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 as 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 than 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
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 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, monodansylpentapeptide (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% CO2.

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/ml) 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 7×105 cells/well in a 24-well plates. Cells were then labeled for 24 hours in medium containing [3H]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; Munafò 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 NH₄Cl 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; Munafò 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 in cells subjected to starvation, indicated 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
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

<table>
<thead>
<tr>
<th></th>
<th>Rab7-WT</th>
<th>Rab7-Q67L</th>
<th>Rab7-T22N</th>
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<tbody>
<tr>
<td>MDC (%)</td>
<td>48.2±5.31</td>
<td>49.28±7.87</td>
<td>66.86±3.24</td>
</tr>
<tr>
<td>MDH (%)</td>
<td>62.57±5.76</td>
<td>54.57±7.66</td>
<td>86.58±5.31</td>
</tr>
<tr>
<td>LC3 (%)</td>
<td>35.52±2.24</td>
<td>31.63±9.2</td>
<td>36.80±2.04</td>
</tr>
<tr>
<td>CathD (%)</td>
<td>51.87±3.15</td>
<td>65.23±8.36</td>
<td>23.87±2.03</td>
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Values are means±s.e.m. from three independent experiments. At least 100 Rab7-decorated vesicles were counted for each condition.
Rab7 is involved in autophagy

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...
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 (Munafò 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 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).

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
Rab7 is involved in autophagy

Rab7 is involved in autophagy 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 were 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 (Munafò 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 depolymerizing agent, known to block fusion between autophagosomes and lysosomes, large autophagic vacuoles accumulate (Marzella et al., 1982; Hoyvik et al., 1986; Munafò 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 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...
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,
Turkey-Kramer test).

The mean Leucine released (in the TCA-soluble supernatant) was calculated as TCA-soluble radioactivity present in the medium were measured. Starvation conditions indicated 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 (Munafò 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 Δypt7 Δapg8 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 (Munafò 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 SKD1E235Q, 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 SKD1E235Q 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 SKD1E235Q. Therefore, it is probable that

![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 [3H]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 means±s.e.m. **Significantly different from the vector under starvation conditions P<0.001 (compared with ANOVA-1 and Turkey-Kramer test).](image-url)
similar to the proposed effect of the mutant SKD1E235Q, 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 shown to be involved in the maturation of autophagosomes with a concomitant decrease in the lysosomal connections between autophagy and endocytosis. Biochem. J. 361-369.


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