Mutant Rab7 Causes the Accumulation of Cathepsin D and Cation-independent Mannose 6–Phosphate Receptor in an Early Endocytic Compartment

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Abstract. Stable BHK cell lines inducibly expressing wild-type or dominant negative mutant forms of the rab7 GTPase were isolated and used to analyze the role of a rab7-regulated pathway in lysosome biogenesis. Expression of mutant rab7N125I protein induced a dramatic redistribution of cation-independent mannose 6–phosphate receptor (CI-MPR) from its normal perinuclear localization to large peripheral endosomes. Under these circumstances ~50% of the total receptor and several lysosomal hydrolases cofractionated with light membranes containing early endosome and Golgi markers. Late endosomes and lysosomes were contained exclusively in well-separated, denser gradient fractions. Newly synthesized CI-MPR and cathepsin D were shown to traverse through an early endocytic compartment, and functional rab7 was crucial for delivery to later compartments. This observation was evidenced by the fact that 2 h after synthesis, both markers were more prevalent in fractions containing light membranes. In addition, both were sensitive to HRP-DAB-mediated cross-linking of early endosomal proteins, and the late endosomal processing of cathepsin D was impaired. Using similar criteria, the lysosomal membrane glycoprotein 120 was not found accumulated in an early endocytic compartment. The data are indicative of a post-Golgi divergence in the routes followed by different lysosome-directed molecules.

The ras-related, rab GTPases serve as important regulators of membrane transport (reviewed in Novick and Zerial, 1997). Rab7 is a representative small GTPase that is localized to late endosomes (Chavrier et al., 1990). Our laboratory has analyzed the function of rab7 and has shown that its activity is required for the efficient transport of molecules to late endosomes (Feng et al., 1995). This was determined using specific mutant forms of rab7 that displayed altered nucleotide binding. One such mutant, rab7N125I, has isoleucine substituted for asparagine in the NKXD region and, consequently, exists preferentially in the nucleotide-free form. Expression of this mutant protein exerted a dominant negative effect on late endocytic transport, as shown by several criteria. rab7N125I caused vesicular stomatitis virus G protein to accumulate in transferrin receptor–positive endosomes and blocked its egress to late endosomes. Quantitative measurements of SV5 hemagglutinin-neuraminidase cleavage showed that rab7N125I caused a twofold decrease in the formation of cleavage fragments generated in late endosomes. Recent studies corroborated our earlier findings and demonstrated that in the absence of rab7 function, delivery of markers to lysosomes for degradation was impaired (Mukhopadhyay et al., 1997; Vitelli et al., 1997). The observation that an activating (rab7Q67L) mutant was partially colocalized with the lysosomal marker lgp120 led to the suggestion that rab7 functions further downstream, in the delivery of molecules from late endosomes to lysosomes (Méresse et al., 1995). Thus, rab7 may in fact regulate membrane flux into and out of late endosomes.

The usefulness of mutant rab GTPases for inducing a transport block at a particular point in the flow of exo- or endocytic membrane traffic has been well documented (Bucci et al., 1992; Riederer et al., 1994; Tisdale et al., 1992; Walworth et al., 1992). Blocking membrane transport at a particular point can in turn be used to examine the consequences for downstream events or interconnected pathways. For example, the existence of transport pathways interconnecting compartments involved in endo- and exocytosis make it of interest to study how the expression of rab7 mutant proteins would impact lysosome biogenesis.

Several different classes of proteins are initially trans-
ported along the exocytic pathway and are segregated in the TGN for delivery to the endocytic pathway. Most soluble lysosomal hydrolases are targeted directly to endosomes via a specific pair of mannose 6–phosphate receptors. Integral lysosomal membrane glycoproteins have distinct targeting signals and may follow a route different from that of the soluble hydrolases (reviewed in Bonifaci et al., 1996; Hunziker and Geuze, 1996). Specialized cell types, such as antigen presenting cells, use these pathways to promote the loading of antigenic peptides, derived on the endocytic pathway, onto newly synthesized MHC class II molecules. Details pertaining to the transport routes and the compartments involved, however, are still being contested. Dissecting the interconnections between compartments and the routes followed by different classes of molecules is important for understanding the biology of these molecules and warrants the application of new strategies.

Most soluble lysosomal enzymes receive a mannose 6–phosphate modification on their carbohydrate side chains during transit through the Golgi (for review see von Figura, 1991). This modification serves as a recognition signal for two receptors involved in targeting these hydrolases to lysosomes. The 275-kD cation-independent mannose 6–phosphate receptor (CI-MPR)\(^1\) appears to be the primary receptor for the major lysosomal aspartyl protease cathepsin D (Pohlmann et al., 1995). CI-MPR can internalize extracellular mannose 6–phosphorylated ligands, and the receptor also functions in the mannose 6–phosphate–independent binding and internalization of insulin-like growth factor II (Dahms, 1996; Kiess et al., 1988; Morgan et al., 1987). The 46-kD cation-dependent (CD) receptor has different ligand specificities from CI-MPR and appears to function only in the transport of newly synthesized lysosomal hydrolases (Dahms et al., 1987; Hoffack and Kornfeld, 1985; Ludwig et al., 1994; Munier-Lehmann et al., 1996). CD-MPR is unable to bind ligands at the cell surface (Stein et al., 1985; Ludwig et al., 1994; Munier-Lehmann et al., 1996). This modification serves as a recognition signal for CD-MPR delivery to early endosomes (Chao et al., 1990), along with the observation that newly synthesized lysosomal hydrolases can be detected in early endosomes (Ludwig et al., 1991; Runquist and Havel, 1991), make it of interest to examine this issue further.

For this purpose, we isolated stable BHK cell lines expressing wild-type and mutant forms of rab7. The newly generated cell lines were then used to test how the expression of dominant negative mutant rab7 protein influenced lysosome biogenesis. Our analysis focused primarily on the localization and targeting of the CI-MPR and one of its ligands, cathepsin D.

### Materials and Methods

#### Cells, Media, and Cell Culture

The BHK cell line (BHK21) was obtained from American Type Culture Collection (Rockville, MD) and grown in complete G-MEM (5% FCS, 2.6 mg/ml tryptose phosphate broth, glutamine, and antibiotics) as described (Feng et al., 1995). BHK21-tTA cells (isolation described below) were cultured in complete G-MEM containing 400 μg/ml Geneticin. Stable BHK-tTA/R7 cell lines were maintained in select G-MEM (complete G-MEM containing 200 μg/ml hygromycin B [Calbiochem-Novabiochem, La Jolla, CA], 3 μg/ml tetracycline, and 400 μg/ml Geneticin). All tissue culture reagents, unless otherwise noted, were purchased from Gibco-BRL (Gaithersburg, MD).

#### Vectors and Constructs

The expression plasmid pUHD15-1, which encodes the chimeric tetracycline-regulated transcription activator tTA, was used as described (Gossen and Bujard, 1992). The entire coding regions of wild-type rab7 (Chavrier et al., 1990) and the rab7N125I mutant (Feng et al., 1995) were each cloned into the XbaI site of the tetracycline-inducible expression plasmid pUHD10-3 (Gossen and Bujard, 1992).

#### Generation of Stable Cell Lines

The BHK-tTA parental cell line was generated by standard calcium phosphate transfection of subconfluent BHK21 cells with pUHD 15-1 (0 μg/6-cm dish). After transfection, the cells were allowed to recover in complete G-MEM for 24 h before passage at 1:2.6 and transfer to complete G-MEM containing 800 μg/ml of Geneticin. After 1 wk, with daily media changes, viable clones were recovered using cloning rings (PCC Scientific, Gaithersburg, MD). Clones were expanded in complete G-MEM containing Geneticin and conditioned media from subconfluent BHK21 cells (1:1). Individual BHK-tTA clones were tested for their ability to induce the expression of luciferase under the control of a tetracycline-sensitive operator (using pUHC 13-3; Gossen and Bujard, 1992). A BHK-tTA clone exhibiting a 10-fold increase in luciferase activity 24 h after transfection was subcloned by limiting dilution in the presence of conditioned media. A subclone expressing <2% of the maximal luciferase activity when cultured in the presence of tetracycline was chosen as the parental BHK-tTA line.

Subconfluent BHK-tTA cells were subjected to calcium phosphate-mediated transfection with 10 μg pUHD 10-3 (containing either wild-type or mutant rab7N125I cDNAs) and 1 μg of plasmid pMiwph (containing the hygromycin resistance gene; Matsuda et al., 1992) to isolate stable lines expressing recombinant rab7 proteins. After recovery in complete G-MEM containing 400 μg/ml Geneticin and 3 μg/ml tetracycline for 24 h, the cells were split 1:2.6 and cultured in select G-MEM containing 400 μg/ml hygromycin B. Individual BHK-tTA/R7 clones were isolated, and overexpression of the rab7 proteins in a tetracycline-regulated manner was monitored by Western blot analysis and immunofluorescence staining. Selected BHK-tTA/R7 clones exhibiting no expression of recombinant rab7 protein in the presence of tetracycline were subcloned twice by limiting dilution. Clonal variations were kept to a minimum by using a single parental line for all second-round transfections.

#### Antibodies

Monoclonal 4F11 directed against the carboxy terminus of rab5 (Bucci et al., 1994; Qiu et al., 1994) and a polyclonal antiserum (R4) directed against the carboxy terminus of rab7 (Qiu et al., 1994) were used for immunoblot

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\(^1\) Abbreviations used in this paper: AP, adaptor protein complex; CD-MPR, cation-dependent mannose 6–phosphate receptor; CI-MPR, cation-independent mannose 6–phosphate receptor; EEA1, early endosome antigen 1; lgp, lysosomal membrane glycoprotein; PNS, postnuclear supernatant.
detection of rab5 and rab7, respectively. Endogenous cathepsin D was de-
tected on immunoblots and by immunoprecipitation using a rabbit anti-
bovine cathepsin D antiserum that was prepared by immunizing rabbits
with purified bovine cathepsin D (Ludwig et al., 1991). This antibody was
able to immunoprecipitate the mature hamster protein, but it failed to de-
tect the mature species on immunoblots. CI-MPR was detected on immu-
noblots using a rabbit anti-bovine CI-MPR antiserum (Ludwig et al.,
1994) and by immunoprecipitation using a rabbit anti-hamster CI-MPR
antiserum kindly provided by Dr. April Robbins (National Institute of Di-
adise and Digestive and Kidney Diseases, Bethesda, MD). Monoconal
4A1 was used to monitor hamster lysosomal glycoprotein (Igp) 120 (a gift
from J. Gruenberg, Université de Genève, Switzerland).

**Immuno-fluorescence Microscopy**

Stable BHK-T/TA7 lines were grown on 15-mm square coverslips. Ex-
pression of recombinant rab7 protein was induced by culture in select
G-MEM without tetracycline for 18 h. Fixation, permeabilization, and
immuno-fluorescence staining were performed as described previously (Feng et
al., 1995). Afinity-purified rabbit anti-bovine CI-MPR was used to detect CI-MPR.

**Enzyme Assays**

Endogenous β-hexosaminidase was measured as described previously
(Riederer et al., 1994). Golgi α-mannosidase II activity was measured as
described previously (Tulsiani et al., 1982).

**Subcellular Fractionation**

Percoll gradient fractionation was performed as described previously with
minor modifications (Czekay et al., 1997). Recombinant rab7 protein ex-
pression was induced by culture in media lacking tetracycline for 18 h.
Where indicated, 10 mM mannose 6-phosphate was also included in the
media during the induction period to prevent secretion-recapture of lys-
osomal hydrolases. Six confluent 15-cm dishes were washed twice in PBS
containing 1 mM MgCl2 and 0.9 mM CaCl2 (PBS*), and the cells were
scraped in PBS* (without MgCl2 and CaCl2) using a windshield wiper
blade to minimize cell damage. From this point on, all buffers contained a
protease inhibitor cocktail (PB/CLAP) consisting of 1 mM PMSF, 1 mM
benzamidine, and 1 mM Protease inhibitor cocktail (PB/CLAP) consisting of
1 mM PMSF, 1 mM benzamidine, and 1 mM Prot.

**Immunoprecipitation and Immunoblotting**

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| Table I. Distribution of Golgi and Plasma Membrane Markers on Percoll Gradients |
|-------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Fraction number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Golgi membrane* | 20.4 | 26.2 | 11.3 | 7.3 | 2.6 | 2.4 | 1.9 | 2.3 | 4.1 | 7.3 | 9.2 | 5.0 |
| Plasma membrane† | 42.3 | 33.5 | 9.9 | 3.8 | 0.6 | 1.6 | 1.6 | 0.6 | 0.1 | 2.2 | 1.6 | 1.1 |

* NBD-sphingolipid fluorescence, percent of total.
† Biotinylated proteins quantified by densitometric scanning, percent of total.
buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.5% SDS) as described previously (Faust et al., 1987). Immunoprecipitates were resolved on SDS-polyacrylamide gels containing 15.0% acrylamide and 0.4% N,N'-methylene bis-acrylamide under nonreducing conditions.

Immunoprecipitation of hamster CI-MPR and hamster lgp120 was performed in RIPA buffer containing 0.1% SDS. For the immunoprecipitation of lgp120, a rabbit anti–mouse IgG linker antibody was included before the addition of protein A–Sepharose. Incubations and washes were performed as described for cathepsin D (Faust et al., 1987). Immunoprecipitates were resolved on SDS-polyacrylamide gels containing 12.5% acrylamide and 0.1% N,N'-methylene bis-acrylamide under nonreducing (CI-MPR) and reducing (lgp120) conditions.

Metabolically labeled cathepsin D, CI-MPR, and lgp120 were visualized by exposing dried gels to BioImaging (Fuji Medical Systems USA, Inc., Stamford, CT) plates or X-OMAT AR film (Eastman Kodak Co., Rochester, NY). Quantitation was performed by measuring photostimulated luminescence on a Bioimager (Fuji) equipped with MacBas software (Fuji).

For immunoblot detection of rab5, rab7, and hamster cathepsin D, samples were resolved on SDS-polyacrylamide gels containing 10.0% acrylamide and 0.27% N,N'-methylene bis-acrylamide under reducing conditions. After electrophoresis, proteins were transferred to PVDF membranes (Millipore, Bedford, MA). For immunoblot detection of CI-MPR, lgp120, and EEA1, samples were resolved on SDS-polyacrylamide gels containing 12.5% acrylamide and 0.1% N,N'-methylene bis-acrylamide under nonreducing (CI-MPR) and reducing (lgp120, EEA1) conditions, followed by transfer to nitrocellulose (Schleicher & Schuell, Keene, NH). Membranes were blocked, washed, and probed as described previously (Feng et al., 1995). HRP-conjugated antibodies were from Amersham (Arlington Heights, IL), and chemiluminescence detection reagents (Supersignal) were from Pierce.

**Results**

**Wild-Type and Mutant Forms of Rab7 Are Inducibly Expressed in Stable BHK Transfectants**

Long-term overexpression of dominant negative forms of the rab proteins can be deleterious to cell viability (van der Sluijs et al., 1992). Therefore, we chose to use a tetracycline-controlled expression system that has been shown to be very tightly regulated (Gossen et al., 1994; Gossen and Bujard, 1992). This system uses a transactivator-tetracycline repressor chimera to regulate expression of the gene of interest from a tetracycline operator and minimal CMV promoter. The hybrid transactivator does not induce transcription in the presence of tetracycline, but upon removal of the drug, the chimeric transactivator binds to the operator and induces expression of the recombinant protein. This system has been used previously to express mutant forms of the dynamin GTPase and the activating rab7Q67L mutant in HeLa HtTA1 cells (Damke et al., 1994, 1995; Méresse et al., 1995).

For our purposes, a new BHK parental line expressing the transactivator-tetracycline repressor chimera was generated. This cell line was subsequently used to derive stable lines that could be induced to overexpress wild-type rab7 or a dominant negative mutant rab7N125I (Fig. 1A). The mutant protein was readily distinguishable from the wild-type protein because of its increased mobility on SDS-PAGE. The cause for this change in migration has not been investigated, but point mutations can cause anomalous migration on SDS-PAGE. Recombinant protein expression was tightly regulated in the newly isolated cell lines. In the presence of tetracycline, and for the first several hours after transfer of the cells to tetracycline-free media, the recombinant protein was not detectable (Fig. 1, A and B). Beginning at 6 h after transfer, however, there was a steady accumulation of the recombinant protein, reaching maximal levels after 48 h (Fig. 1B; data not shown). The recombinant rab proteins were overexpressed modestly (approximately threefold) after 18 h, obviating concerns that grossly overexpressed protein might have pleiotropic effects (Fig. 1B). This level of mutant protein expression was sufficient to cause a 2.5-fold decrease in CI-MPR–mediated internalization when compared to duplicate samples cultured in the presence of tetracycline (Press, B., and A. Wandinger-Ness, manuscript in preparation). This is consistent with what was observed after transient overexpression (Feng et al., 1995). The 18-h induction period was also short enough to avoid compensatory changes caused by long-term overexpression of deleterious proteins. For these reasons, recombinant protein expression was induced consistently for 18 h in all subsequent experiments.

**Overexpression of Mutant Rab7N125I Alters the Steady-State Distribution of CI-MPR, but Not That of Lgp120**

The first hint that the expression of a dominant negative

![Figure 1](image-url)
CI-MPR /lgp120

rab7wt

rab7N125I

Figure 2. CI-MPR distribution is altered in cells expressing a mutant form of rab7. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type (rab7wt) or mutant rab7N125I proteins. Cells were then fixed and stained with an affinity-purified polyclonal antibody directed against CI-MPR (left) or a mouse mAb against lgp120 (right). Antibody complexes were visualized with appropriate secondary antibodies conjugated to Texas red or FITC.

A Dominant Negative Rab7 Mutant Causes CI-MPR and Its Ligands to Accumulate in Light Membrane Fractions

Individual subcellular compartments are readily separated on Percoll gradients, providing a convenient means of monitoring the proteins or enzyme activities associated with various membrane fractions. To analyze the subcellular distribution of CI-MPR and its ligands, it was essential to use conditions that could resolve early and late endosomes. This was made possible using iso-osmotic homogenization and Percoll solutions prepared without sucrose (Czekay et al., 1997). Under these conditions, rab5-positive early endosomes were routinely recovered near the top (fractions 1–3) of a 20% Percoll gradient and were well resolved from rab7-positive late endosomes present in the denser fractions (5–12) (Fig. 3 A). The profile of a peripheral membrane protein EEA1, recently shown to be associated with early endosomes and recognized by human autoimmune serum (Mu et al., 1995), overlapped with that of rab5 (Fig. 3 A). Thus, two independent markers confirmed that early endosomes were confined to the top of the gradient. The profile of integral lgp120 established that lysosomes were confined to the densest fractions (Fig. 3 A). Additional markers were used to demonstrate that the Golgi was confined to fractions 1–4 and the plasma membrane was confined to fractions 1–3 (see Table I). The results presented in Fig. 3 A, using cells overexpressing wild-type rab7, were identical to those obtained with control BHK cells (not shown).

When the fractionation was performed with cells induced to overexpress the dominant negative mutant rab7N125I protein, the distribution of the markers for various endocytic compartments was largely unchanged. Lysosomes marked by lgp120 were still found primarily in the densest fractions (Fig. 3 B). Late endosomes marked by the endogenous rab7 protein remained in the lower third of the gradient (Fig. 3 B, wt). The majority of early endosomes were detected in the top three to four gradient fractions, based on the distributions of both rab5 and EEA1 markers (Fig. 3 B). A second peak of rab5-containing endosomes was also observed at slightly higher densities. This modest alteration in the density of a subset of early endosomes most likely results from the transport block induced by expression of the mutant rab7N125I. Increased protein accumulation in early endosomes would be expected under these circumstances and could affect early endosome density. In spite of these differences, there was never any overlap between early and late endosomes in the top four gradient fractions.

It is noteworthy that the overexpressed mutant rab7N125I protein was recovered in the uppermost gradient fractions (Fig. 3 B, N125I). This could be caused by a propensity of the mutant protein to be cytosolic and associated with structures other than late endosomes, including vesicles aligned on the actin cytoskeleton (Wandinger-Ness, A., unpublished observation). We do not believe that this represents a subpopulation of late endosomes because there was no trace of any endogenous, wild-type rab7 protein (distinguished by its slower mobility) associated with these fractions.

The gradient fractions shown in Fig. 3 A were also assayed for CI-MPR and its ligands. As expected, CI-MPR was found to be associated with fractions containing rab7-positive late endosomes (fractions 6–12). Lysosomal hydrolases known to be targeted by CI-MPR (including β-hexosaminidase and cathepsin D) cofractionated with rab7 and lgp120 markers, indicative of their late endosomal/lysosomal localization under steady-state conditions (Fig. 3, A and C, closed squares). The cathepsin D detected by immunoblotting is the intermediate form of the protein, and it represents a major species of the protein that is

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present in hamster cells under steady-state conditions (Isidoro et al., 1991). The antibody used for these studies was unable to detect the mature hamster cathepsin D protein on immunoblots.

The situation was remarkably different when the steady-state distributions of both the CI-MPR and its ligands were examined after overexpression of the mutant rab7N125I protein. Approximately 40–50% of the receptor and ligands (β-hexosaminidase and cathepsin D) were now found to be associated with the top four gradient fractions containing Golgi and early endosomes, but devoid of late endosomes, as monitored by the distribution of the wild-type rab7 protein (Fig. 3, B and C, open squares). Clearly, this shift in the hydrolase distributions cannot be accounted
for by a change in lysosome density because the lysosomal membrane protein lgp120 was still present exclusively in the densest fractions (Fig. 3B). These results showed that the expression of the mutant rab7N125I protein clearly perturbed the lysosomal accumulation of some markers while leaving others unaffected. One unresolved question concerned the issue as to whether the light membranes containing the lysosomal hydrolases were derived from the Golgi or endosomes.

Expression of Mutant Rab7N125I Causes Lysosomal Hydrolases to Accumulate in Endosomes

DAB can be used to cross-link endocytic compartments that contain internalized HRP (Ajioka and Kaplan, 1987; Courtoy et al., 1984, 1988). After the formation of a dense, cross-linked reaction product within their lumen, endosomes are readily sedimented at low speed. The time required for HRP to fill early endosomes is on the order of min, and HRP does not reach Golgi compartments during incubation periods of <1 h (data not shown). Therefore, this method provided a convenient means to distinguish between marker residence in Golgi membranes or endosomes.

Cells were induced to express rab7N125I and were maintained continuously in medium containing mannose 6–phosphate to prevent recapture of any secreted enzymes. HRP was internalized for 30 min to ensure that the endosomes would be well labeled. After Percoll gradient fractionation, the top four fractions were collected and divided into two equal aliquots. One aliquot was subjected to DAB cross-linking (+), and the other aliquot was left untreated (−) as a control. After the removal of cross-linked material by centrifugation, the supernatant fractions were analyzed. (A) Schematic outline of the experiment. (B) Immunoblots for hamster cathepsin D (cath) (P, procathepsin; I, intermediate form) and rab5. (C) The activity of α-mannosidase II (Golgi marker, units × 1) and β-hexosaminidase (units x 1.5) were determined enzymatically, while NBD-sphingolipids (a trans-Golgi marker) were monitored fluorometrically (relative fluorescence ×40). The activities measured in each of the top three gradient fractions with (+) or without (−) DAB treatment have been plotted as averaged values with SD shown.

Figure 4. Light membranes containing CI-MPR and its ligands are derived from the endocytic pathway and not from the Golgi complex. Stable BHK fibroblasts were induced to overexpress the mutant rab7N125I protein by culture in the absence of tetracycline for 18 h. Mannose 6–phosphate was included in the culture medium during this induction period to prevent the reinternalization of secreted ligands. HRP (5 mg/ml) was internalized for 30 min, and the Golgi was labeled with NBD-ceramide, as described in Materials and Methods. PNS were prepared and fractionated on Percoll gradients. The top four fractions were divided into two aliquots; one aliquot was subjected to DAB cross-linking (+), and the other aliquot was left untreated (−) as a control. After the removal of cross-linked material by centrifugation, the supernatant fractions were analyzed. (A) Schematic outline of the experiment. (B) Immunoblots for hamster cathepsin D (cath) (P, procathepsin; I, intermediate form) and rab5. (C) The activity of α-mannosidase II (Golgi marker, units × 1) and β-hexosaminidase (units x 1.5) were determined enzymatically, while NBD-sphingolipids (a trans-Golgi marker) were monitored fluorometrically (relative fluorescence ×40). The activities measured in each of the top three gradient fractions with (+) or without (−) DAB treatment have been plotted as averaged values with SD shown.
dase II and NBD-sphingolipids, were largely unaffected by the cross-linking and remained soluble; their activity decreased <6% in the presence of DAB (Fig. 4 C). These results strongly indicate that the hydrolases recovered from the top of the gradient were primarily associated with endosomes and not the Golgi.

**Overexpression of Mutant Rab7N125I Impairs the Endosomal Processing of the Lysosomal Hydrolase Cathepsin D**

Cathepsin D processing was analyzed in an effort to pinpoint the site of lysosomal enzyme delivery from the TGN. Cathepsin D is the major aspartyl protease of lysosomes and a preferred ligand for CI-MPR (Ludwig et al., 1994; Pohlmann et al., 1995). Cathepsin D is initially synthesized in the ER as an inactive proenzyme (∼53 kD) that is subsequently converted into an active, single-chain intermediate (∼46 kD) (Delbrück et al., 1994; Richo and Conner, 1994; Rijnboutt et al., 1992). Processing to the single chain form most likely occurs in late endosomes. In specialized cell types, activation may even occur in early endosomes (Diment et al., 1988). A final cleavage in the lysosome generates the two-chain mature form, which consists of one 14-kD (M_L) and one 31-kD (M_H) subunit. Thus, the processing of cathepsin D can be used to gauge the progress of its intracellular transport.

Immunoprecipitation of endogenous hamster cathepsin D from pulse-labeled cells after various chase periods (from 0 to 4 h) revealed that processing to the intermediate form began ∼1 h after synthesis, and formation of the lysosomal mature forms was detected within a 2-h chase period (Fig. 5 A, rab7wt lanes). After a 4-h chase period, the procathepsin D species was mostly processed, and the intermediate and mature forms prevailed. This is in agreement with other studies performed on the processing of hamster cathepsin D (Isidoro et al., 1991). Expression of the mutant rab7N125I protein resulted in a kinetic delay in the processing of procathepsin D to the intermediate species, and formation of the mature species was undetectable even after a 4-h chase (Fig. 5 A, rab7N125I lanes). Alterations in cathepsin D processing persisted and were detectable in cells that were metabolically labeled for as long as 6 h (Press, B., and A. Wandinger-Ness, manuscript in preparation). Procathepsin D levels were elevated by three- to fourfold, while the levels of the mature species were similarly decreased in cells expressing rab7N125I as compared to cells expressing wild-type rab7. This is consistent with the fact that procathepsin D was clearly discernible in cells expressing rab7N125I, even at steady state (Figs. 3 B and 4 B).

The Golgi processing of the carbohydrate chains on CI-MPR (Fig. 5 B) and lgp120 (Fig. 7 E; data not shown) was identical in both cell lines, serving as a strong indication that expression of the mutant rab7N125I protein had no effect on exocytic transport (Fig. 5 B). This finding agrees

![Figure 5. Rab7N125I expression leads to diminished cathepsin D processing in endosomes, but Golgi processing remains normal.](image)

(A) Processing of cathepsin D to the intermediate species is kinetically delayed, and formation of the mature protein is inhibited in cells overexpressing rab7N125I. (B) Acquisition of complex carbohydrates by CI-MPR in the Golgi follows similar kinetics in cells expressing wild-type or mutant rab7N125I proteins. Cells were metabolically labeled and harvested as described above. Hamster CI-MPR was immunoprecipitated as described in Materials and Methods. Immunoprecipitates were resolved by SDS-PAGE, and positions of procathepsin D (P), the single chain intermediate (I) form of the enzyme, and the heavy and light chains of the two-chain mature form (M_H, M_L) of cathepsin D are indicated. Data shown are representative of three independent trials.
with our previous results showing that rab7N125I had no effect on vesicular stomatitis virus G protein transport along the exocytic pathway (Feng et al., 1995).

Evidence for the Direct Intracellular Delivery of CI-MPR and Newly Synthesized Cathepsin D from the TGN to an Early Endocytic Compartment

To distinguish whether targeting from the TGN entailed direct delivery to an early endocytic compartment, the transport of newly synthesized molecules was analyzed in both wild-type and mutant cell lines. In this regard, it was first important to establish a meaningful time point for the gradient fractionation of metabolically labeled samples (i.e., sufficient time for exit from the TGN to have occurred). Analysis of the kinetics of CI-MPR and cathepsin D processing revealed that a 2-h chase period was sufficient to allow CI-MPR to acquire Golgi-specific carbohydrate modifications and cathepsin D to become processed in endocytic compartments (Figs. 5, A and B). On the basis of this information, cells were induced to express rab7 wild-type or rab7N125I proteins for 18 h, and they were subjected to a 2-h chase period after brief metabolic labeling. Postnuclear supernatants were prepared and subjected to Percoll gradient fractionation. Individual fractions were collected and analyzed as pools (I–III) to facilitate sample handling and protein detection. Cathepsin D was immunoprecipitated from each set of pooled fractions. The differences between the two cell lines in the endosomal processing of cathepsin D were once again apparent. The mature species were only observed in cells expressing the wild-type protein, and these forms were primarily in pool III, concordant with their formation in lysosomes. The procathepsin D species was prevalent in cells expressing rab7N125I and was enriched in the top gradient fractions (pool I), consistent with its presence in the Golgi and/or early endocytic structures. The distribution of the intermediate form was of primary interest because it is diagnostic of cathepsin D in endosomes.

Quantitative analysis revealed that in cells expressing wild-type rab7, the majority (59.5%) of the intermediate form of cathepsin D was associated with the densest gradient fractions in pool III (Fig. 6A, rab7wt lanes). The remainder was nearly equally distributed between pools I (18.3%) and II (22.2%). This distribution is consistent with formation of the intermediate species in endosomes. Lysates derived from cells expressing mutant rab7N125I exhibited a remarkably distinct profile for the intermediate species (Fig. 6A, rab7N125I lanes). In this case, the majority (54%) of intermediate cathepsin D was recovered in pool I together with procathepsin D. The amount of the intermediate form detected in pool III (27.7%) decreased accordingly, while the amount in pool II (18.3%) was similar to that found in pool II using cells expressing wild-type rab7.

Immunoprecipitation of CI-MPR from the same fractions revealed that the newly synthesized receptor also exhibited an altered distribution in cells expressing mutant rab7N125I protein (Fig. 6B). Normally, the majority (56.8%) of the receptor was detected in pool III. Pool II contained somewhat less CI-MPR (32.2%), and pool I had low but detectable levels (11.0%). In cells expressing mutant rab7N125I, the amount of CI-MPR present at the top of the gradient increased threefold (41%), and the receptor in the densest fractions decreased correspondingly (23.0%). These data highlight the increased presence of cathepsin D and CI-MPR in light membrane fractions upon expression of mutant rab7N125I protein.

If the newly synthesized proteins associated with these light membrane fractions are primarily contained in endosomes, they would be expected to exhibit an increased sensitivity to DAB cross-linking, as demonstrated above for the proteins accumulated at steady state. To examine this issue, cells were induced to express the wild-type or mutant rab7 proteins as before, and were then metabolically labeled and incubated for chase periods of 30 and 120 min. HRP was internalized during the last 10 min of each chase period, and samples were subjected to DAB cross-linking, as detailed in Materials and Methods. Replicate samples were left untreated as controls. Quantitative immunoprecipitations of cathepsin D and CI-MPR were conducted in triplicate for each time point, and results from a representative experiment are shown. After a 30-min chase period, the procathepsin D species prevailed in cells expressing either form of rab7, and this species was insensitive to DAB cross-linking (Fig. 7A). At this time point, the procathepsin D was most likely still in the Golgi and, therefore, inaccessible to HRP. After a 120-min chase period, the inter-

Figure 6. Rab7N125I expression causes newly synthesized CI-MPR and immature cathepsin D to accumulate in light membrane fractions. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type and mutant rab7 proteins. Cells were metabolically labeled and, after a 2-h chase period, were subjected to Percoll gradient fractionation, as described in Materials and Methods. Before immunoprecipitation, fractions were pooled as follows: pool I, fractions 1–4; pool II, fractions 5–8; and pool III, fractions 9–12. (A) Cathepsin D or (B) CI-MPR were immunoprecipitated from pooled fractions as described in Materials and Methods. Immunoprecipitates were resolved by SDS-PAGE, and positions of procathepsin D (P), the single chain intermediate (I) form of the enzyme, and the heavy and light chains of the two-chain mature form (M_H, M_L) of cathepsin D are indicated. The data shown are representative of two independent trials.
mediate and mature forms of cathepsin D predominated in cells expressing wild-type rab7, and these species were also insensitive to DAB cross-linking (Fig. 7, A and B). In contrast, the intermediate form of cathepsin D was clearly sensitive to DAB cross-linking after a 120-min chase period in cells expressing rab7N125I (Fig. 7 A). Quantitation showed that the intermediate form was decreased by 40% in the sample treated with DAB (Fig. 7 B). Again, processing to the mature forms did not occur in this cell line within this time frame.

Quantitative immunoprecipitations of CI-MPR yielded analogous results. At the 30-min time point, the immature form of CI-MPR was insensitive to cross-linking in both cell lines because it had not yet traversed through the Golgi (Fig. 7 C). The mature CI-MPR prevalent at the 120-min time point was sensitive to cross-linking, but only in cells expressing mutant rab7N125I protein (Fig. 7 C). In these cells, the mature receptor was decreased by 43% after DAB cross-linking (Fig. 7 D), concuring with the results obtained for the intermediate cathepsin D species.

These DAB–cross-linking experiments using metabolically labeled samples were extremely difficult to perform and, as a result, some of the standard deviations were large (Fig. 7, B and D). Calculation of the Student’s t distribution,
with a 90% confidence limit, indicated that the differences in cathepsin D and CI-MPR signals (±DAB cross-linking) upon rab7N125I expression were statistically significant.

The results presented in this section are consistent with the interpretation that the newly synthesized proteins are initially delivered from the TGN to an early endocytic compartment, where they accumulate in cells expressing mutant rab7N125I. Here, slow conversion of procathepsin D to the intermediate form can still take place when transport to late endosomes is diminished. Such early structures would readily be loaded with HRP during a 10-min internalization period and could account for the sensitivity of the newly synthesized proteins to HRP/DAB–mediated cross-linking. On the other hand, when egress from this compartment is normal (as is the case in cells expressing wild-type rab7), these proteins are rapidly transported to late endocytic compartments and are no longer sensitive to DAB cross-linking.

There are numerous indications that lysosomal membrane glycoproteins are transported from the Golgi to lysosomes via a route distinct from that used by CI-MPR and its ligands (Brown et al., 1986; Griffiths et al., 1988; Harter and Mellman, 1992; Mathews et al., 1992). The finding that expression of rab7N125I had no apparent impact on the steady-state distribution of lgp120 (Figs. 2 and 3, A and B) is in keeping with this. Therefore, we also examined the sensitivity of newly synthesized lgp120 to DAB cross-linking. After brief metabolic labeling and a 30-min chase period, a significant fraction of immature lgp120 was still detectable in both cell lines, but the fully glycosylated mature species was also clearly evident (Fig. 7 E). Neither the immature nor the mature forms of lgp120 were sensitive to DAB cross-linking at either time point examined in cells expressing wild-type rab7 (Fig. 7, E and F). In contrast to what was observed for CI-MPR and cathepsin D, lgp120 remained insensitive to DAB cross-linking, even in cells expressing the mutant rab7N125I protein. Thus, lgp120 served as a useful control for the DAB cross-linking studies. It is a marker that is transported to lysosomes, yet it was apparently unaffected in its targeting by expression of the mutant rab7N125I protein. These findings also serve as an indication that lgp120 is likely to follow an independent route to lysosomes.

**Discussion**

**Inducible Rab7 Cell Lines Facilitate the Analysis of Membrane Transport Pathways**

We have isolated stable BHK cell lines that inducibly express wild-type or a dominant negative mutant form of rab7. Dominant negative mutant forms of the rab proteins have been shown to interfere with specific membrane transport pathways (for reviews see Stow, 1995; Zerial and Stenmark, 1993). This feature can be exploited to generate mammalian cells with specific defects in membrane transport. In effect, this makes it possible to circumvent the difficulties associated with isolating transport mutants in mammalian cells. In the case of the yeast *Saccharomyces cerevisiae*, such mutants (sec and vps) have been invaluable both for the elucidation of interconnected pathways and the identification of important components of the transport machinery (Comibear and Stevens, 1995; Ferro-Novick et al., 1984; Novick et al., 1980; Rothman et al., 1989).

We showed previously that the transient overexpression of dominant mutant forms of rab7 inhibited the late endocytic transport of endocytosed molecules (Feng et al., 1995). One drawback of this transient vaccinia expression system is the limited time course over which the effects of the rab7 mutants could be evaluated. We show here that the stable cell lines provide an ideal system for examining the effects of dominant negative rab7 protein expression on lysosome biogenesis. In this work, we have addressed a question relevant to the trafficking of CI-MPR and its associated ligands. It is anticipated that these cell lines will also be more broadly useful to dissect the routes followed by other molecules on the exocytic and endocytic circuits.

**Rab7 Function in Late Endocytic Membrane Transport**

The yeast homologue of rab7, Ypt7p, has been shown to function at a later step in transport to the vacuole and in homotypic vacuole fusion events (Haas et al., 1995; Schimmel and Riezman, 1993). Studies in mammalian cells using the rab7Q67L mutant led to the suggestion that rab7 may play a role in transport from late endosomes to lysosomes (Méresse et al., 1995). Therefore, it is possible that rab7 is required for two transport steps, leading from early to late endosomes (Feng et al., 1995) and from late endosomes to lysosomes. The results shown here cannot exclude this possibility. They do, however, exclude the possibility that rab7 functions only in late endosome to lysosome transport. Had this been the case, the lysosomal hydrolases would have been expected to accumulate in late endosomes and not in early endosomes, as shown by the fractionation and HRP/DAB–cross-linking experiments. The cleavage profile of newly synthesized cathepsin D further demonstrates that this is not the case, otherwise there should not have been a delay in proenzyme processing, as seen in the pulse-chase study.

Several controls excluded the possibility that the mutant rab7 protein had pleiotropic inhibitory effects on membrane transport. First, expression of the mutant rab7N125I protein had no effect on exocytosis (Feng et al., 1995). Second, the kinetics of CI-MPR and lgp120 synthesis and carbohydrate processing (serving as a measure of transport from the ER through the Golgi) appeared to be identical in cells expressing wild-type or mutant forms of rab7 (Fig. 5 B, 7 E: data not shown). The short-term internalization kinetics of fluid phase markers such as HRP were identical in both cell lines (Press, B., and A. Wandinger-Ness, manuscript in preparation). Finally, molecules transported to lysosomes by a mannose 6–phosphate–independent route, such as lgp120, were unperturbed by expression of rab7N125I.

**Intracellular Trafficking of CI-MPR and Cathepsin D**

The results presented here support the view that a significant fraction of CI-MPR and cathepsin D traffic from the TGN to late endosomes/lysosomes via early endosomes. Several lines of evidence are consistent with this conclusion. The first indication came from the observation that the overexpression of the mutant rab7 proteins caused the CI-MPR to accumulate in large peripheral vesicles, while lysosomes characterized by lgp120 staining appeared morphologically unaffected. Further support was obtained...
through cell fractionation studies, which showed that not only the receptor, but also several of its ligands accumulated in early endocytic structures when the mutant rab7N125I protein was expressed. This assessment was based on CI-MPR and cathepsin D comigration with rab5 and EEA1, established markers of early endosomes, as well as the sensitivity of CI-MPR and cathepsin D to HRP/DAB–mediated cross-linking of endosomal proteins. Even newly synthesized receptor and cathepsin D were found to be increasingly prevalent in early endocytic compartments upon expression of the mutant rab7N125I protein. Therefore, slowed delivery of newly synthesized molecules to late endocytic compartments, caused by mutant rab7N125I expression, seems to be a likely explanation for the dramatic accumulation of receptor and ligands in early endocytic compartments at steady state. Such an interpretation is further corroborated by the fact that processing of pro-cathepsin D to the late endosomal intermediate form was noticeably diminished in cells expressing mutant rab7N125I protein.

Our proposal that a significant portion of CI-MPR and bound ligands exiting the TGN are initially targeted to early endosomes is in agreement with the finding that 20–40% of the newly synthesized hydrolases in NRK cells were detected in early endosomes (Ludwig et al., 1991). It is important to note that the bulk of the receptor–ligand complexes are not expected to dissociate in late endosomes because the pH is not low enough (Borden et al., 1990). Thus, the receptor could still facilitate sorting of the hydrolases from molecules destined for recycling. In this scenario, either rapid transport out of early endosomes or slow return to the TGN could account for the steady-state prevalence of CI-MPR in late endosomes.

We believe that we can exclude a number of alternative interpretations of the data. For example, Golgi membranes cofractionated with early endosomes at the top of our gradients, raising the possibility that CI-MPR and cathepsin D accumulated in the Golgi when the rab7 mutants were expressed. This is unlikely because a significant fraction of the hydrolase activity present at the top of the gradient under steady-state conditions could be depleted by HRP/DAB–mediated cross-linking of endosomes. In contrast, <6% of the Golgi markers were depleted under identical conditions. Furthermore, newly synthesized CI-MPR and cathepsin D were sensitive to HRP/DAB–mediated cross-linking only at time points that were consistent with their exit from the Golgi. The kinetics of carbohydrate acquisition by CI-MPR and lgp120 in cells expressing mutant rab7N125I also give no indication for any increase in Golgi residence time. It is unlikely that the early endosomal pool of hydrolases arose by reinternalization of secreted molecules because key experiments were performed with mannose 6–phosphate present in the media to minimize secretion and recapture.

**Multiple Routes Leading from the TGN to Lysosomes**

It is clear that integral lysosomal membrane proteins and MPRs have different sorting signals (Bonifacino et al., 1996; Rohrer et al., 1996; Schweizer et al., 1996). This is understandable because CI-MPR must recycle back to the TGN from the late endosome, while lysosomal membrane proteins traffic onto lysosomes from late endosomes. It has been less clear, however, whether the two groups of molecules are initially shuttled from the TGN in the same or distinct carrier vesicles. Recent studies on the transport of integral membrane proteins and soluble enzymes to the vacuole provide clear evidence for the existence of two distinct routes in yeast (Cawles et al., 1997b; Piper et al., 1997). The soluble enzyme carboxypeptidase Y, in association with a specific membrane receptor, exits the Golgi and is first delivered to a prevacuolar compartment. From here, the protein is then transported to the vacuole. The membrane protein alkaline phosphatase bypasses the prevacuolar compartment and depends on a unique set of transport machinery for delivery to the vacuole (Cawles et al., 1997a; Piper et al., 1997).

The results presented here concur with the scenario emerging from the studies in yeast. Our results show that CI-MPR and associated ligands follow a pathway that is distinct from that used by lgp120. This is evidenced by the fact that the rab7N125I mutant perturbed the steady-state distributions of CI-MPR and associated soluble hydrolases, but had no effect on the lgp120 profile. Furthermore, while both newly synthesized cathepsin D and CI-MPR could be shown to be sensitive to DAB cross-linking in cells expressing rab7N125I, lgp120 remained insensitive to DAB cross-linking at all time points tested. Further confirmation depends on the identification of the machinery that allows the formation of two distinct transport vesicles from the Golgi. Specific adaptor protein complexes (AP-1) have been shown to be recruited to the TGN by CI-MPR (Le Borgne and Hoflack, 1997). These complexes promote clustering and inclusion into TGN-derived, clathrin-coated vesicles, which then shuttle their cargo to the endocytic pathway (Bonifacino et al., 1996; Robinson, 1994). A third adaptor protein complex (AP-3) has now been identified in yeast and mammalian cells (Cawles et al., 1997a; Dell’Angelica et al., 1997; Simpson et al., 1996). Yeast AP-3 has been shown to be involved in the vacuolar targeting of membrane-bound alkaline phosphatase. Mammalian AP-3 is therefore an excellent candidate for regulating the inclusion of lysosomal membrane proteins into distinct cargo vesicles from the TGN.

**Potential Significance of CI-MPR and Hydrolase Delivery to Early Endosomes**

It is interesting to consider the potential significance of CI-MPR and lysosomal hydrolase delivery to early endosomes in light of their involvement in various aspects of cellular growth control and differentiation. It is well known that CI-MPR also binds IGF II and, as a consequence, may participate in signal transduction cascades (Murayama et al., 1990). Cell surface CI-MPR and secreted cathepsins have also been implicated in cell migration and the remodeling of the extracellular matrix (Rozhin et al., 1987). These processes are critical during normal cellular differentiation (O’Brien et al., 1991; Szelenyi and Rotwein, 1991), and anomalous expression of lysosomal enzymes in tumors has been suggested to promote invasion (Sleat et al., 1995; Zhao et al., 1993). Increased extracellular levels of various cathepsins and other lysosomal hydrolases have been correlated with metastasis and a
number of human tumors (Boyer and Tannock, 1993; Braulke et al., 1992; Sleat et al., 1995; Sloane et al., 1991; Zhao et al., 1993). Even cell death was recently shown to be influenced by lysosomal hydrolases in a study showing cathepsin D involvement in IFN-γ-mediated apoptosis (Deiss et al., 1996).

One interpretation of these data is that the balance between extra- versus intracellular pools of hydrolases is highly regulated. Transport of CI-MPR and its ligands to early endosomes may increase cellular capacity for modulating the fraction of these molecules delivered to the cell surface versus the amount transported toward late endosomes and lysosomes. In this scenario, at least two levels of regulation could be operative, the first being at the level of CI-MPR-mediated targeting and transport. This might well be influenced by transient signals that are known to modify the cytoplasmic domain of CI-MPR, such as phosphorylation (Méresse and Hoflack, 1993) or palmitoylation (Schweizer et al., 1996). A second level of regulation could control membrane flow to late stages of the endocytic pathway, as has been shown to be the case for PDGF receptors and B cell receptors (Joly et al., 1995; Sphetner et al., 1996; Xu et al., 1996). Their increased transport to late endosomes has been shown to involve activation of phosphatidylinositol 3-kinases and small GTPases. Regulation at this level would most likely be influenced by extracellular factors and signaling cascades (Braulke et al., 1990). In this regard, it is interesting to note that transport of lysosomal hydrolases to late endosomes or lysosomes, where removal of the mannose 6-phosphate signal occurs, was shown to be modulated by extracellular serum levels (Einstein and Gabel, 1989, 1991).

In summary, the mutant forms of rab proteins are useful tools for unraveling the intracellular transport pathways followed by any molecule of interest. Here, mutant forms of rab7 were used to provide evidence for the involvement of early endosomes in the trafficking of CI-MPR and associated lysosomal hydrolases to later endocytic compartments. The transport of lysosomal membrane proteins appears to occur via a different route. In light of this finding, it will be of interest to determine how biosynthetic transport from the TGN to different endosomes is regulated and how this may, in turn, be correlated with the regulation of biological activity.

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