC (Munafo and Colombo, 2001). Therefore, it is likely that overexpression of Rab7T22N prevents the normal progression of the autophagic pathway causing the accumulation of large autophagosomes, indicating that a functional Rab7 is crucial for this intracellular transport event.

As mentioned above we have observed that in cells overexpressing the dominant negative mutant Rab7T22N, larger autophagosomes accumulate under starvation conditions. A similar result has been observed in yeast cells lacking ypt7, the yeast homologue of Rab7. Starved Δypt7 cells have many fragmented vacuoles and many more autophagosomes than wild-type cells (Kirisako et al., 1999), indicating that depletion of YPT7 causes the accumulation of autophagosomes in the cytoplasm. Interestingly, they have observed that in the double mutant Δypt7Δapg8 cells did not accumulate autophagosomes in the cytosol. LC3 is one of the mammalian homologues of the yeast protein apg8/Aut7 (Kabeya et al., 2000). LC3 is associated with the autophagosome membranes after processing by a N-ethylmaleimide-sensitive cysteine protease. We have recently shown that treatment with NEM prevents the change in distribution of GFP-LC3 from cytosol to a membrane that is induced by amino acid deprivation (Munafó and Colombo, 2002). Interestingly, we have observed that NEM treatment of cells overexpressing Rab7T22N completely blocks the accumulation of the large autophagosomes. Furthermore, EGFP-Rab7T22N was not detected on the limiting membrane of autophagic vacuoles labeled with MDC. Taken together, our results suggest that, similar to the yeast system, normal processing of LC3 (Apg8/Aut7) is required for the formation of autophagosomes even in cells overexpressing the dominant negative mutant of Rab7.

The effects of Rab7 T22N are very reminiscent of the overexpression of a dominant-negative mutant of the protein SKD1, an ATPase involved in endosomal transport (Yoshimori et al., 2000). In cells overexpressing the mutant SKD1^{E235Q}, accumulation of autophagosomes was observed whereas the number of autolysosomes decreased, suggesting that this mutant blocks the transition from autophagosomes to autolysosomes (Nara et al., 2002). Furthermore, overexpression of SKD1^{E235Q} significantly increased the amounts of processed LC3, confirming the accumulation of autophagosomes. This is consistent with our observation that Rab7T22N induces the accumulation of large LC3-labeled autophagosomes. Finally, Yoshimori and collaborators (Nara et al., 2002) have also reported that delivery of an endosomal lipid from late endosomes to autophagosomes was inhibited by overexpression of SKD1^{E235Q}. Therefore, it is probable that

similar to the proposed effect of the mutant SKD1^{E235Q}, lack of a functional Rab7 may prevent the formation of intermediate autophagic vacuoles called amphisomes (Berg et al., 1998). Our experiments with internalized dextran indicate that in cells overexpressing the Rab7 dominant negative mutant, autophagosomes are somewhat accessible to early endocytic markers but not to a marker internalized in a late endosome/lysosome compartment, indicating that fusion with this compartment was altered. It is possible that alteration in fusion with late endosomes may prevent autophagosomes acquiring some components necessary for fusion with lysosomes. Indeed, our results indicate that autophagic degradation of long-lived proteins was impaired in cells overexpressing Rab7T22N, suggesting that maturation to autolysosomes was hampered. Furthermore, we cannot discard the possibility that the lysosomes formed in cells overexpressing Rab7T22N are deficient in their proteolytic capacity (Press et al., 1998; Bucci et al., 2000) and, as a consequence, autophagic degradation is severely suppressed. Nevertheless, our results clearly indicate that a functional Rab7 is required for the normal progression of the autophagic pathway. Furthermore, in a very recent publication it has been shown that in *Drosophila* autophagic cell death, Rab7 is one of the genes upregulated (Gorski et al., 2003), suggesting the involvement of this protein in autophagy.

In summary, we present evidence that Rab7 is an essential participant in the autophagic pathway since this protein was associated with autophagic vacuoles, and that overexpression of a Rab7 dominant negative mutant led to the accumulation of autophagosomes with a concomitant decrease in the degradation of long-lived proteins. Thus, our results suggest that Rab7 is involved in maturation of autophagosomes since autophagosomes cannot become competent to fuse with lysosomes in cells overexpressing Rab7T22N. Future experiments will be directed at determining the precise role of Rab7 in autophagolysosome formation and to identify other components of the machinery involved in the maturation process of autophagosomes.

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References

Berg, T. O., Fengsrud, M., Stromhaug, P. E., Berg, T. and Seglen, P. O. (1998). Isolation and characterization of rat liver amphisomes. Evidence for fusion of autophagosomes with both early and late endosomes. *J. Biol. Chem.* **273**, 21883-21892.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J. and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell.* **11**, 467-480.

Biederbick, A., Kern, H. F. and Elsasser, H. P. (1995). Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur. J. Cell Biol.* **66**, 3-14.

Blommaart, E. F., Krause, U., Schellens, J. P., Vreeling-Sindelarova, H. and Meijer, A. J. (1997). The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur. J. Biochem.* **243**, 240-246.

Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K. and Zerial, M. (1990). Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* **62**, 317-329.

Chavrier, P. and Goud, B. (1999). The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol.* **11**, 466-475.

Cutler, N. S., Heitman, J. and Cardenas, M. E. (1999). TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals. *Mol. Cell Endocrinol.* **155**, 135-142.

Cutler, N. S., Pan, X., Heitman, J. and Cardenas, M. E. (2001). The TOR signal transduction cascade controls cellular differentiation in response to nutrients. *Mol. Biol. Cell* **12**, 4103-4113.

Dirac-Svejstrup, A. B., Sumizawa, T. and Pfeffer, S. R. (1997). Identification of a GDI displacement factor that releases endosomal Rab GTPases from Rab-GDI. *EMBO J.* **16**, 465-472.

Dunn, W. A., Jr (1990a). Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* **110**, 1923-1933.

Dunn, W. A., Jr (1990b). Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J. Cell Biol.* **110**, 1935-1945.

Feng, Y., Press, B. and Wandinger-Ness, A. (1995). Rab7: an important regulator of late endocytic membrane traffic. *J. Cell Biol.* **131**, 1435-1452.

Gordon, P. B. and Seglen, P. O. (1988). Prelysosomal convergence of autophagic and endocytic pathways. *Biochem. Biophys. Res. Commun.* **151**, 40-47.

Gordon, P. B., Hoyvik, H. and Seglen, P. O. (1992). Prelysosomal and lysosomal connections between autophagy and endocytosis. *Biochem. J.* **283**, 361-369.

Gorski, S. M., Chittaranjan, S., Pleasance, E. D., Freeman, J. D., Anderson, C. L., Varhol, R. J., Coughlin, S. M., Zuyderduyn, S. D., Jones, S. J. and Marra, M. A. (2003). A SAGE approach to discovery of genes involved in autophagic cell death. *Curr. Biol.* **13**, 358-363.

Haas, A., Scheglmann, D., Lazar, T., Gallwitz, D. and Wickner, W. (1995). The GTPase Ypt7p of Saccharomyces cerevisiae is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance. *EMBO J.* **14**, 5258-5270.

Hoyvik, H., Gordon, P. B. and Seglen, P. O. (1986). Use of a hydrolysable probe, [14C]lactose, to distinguish between pre- lysosomal and lysosomal steps in the autophagic pathway. *Exp. Cell Res.* **166**, 1-14.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19**, 5720-5728.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J. Cell Biol.* **147**, 435-446.

Klionsky, D. J. and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717-1721.

Klionsky, D. J. and Ohsumi, Y. (1999). Vacuolar import of proteins and organelles from the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 1-32.

Liou, W., Geuze, H. J., Geelen, M. J. and Slot, J. W. (1997). The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J. Cell Biol.* **136**, 61-70.

Martinez, O. and Goud, B. (1998). Rab proteins. *Biochim. Biophys. Acta Mol. Cell Res.* **1404**, 101-112.

Marzella, L., Ahlberg, J. and Glaumann, H. (1982). Isolation of autophagic vacuoles from rat liver: morphological and biochemical characterization. *J. Cell Biol.* **93**, 144-154.

Meresse, S., Gorvel, J. P. and Chavrier, P. (1995). The rab7 GTPase resides on a vesicular compartment connected to lysosomes. *J. Cell Sci.* **108**, 3349-3358.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y. and Yoshimori, T. (2001). Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J. Cell Biol.* **152**, 657-668.

Mohrmann, K. and van der Sluijs, P. (1999). Regulation of membrane transport through the endocytic pathway by rabGTPases. *Mol. Membr. Biol.* **16**, 81-87.

Munafo, D. B. and Colombo, M. I. (2001). A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J. Cell Sci.* **114**, 3619-3629.

Munafo, D. B. and Colombo, M. I. (2002). Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24. *Traffic* **3**, 472-482.

Nara, A., Mizushima, N., Yamamoto, A., Kabeya, Y., Ohsumi, Y. and Yoshimori, T. (2002). SKD1 AAA ATPase-dependent endosomal transport is involved in autolysosome formation. *Cell Struct. Funct.* **27**, 29-37.

Niemann, A., Baltes, J. and Elsässer, H. P. (2001). Fluorescence properties

and staining behavior of monodansylpentane, a structural homologue of the lysosomotropic agent monodansylcadaverine. *J. Histochem. Cytochem*. **49**, 177-185.

Noda, T. and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* **273**, 3963-3966.

Ohashi, M., Miwako, I., Nakamura, K., Yamamoto, A., Murata, M., Ohnishi, S. and Nagayama, K. (1999). An arrested late endosome-lysosome intermediate aggregate observed in a Chinese hamster ovary cell mutant isolated by novel three-step screening. *J. Cell Sci.* **112**, 1125-1138.

Petiot, A., Ogier-Denis, E., Blommaart, E. F., Meijer, A. J. and Codogno, P. (2000). Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.* **275**, 992-998.

Press, B., Feng, Y., Hoflack, B. and Wandinger-Ness, A. (1998). Mutant Rab7 causes the accumulation of cathepsin D and cation-independent mannose 6-phosphate receptor in an early endocytic compartment. *J. Cell Biol*. **140**, 1075-1089.

Punnonen, E. L., Autio, S., Kaija, H. and Reunanen, H. (1993). Autophagic vacuoles fuse with the prelysosomal compartment in cultured rat fibroblasts. *Eur. J. Cell Biol*. **61**, 54-66.

Rubino, M., Miaczynska, M., Lippe, R. and Zerial, M. (2000). Selective membrane recruitment of EEA1 suggests a role in directional transport of clathrin-coated vesicles to early endosomes. *J. Biol. Chem*. **275**, 3745-3748.

Simonsen, A., Wurmser, A. E., Emr, S. D. and Stenmark, H. (2001). The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol*. **13**, 485-492.

Soldati, T., Shapiro, A. D., Svejstrup, A. B. and Pfeffer, S. R. (1994). Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. *Nature* **369**, 76-78.

Tallóczy, Z., Jiang, W., Virgin, H. W., Leib, D. A., Scheuner, D., Kaufman, R. J., Eskelinen, E. and Levine, B. (2002). Regulation of starvation- and virus-induced autophagy by the eIF2α kinase signaling pathway. *Proc. Natl. Acad. Sci. USA* **99**, 190-195.

Tooze, J., Hollinshead, M., Ludwig, T., Howell, K., Hoflack, B. and Kern, H. (1990). In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. *J. Cell Biol.* **111**, 329-345.

van Sluijters, D. A., Dubbelhuis, P. F., Blommaart, E. F. and Meijer, A. J. (2000). Amino-acid-dependent signal transduction. *Biochem. J.* **351**, 545-550.

Ullrich, O., Horiuchi, H., Bucci, C. and Zerial, M. (1994). Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature* **368**, 157-160.

Vitelli, R., Santillo, R., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C. B. and Bucci, C. (1997). Role of the small GTPase Rab7 in the late endocytic pathway. *J. Biol. Chem*. **72**, 4391-4397.

Waters, M. G. and Pfeffer, S. R. (1999). Membrane tethering in intracellular transport. *Curr. Opin. Cell Biol*. **11**, 453-459.

Wurmser, A. E., Sato, T. K. and Emr, S. D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J. Cell Biol*. **151**, 551-562.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R. and Tashiro, Y. (1998). Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct. Funct*. **23**, 33-42.

Yoshimori, T., Yamagata, F., Yamamoto, A., Mizushima, N., Kabeya, Y., Nara, A., Miwako, I., Ohashi, M., Ohsumi, M. and Ohsumi, Y. (2000). The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol. Biol. Cell*. **11**, 747-763.

Zerial, M. and McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol*. **2**, 107-117.

Zuk, P. A. and Elferink, L. A. (1999). Rab15 mediates an early endocytic event in Chinese hamster ovary cells. *J. Biol. Chem*. **274**, 22303-22312.