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-

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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 Bonifacino 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; Hoflack 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., 1987). Thus, although the two receptors both bind mannose 6–phosphorylated ligands, there are a number of functional distinctions between them.

It is generally agreed that the targeting of both MPRs (in association with their newly synthesized ligands) to endosomes occurs via an intracellular pathway (Klumperman et al., 1993). It is somewhat less clear, however, whether targeting from the TGN occurs primarily to early or late endosomes or even equally to both. At steady state, both receptors are principally localized to late endosomes (Bleekemolen et al., 1988; Griffiths et al., 1988). Here, the receptors dissociate from their ligands and may recycle back to the Golgi (Brown et al., 1986; Duncan and Kornfeld, 1988; Goda and Pfeffer, 1988; Rohrer et al., 1995). These observations led to the suggestion that receptor–ligand complexes are targeted directly from the TGN to late endosomes (Griffiths et al., 1988). Circumstantial evidence 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 pUHD 15-1, 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 (6 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 (PGC 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 pMiwhph (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

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 detected 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 detect the mature species on immunoblots. CI-MPR was detected on immunoblots 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 Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Monoclonal 4A1 was used to monitor hamster lysosomal glycoprotein (lgp) 120 (a gift from J. Gruenberg, Université de Genève, Switzerland).

Immunofluorescence Microscopy

Stable BHK-tTA/R7 lines were grown on 15-mm square coverslips. Expression of recombinant rab7 proteins was induced by culture in select G-MEM without tetracycline for 18 h. Fixation, permeabilization, and immunofluorescence staining were performed as described previously (Feng et al., 1995). Affinity-purified rabbit anti–bovine CI-MPR was used to detect CI-MPR. mAb 4A1 was used to detect lgp120. Secondary antibodies and detection reagents were from Vector Laboratories (Burlingame, CA). Samples were viewed with an Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

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 expression 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 lysosomal hydrolases. Six confluent 15-cm dishes were washed twice in PBS containing 1 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS^+), and the cells were scraped in PBS^- (without $MgCl_2$ and $CaCl_2$) 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 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A (Sigma Immunochemicals, St. Louis, MO). Cells were pelleted by centrifugation at 4000 rpm in a rotor (model SA600; Sorvall) for 10 min at 4°C. The cell pellet was resuspended in 10 ml homogenization buffer (HB; 20 mM Hepes, pH 7.4, 150 mM NaCl, and 2 mM $CaCl_2$). The cells were again collected by centrifugation and resuspended in three-pellet volumes of HB. The suspension was gently passed through a 27-gauge needle eight times. Cell debris and nuclei were removed by two successive centrifugation steps at 2,500 rpm for 5 min in a microfuge at 4°C. Under these conditions, ≤30% of the endocytic markers rab5, rab7, lgp, and cathepsin D were recovered in the postnuclear supernatant (PNS). Such gentle homogenization conditions resulted in a high fraction of unbroken cells that decreased the apparent recovery of markers in the PNS. More vigorous homogenization was found to compromise the integrity of the endocytic organelles in agreement with the findings of Gruenberg and coworkers (Bomsel et al., 1990; Gorvel et al., 1991). The resulting PNS (~1.2 ml) was overlaid onto 11 ml of a 20% (wt/vol) Percoll solution prepared in TBS buffer (10 mM Tris, pH 8.0, and 150 mM NaCl). The samples were subjected to centrifugation at 4°C for 50 min in a 70.1Ti fixed angle rotor (Beckman Instruments, Fullerton, CA) at 20,000 *g*. 1-ml fractions were collected from the top of the gradient using a Buchler Auto Densi-Flow IIC pump (Lenexa, KS). CHAPS detergent (Pierce Chemical Co., Rockford, IL) was added to each fraction to a final concentration of 10 mM and incubated for 1 h on ice to solubilize all membranes. Subsequently, the Percoll was removed by centrifugation for 1 h at 100,000 *g* in a TLA 100.2 rotor (Beckman), and the fractions were concentrated to 100 μl using a Centricon 10 concentrator (Amicon, Beverly, MA). To remove all traces of Percoll, the concentrated samples were subjected to a final centrifugation for 1 h at 100,000 *g* in a TLA 100.2 rotor. Individual fractions were analyzed for rab5, rab7, hamster cathepsin D, early endosome antigen 1 (EEA1), CI-MPR, and lgp120 by immunoblot. Lysosomal hydrolase activities were measured enzymatically.

Metabolic Labeling and Analysis of Newly Synthesized Proteins

To monitor the synthesis and processing of newly synthesized cathepsin D and CI-MPR, cells were metabolically labeled with 0.4 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (Trans-label; ICN Biomedicals, Inc., Costa Mesa, CA) for 15 min and were transferred to complete G-MEM containing 10 mM mannose 6–phosphate for various lengths of time as described previously (Richo and Conner, 1994).

The distributions of newly synthesized cathepsin D and CI-MPR were monitored by Percoll gradient fractionation. Three confluent 15-cm dishes were incubated for 30 min in DME lacking methionine and cysteine (and containing dialyzed serum) to deplete intracellular levels of these amino acids. Cells were then metabolically labeled as described above and transferred to medium containing excess unlabeled amino acids for a period of 2 h. Subsequently, the metabolically labeled cells were cooled on ice, pooled with three 15-cm dishes of unlabeled cells, and PNS were prepared and subjected to fractionation as described above. Before immunoprecipitation, solubilized fractions (containing 1% Triton X-100 instead of CHAPS detergent) were pooled as follows: pool I, fractions 1–4; pool II, fractions 5–8; and pool III, fractions 9–12.

NBD-sphingolipids were used as specific markers for the Golgi and TGN, as described previously (Lipsky and Pagano, 1985). Briefly, cells were labeled with NBD-ceramide at 20°C for 1 h, allowing conversion to NBD-sphingomyelin and NBD-glycosyl ceramide in the Golgi and blocking transport to the cell surface (van Meer et al., 1987). Before homogenization and fractionation, the cells were back-exchanged at 4°C with delipidated BSA to remove any unconverted NBD-ceramide. The NBD-sphingolipid distribution on the Percoll gradients was determined by measuring the fluorescence (Ex λ: 465 nm and Em λ: 572 nm) of individual fractions (see Table I).

The distribution of plasma membrane was determined by biotinylating cell surface proteins at 4°C before fractionation and detecting the labeled proteins after blotting with streptavidin-conjugated HRP (see Table I).

DAB Cross-Linking of HRP-containing Endosomes

For DAB cross-linking experiments performed on Percoll gradient fractions, HRP (5 mg/ml) was internalized for 30 min, and the cells were extensively washed immediately before lysis and gradient fractionation. Under the conditions used, the internalized HRP was confined to endocytic compartments and very little reached the Golgi (see Fig. 4 *B*; data not shown). Individual fractions were divided into two aliquots, one aliquot was treated with DAB (0.4 mg/ml, final) and hydrogen peroxide (0.0225% final) for 1 h at 4°C in the dark to induce cross-linking (Courtoy et al., 1984), and the second aliquot was left untreated. The cross-linked products were removed by centrifugation at 2,500 rpm in an Eppendorf microfuge, and the supernatant fractions were analyzed using fluorimetry, enzyme activity assays, or immunoblotting.

For DAB cross-linking experiments performed on pulse-labeled cells, HRP (5 mg/ml) was internalized during the final 10 min of the chase period (to label early endocytic compartments only), after which time, the cells were transferred to ice and washed extensively. Cross-linking was induced as described above, cells were lysed with the addition of Triton X-100, and precipitated material was removed by centrifugation. Supernatant fractions were analyzed by quantitative immunoprecipitation. Replicate control samples were incubated in the absence of added DAB and hydrogen peroxide, but were otherwise treated the same as the experimental samples. Additional control experiments (not shown) confirmed that no cross-linking occurred when DAB or hydrogen peroxide were added individually to samples containing HRP.

Immunoprecipitation and Immunoblotting

Immunoprecipitation of hamster cathepsin D was performed in RIPA

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	1.1	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. 1 *A*). 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. 1 *B*; 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. 1 *B*). 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

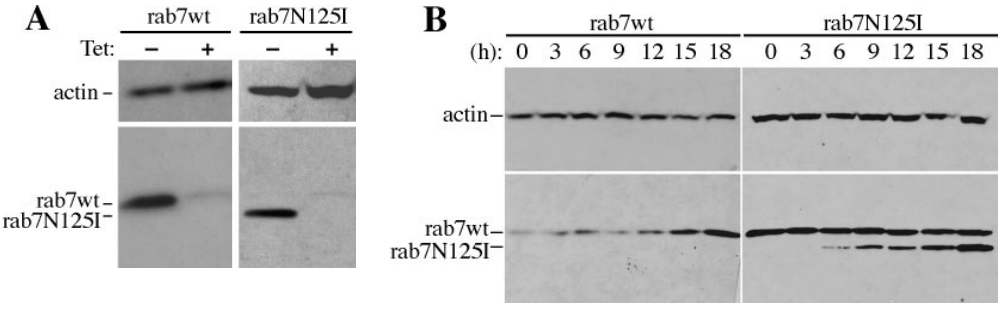


Figure 1. Wild-type and mutant forms of rab7 are inducibly expressed in stable transfectants. Stable BHK fibroblasts were cultured in the absence of tetracycline (*−Tet*) for various times to induce the expression of recombinant rab7 proteins. Duplicate control samples were maintained continuously in media containing 3 μg/ml tetracycline (*+Tet*). (*A*) Cell lysates prepared 24 h after transfer to medium lacking tetracycline compared to control samples. (*B*) A representative time course of rab7 induction upon transfer of cells to medium lacking tetracycline for 0–18 h. Immunoblots were probed with antibodies against rab7 and actin (as a control for protein loading). In all cases, an affinity-purified anti-rab7 antibody was used. The antibody was typically diluted to detect primarily the overexpressed rab7 protein. In the case of the rab7N125I samples shown in *B*, slightly higher levels of antibody were used to allow simultaneous detection of the endogenous wild-type rab7 protein and the induction of the mutant protein. *rab7wt*, cells expressing wild-type rab7; *rab7N125I*, cells expressing a mutant form of rab7. An immunoblot for actin served as a control for protein loading.

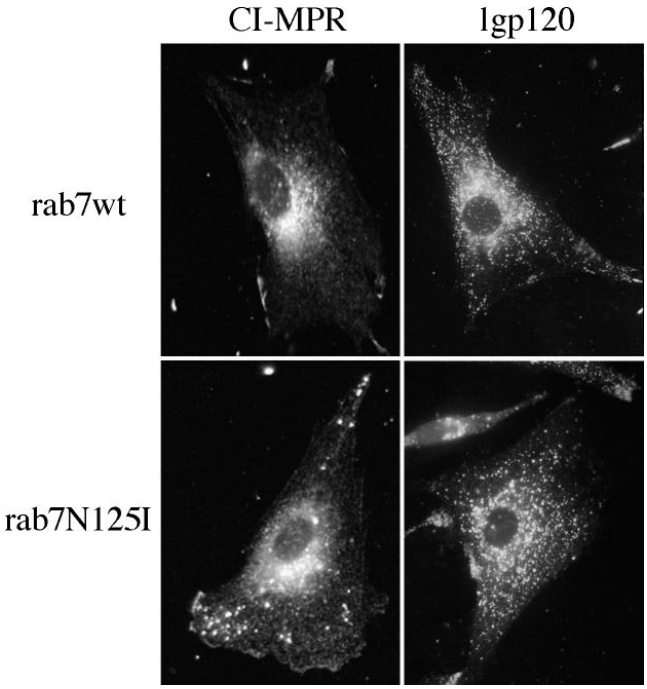


Figure 2. CI-MPR distribution is altered in cells expressing a mutant form of rab7. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type (*rab7wt*) or mutant rab7N125I proteins. Cells were then fixed and stained with an affinity-purified 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.

rab7 mutant might perturb lysosomal transport came from an examination of the subcellular localization of CI-MPR in the newly isolated cell lines. The distribution of CI-MPR was assessed by immunofluorescence microscopy. Normal, perinuclear staining of the CI-MPR was evident in cells expressing wild-type rab7 (Fig. 2, *top left*). Strikingly, when mutant rab7N125I protein was overexpressed, a significant fraction of the CI-MPR was localized in large peripheral vesicles (Fig. 2, *bottom left*). Identical results were also obtained using a stable line expressing a second dominant negative mutant, rab7T22N (data not shown).

In contrast, the distribution of lgp120 was not noticeably altered in cells expressing mutant rab7N125I as compared to control cells or those expressing wild-type rab7 (Fig. 2, *right*). To further evaluate the alteration in CI-MPR distribution and to determine its impact on the trafficking of molecules to lysosomes, cell fractionation studies were conducted.

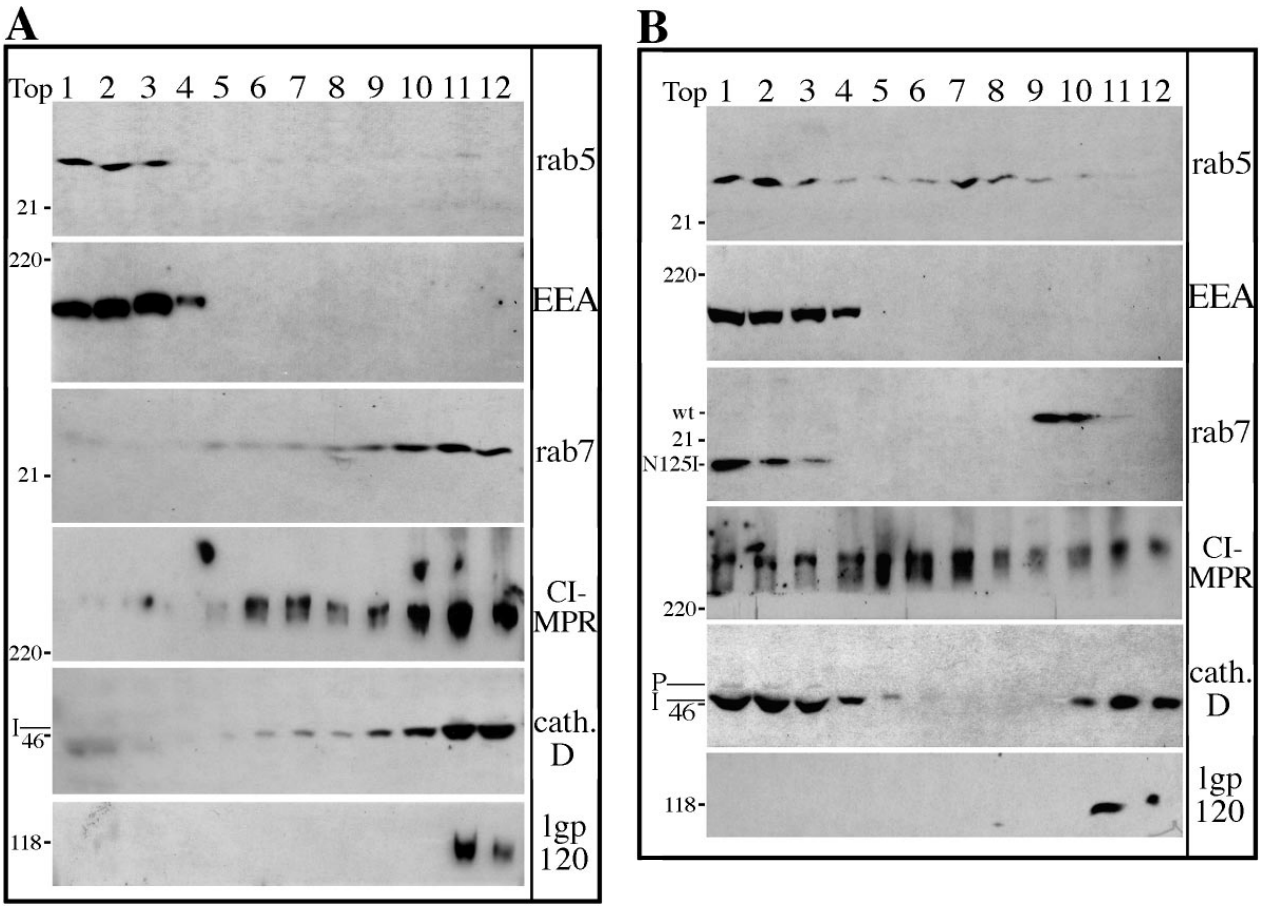
A Dominant Negative Rab7 Mutant Causes CI-MPR and Its Ligands to Accumulate in Light Membrane Fractions

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



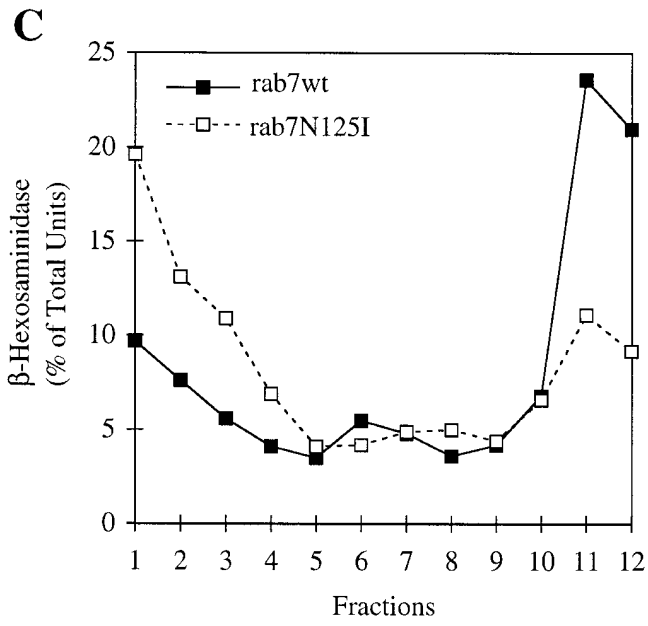


Figure 3. CI-MPR and its ligands accumulate in light membranes in cells expressing the mutant protein rab7N125I. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type and mutant rab7 proteins. PNS were prepared and fractionated on Percoll gradients, as described in Materials and Methods. Percoll gradient fractions derived from cells (*A*) overexpressing wild-type rab7 and (*B*) overexpressing rab7N125I. The distribution of early endosomes was monitored using rab5 and EEA1 as markers. Late endosomes were detected using wild-type rab7 (*wt*) as a marker; N125I denotes the overexpressed mutant protein. Lysosomes were detected using lgp120 as a marker. The distributions of CI-MPR and hamster cathepsin D (*P*, procathepsin D; *I*, intermediate form) were followed using specific antibodies. All immunoblot analysis was conducted as detailed in Materials and Methods. (*C*) The distribution of β-hexosaminidase was measured enzymatically after Percoll gradient fractionation of cells expressing wild-type rab7 (*closed squares*, *solid line*) or rab7N125I (*open squares*, *dashed line*) proteins. All immunoblots are representative examples of data from four independent trials. The β-hexosaminidase activities represent average values of three separate experiments. SD values have been omitted for clarity. The average SD was ±0.94 for the wild-type rab7 samples and ±0.54 for the rab7N125I samples.

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

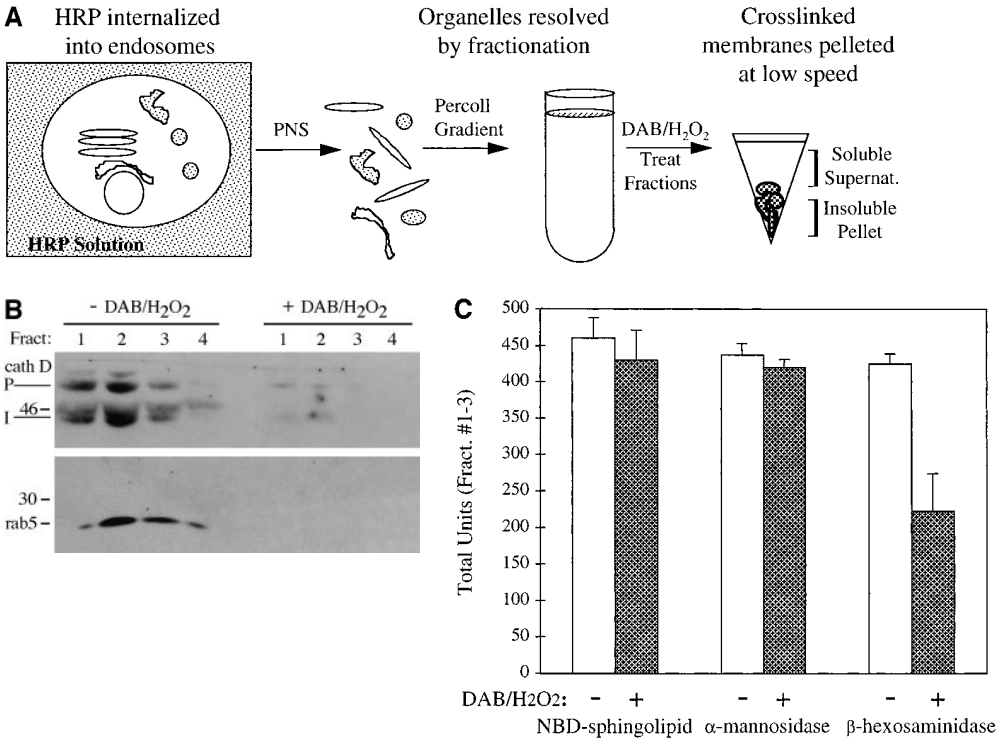


Figure 4. Light membranes containing CI-MPR and its ligands are derived from the endocytic pathway and not from the Golgi complex. Stable BHK fibroblasts were induced to overexpress the mutant rab7N125I protein by culture in the absence of tetracycline for 18 h. Mannose 6–phosphate was included in the 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.

for by a change in lysosome density because the lysosomal membrane protein lgp120 was still present exclusively in the densest fractions (Fig. 3 *B*). 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 second was left untreated as a control. After a short incubation period, cross-linked membranes were removed by low speed centrifugation (see schematic, Fig. 4 *A*). The resulting soluble fraction was assayed for various markers because the insoluble nature of the cross-linked material makes analysis of the pellet fractions intractable. Cathepsin D, present in the top four gradient fractions without DAB treatment, was significantly depleted after DAB cross-linking, as was the early endosome marker rab5 (Fig. 4 *B*). Inclusion of mannose 6–phosphate in the culture medium was noted to cause an increase in procathepsin D levels, perhaps reflecting its premature dissociation from CI-MPR in early endosomes caused by the presence of mannose 6–phosphate in the endocytic system. The effect of DAB cross-linking on cathepsin D was dramatic, but it could not be quantified because of the use of chemiluminescence detection. Therefore, a quantitative measure of the fraction of lysosomal enzymes depleted by DAB cross-linking was obtained by assaying the activity of β-hexosaminidase in the top three fractions. A reduction in activity of >40% was measured after DAB cross-linking (Fig. 4 *C*). Because the maximal depletion of endocytic markers that can be achieved after DAB cross-linking is in the vicinity of 70–80%, a significant fraction of the β-hexosaminidase can be considered sensitive to DAB cross-linking (Futter et al., 1996). In marked contrast, two Golgi markers, α-mannosi-

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

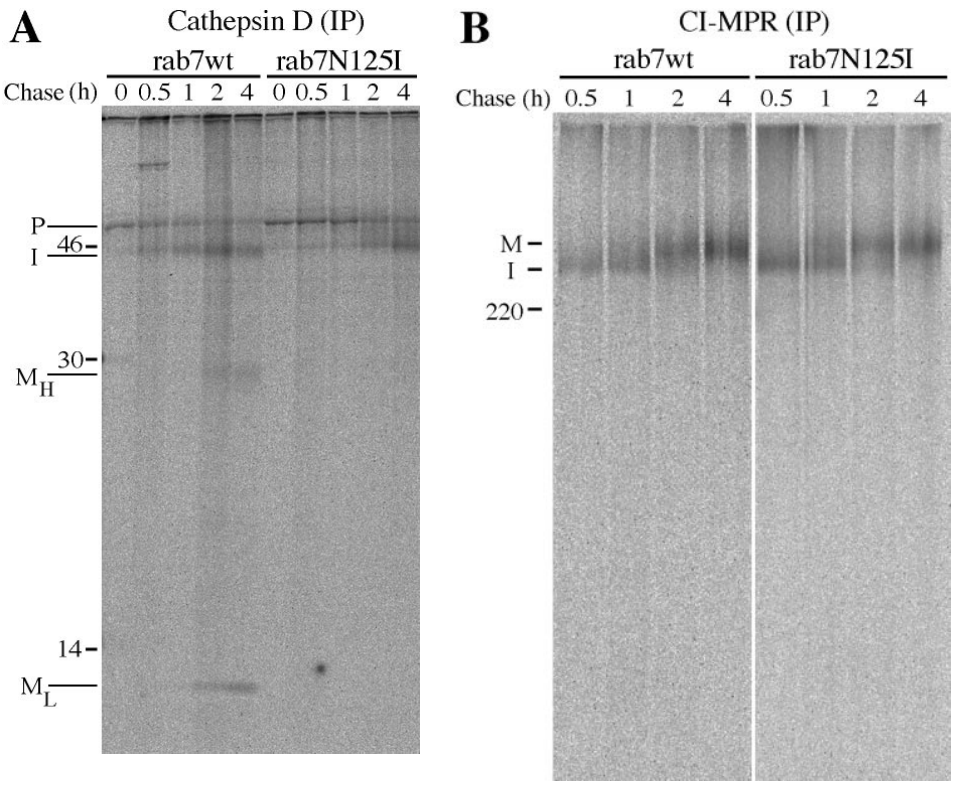


Figure 5. Rab7N125I expression leads to diminished cathepsin D processing in endosomes, but Golgi processing remains normal. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type and mutant rab7 proteins. (*A*) Processing of cathepsin D to the intermediate species is kinetically delayed, and formation of the mature protein is inhibited in cells overexpressing rab7N125I. Cells were metabolically labeled and then transferred to medium containing excess unlabeled amino acids and mannose 6–phosphate to prevent reinternalization of secreted ligand. After the indicated times, cells were collected and hamster cathepsin D 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. (*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 detailed in Materials and Methods, and immunoprecipitates were resolved by SDS-PAGE. Positions of the immature (*I*) and mature (*M*) forms of the receptor 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. 6 *A*, *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 cathepsin D species (Fig. 6 *A*, *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. 6 *B*). 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.

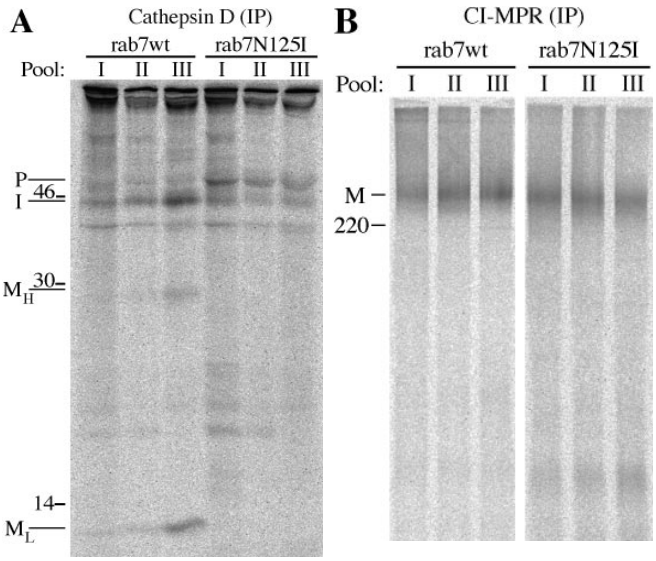


Figure 6. Rab7N125I expression causes newly synthesized CI-MPR and immature cathepsin D to accumulate in light membrane fractions. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type and mutant rab7 proteins. Cells were metabolically labeled 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.

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. 7 *A*). 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-

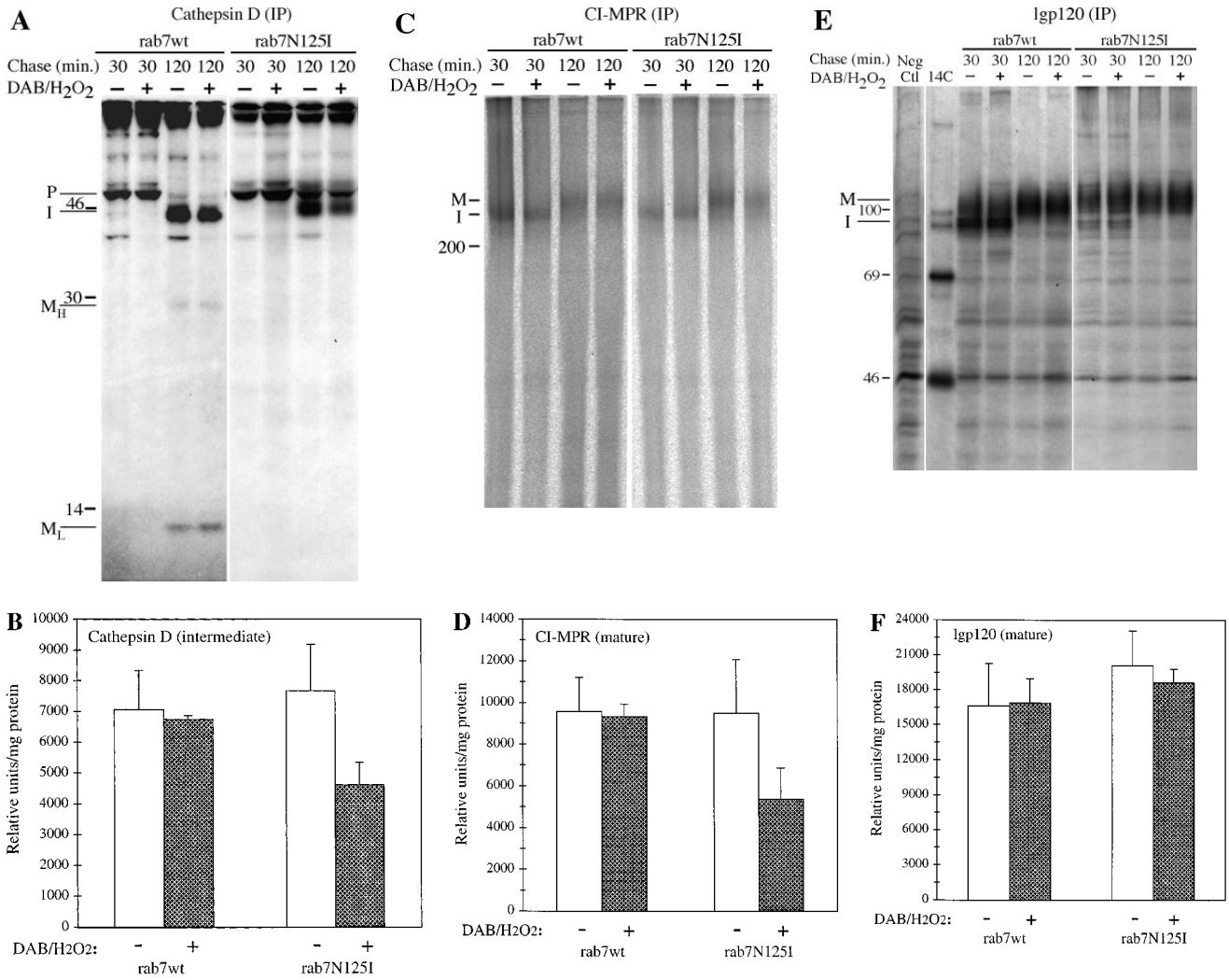


Figure 7. Newly synthesized CI-MPR and immature cathepsin D are present in early endocytic compartments in cells expressing rab7N125I. Stable BHK fibroblasts were cultured in the absence of tetracycline for 18 h to allow for overexpression of wild-type and mutant rab7 proteins. Cells were metabolically labeled and incubated for 30 or 120 min in medium containing excess unlabeled amino acids. During the final 10 min of the chase period, HRP was added to a final concentration of 5 mg/ml. Cells were subsequently cooled on ice, and individual dishes were subjected to DAB cross-linking (+) and controls were left untreated (−) (details in Materials and Methods). After cell lysis and removal of cross-linked material by centrifugation, immunoprecipitation of (*A*) hamster cathepsin D, (*C*) hamster CI-MPR, or (*E*) hamster lgp120 was carried out as detailed in Materials and Methods. Positions of procathepsin D (*P*), the single chain intermediate (*I*) form of the enzyme, the heavy and light chains of the two-chain mature form (M_H, M_L) of cathepsin D, and the immature (*I*) and mature (*M*) forms of CI-MPR and lgp120 are indicated. Quantitation of the amounts of (*B*) intermediate cathepsin D, (*D*) mature CI-MPR, and (*F*) mature lgp120 immunoprecipitated with (+) or without (−) DAB cross-linking. Samples were derived from cell lysates overexpressing wild-type (*wt*) rab7 or mutant rab7N125I proteins, as indicated. All values were normalized to total protein and are the average of triplicate determinations (+SD) from one of three representative and independent experiments.

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*), concurring 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 later 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 (Conibear 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; Schimmöller 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 procathepsin 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 early 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 (Cowles et al., 1997*b*; 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 (Cowles et al., 1997*a*,*b*; 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 (Cowles et al., 1997*a*; 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; Szebenyi 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; Shpetner 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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