Syntaxin 7 Mediates Endocytic Trafficking to Late Endosomes*

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The lysosome functions are ensured by accurate membrane trafficking in the cell. We found that mouse syntaxin 7 could complement yeast vam3 and pep12 mutants defective in docking/fusion to vacuolar and prevacuolar membranes, respectively. Immunohistochemical studies showed that syntaxin 7 is localized to late endosomes, but not to early endosomes. Induced expression of mutant syntaxin 7 blocked endocytic transport from early to late endosomes but did not block the transport of cathepsin D and lamp-2 from the trans-Golgi network to lysosomes. Thus, syntaxin 7 mediates the endocytic trafficking from early endosomes to late endosomes and lysosomes. These results also suggest that the biosynthetic pathway utilizes a different machinery from that of the endocytic pathway in the docking/fusion to late endosomes.

Lysozymes are inside acidic organelles enriched with hydrolases (for reviews, see Refs. 1 and 2). They degrade macromolecules internalized through endocytosis and cytoplasmic components that are no longer useful. Specific delivery of the constituent molecules is essential for the functional integrity of lysozymes. Lysosomal proteins from the endoplasmic reticulum are sorted in the trans-Golgi network, introduced into AP1-1- and/or AP-3-complex-coated vesicles, and then transported to endosomes (3–8). Soluble lysosomal hydrolases bind to mannose 6-phosphate receptors in the trans-Golgi network, and the hydrolase mannose 6-phosphate receptor complexes are transported to the endosomes by clathrin/AP-1-coated vesicles (5, 9). Cell surface proteins destined for lysozymes are internalized in clathrin/AP-2-coated vesicles and then transported to early endosomes (7, 10). The internalized molecules are selectively incorporated within COP-I-coated transport intermediates in the early endosomes and then delivered to late endosomes (11–13). Despite the progress in our understanding of trafficking events in the early stages of the endocytic and biosynthetic pathways, little is known regarding how these pathways are organized and regulated en route to lysosomes.

The yeast vacuole is a lysosome-like compartment (14, 15). Biogenesis and assembly of this organelle have been studied extensively by means of biochemical and genetic approaches. Vam3p (16) and Pep12p (17) are syntaxin-related proteins (18, 19) acting on vacuoles and prevacuolar/late endosomal compartments, respectively. Detailed analyses of yeast mutants have suggested that Vam3p is involved in multiple membrane fusion events in vacuoles: biosynthetic transport via prevacuolar compartments, AP-3-dependent transport, endocytic transport, autophagy, and homotypic fusion (20–23). Pep12p is required for the transport from the trans-Golgi network to the prevacuolar compartments (17, 24, 25). vam3 and pep12 mutants have been useful for identifying Arabidopsis thaliana vacuolar/prevacuolar syntaxins: two plant proteins (AtVam3p and aPep12p) were identified from their functional complementation in yeast vam3 and pep12 mutants, respectively (26, 27). In a similar fashion, we found that the mouse syntaxin 7 cDNA cloned in this study could complement vam3 and pep12 mutants. These exciting results prompted us to study the role(s) of syntaxin 7 in the biogenesis of lysosomes and/or late endosomes in mammalian cells. Immunofluorescence and electron microscopy revealed that syntaxin 7 is localized to late endosomes. Moreover, we demonstrated that syntaxin 7 is involved in the endocytic trafficking to late endosomes. These results indicate that the functional site of syntaxin 7 molecules is late endosomes.

EXPERIMENTAL PROCEDURES

Antibodies—The monoclonal antibodies for lamp-2 (rat clone GL2A7) and EEA1 (mouse clone 14) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa) and Transduction Laboratories (Lexington, KY), respectively. Goat polyclonal antibodies for cathepsin D were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cy3- and FITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Anti-rab7 antibodies were prepared by immunizing a guinea pig with a synthetic peptide based on the carboxyl-terminal sequence (Asn-172 to Cys-208) of mouse rab7. Anti-syntaxin 7 antibodies were prepared by immunizing a rabbit with the bacterially expressed cytoplasmic domain (Met-1 to Cys-239) of mouse syntaxin 7, followed by affinity purification.

cDNA and Plasmids—The GenBankTM database of expressed sequence tags was searched using the human and rat syntaxin 7 sequences (28, 29). The mouse expressed sequence tag clone, accession number AA014394, contained the entire open reading frame of syntaxin 7. The sequence data has been deposited in the DDBJ/GenBank™/EBI Data Bank (accession number AB019213). The entire coding region was subcloned into yeast expression plasmid pKT10 (30) to generate pKT-syn7. The inducible expression plasmid pInd-syn7ATMD was constructed by the introduction of a polymerase chain reaction fragment coding for the cytoplasmic domain (Met-1 to Cys-239) of mouse syntaxin 7 into the multiple cloning site of pIND (Invitrogen).

Cell Culture and Yeast Experiments—NIH3T3 and NRK cells were grown in Dulbecco's modified Eagle's medium and F-12 medium, respectively, containing 10% fetal calf serum. EC3T3 cells (pPyRKR-transfected NIH3T3 cells; Invitrogen) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, nonessential amino acids, and 0.4 mg/ml Zeocin (Invitrogen). Individual clones of EC3T3 transfected with pIND (control plasmid) and pIND-syn7ATMD (mutant syntaxin 7 expression plasmid) were isolated and then maintained in the presence of 0.4 mg/ml Zeocin and 0.5 mg/ml Geneticin. Cell
culture reagents were obtained from Life Technologies, Inc. Yeast cell culture, transformation, and Western blotting were performed as described previously (31). Yeast cells were observed under an Olympus BX50 microscope equipped with differential interference contrast optics. The yeast strains used were YPH499 (Ref. 30), MHS3–4D (YPH499, \( \Delta \)vam3::HIS3), and MHS3–3A (YPH499, \( \Delta \)pep12::LEU2).

Fluorescence Microscopy—Cells were fixed with 2% paraformaldehyde and then permeabilized in phosphate-buffered saline containing 1% bovine serum albumin, 0.4% saponin, and 2% normal goat serum. The cells were incubated with the antibodies against syntaxin 7, lamp-2, rab7, or EEA1 and then reacted with fluorescence-labeled secondary antibodies. After washing extensively with phosphate-buffered saline, samples were mounted in VectaShield (Vector Laboratories, Burlingame, CA) and then visualized using a laser scanning confocal imaging system (LSM510; Carl Zeiss). To analyze the endocytic trafficking of FITC-dextran, cells were cultured in the presence of 10 \( \mu \)M muristerone A for 60 h before the addition of FITC-dextran. The cells were labeled with 1 mg/ml FITC-dextran (70 kDa; Molecular Probes) for 1 h, chased for 0 or 6 h, and then fixed and immunostained with anti-lamp-2 antibody. For preloading of FITC-dextran, cells were cultured with FITC-dextran overnight and then cultured in the absence of FITC-dextran overnight before the addition of muristerone A.

Electron Microscopy—Cells were fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate buffer, pH 7.4, and postfixed in 1% OsO4 and 0.1 M cacodylate buffer, pH 7.4. Ultra-thin sections were double-stained with uranyl acetate and lead citrate and observed under a Hitachi H7000 electron microscope. For immunoelectron microscopy, the pre-embedding silver enhancement immunogold method (32) was used, with a slight modification. Cells were fixed in 4% paraformaldehyde, cryo-protected with 35% sucrose and 14% glycerol, and frozen in liquid nitrogen. The samples were thawed and reacted with anti-syntaxin 7 antibodies, followed by incubation with colloidal gold-conjugated sec-

![Fig. 1. Syntaxin 7 complements yeast vam3 and pep12 mutants.](image1)

![Fig. 2. Syntaxin 7 is co-localized with rab7 and lamp-2.](image2)
ondary antibodies. The gold labeling was intensified using a silver enhancement kit (Nano Probes). For double staining, cells were reacted with anti-syntaxin 7 and anti-lamp-2 antibodies, then reacted with anti-rabbit IgG labeled with colloidal gold and biotinylated anti-rat IgG, and finally reacted with horseradish peroxidase-conjugated streptavidin. Horseradish peroxidase labeling was visualized by means of the 3,3′-diaminobenzidine tetrahydrochloride reaction.

**Cell Fractionation—** Stable transfectants were cultured in the presence of 10 μM muristerone A for 60 h, washed with phosphate-buffered saline, and then lysed in 10 mM HEPES-KOH containing 1 mM phenylmethylsulfonyl fluoride and the Complete™ proteinase inhibitor mixture (Roche Molecular Biochemicals). Homogenization was performed by 10 passages through a 26-gauge needle. Postnuclear supernatants were centrifuged at 100,000 × g for 30 min. The resulting supernatants and pellets were subjected to polyacrylamide gel electrophoresis and immunoblotting and probed with anti-syntaxin 7 antibodies.

**Metabolic Labeling—** Cells were cultured in the presence of 10 μM muristerone A for 60 h and then transferred to Dulbecco’s modified Eagle’s medium containing 10 μM muristerone A but lacking methionine and cysteine, followed by a 45-min incubation. The cells were labeled with 3.7 MBq/ml Tran35S-label (ICN) for 1 h and then chased for 0, 1.5, or 3 h in fresh medium containing 10% fetal calf serum and 0.25 mg/ml unlabeled methionine and cysteine. The cells were lysed in 50 mM Tris-Cl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, and the Complete™ proteinase inhibitor mixture. Cathepsin D was immunoprecipitated from the lysates using an excess amount of antibodies, subjected to polyacrylamide gel electrophoresis, and then visualized with a phosphorimaging analyzer (BAS1000; FujiFilm, Tokyo, Japan).

**RESULTS**

**Complementation of Yeast vam3 and pep12 Mutants by Syntaxin 7—** Yeast vam3 mutants (Δvam3) lack normal vacuoles but accumulate small vacuole-related structures, as shown previously (16). The introduction of syntaxin 7 restored the vacuolar morphology of the Δvam3 mutant (Fig. 1, a–c), indicating that syntaxin 7 has a similar function to Vam3p. Whether or not syntaxin 7 also complements the pep12 mutation (Δpep12) became a matter of interest because Pep12p is homologous to...
Because the Δpep12 mutation did not affect the vacuolar morphology (17, 33), we examined the maturation of vacuolar luminal proteins. Abolition of PEP12 function results in retardation of the maturation of vacuolar proteins including carboxypeptidase Y (CPY) and alkaline phosphatase (ALP) because of a defect in transport to vacuoles (17, 33). Thus, Δpep12 cells accumulate the precursor proteins of CPY and ALP and contain only small amounts of the mature proteins. When syntaxin 7 was expressed in the Δpep12 mutant, CPY and ALP became mature forms similar to the wild type (Fig. 1d, + Syntaxin 7), indicating that syntaxin 7 has a function similar to Pep12p as well as Vam3p.

Syntaxin 7 Is Localized to Late Endosomes—The results with yeast mutants clearly suggest that syntaxin 7 is involved in the biogenesis of lysosomes and/or late endosomes. To confirm this notion, we examined the intracellular localization of syntaxin 7 by means of immunofluorescence microscopy. Numerous small organelles were identified in the perinuclear region on staining with anti-syntaxin 7 antibodies (Fig. 2, a, d, and g). The immunostaining pattern of syntaxin 7 overlapped those of rab7 (34) and lamp-2 (35), which are marker proteins for late endosomes, and of both late endosomes and lysosomes, respectively (Fig. 2, top and middle panels). Essentially all compartments containing syntaxin 7 were also positive for rab7. In contrast, the distribution of early endosomal protein EEA1 (36, 37) was clearly different from that of syntaxin 7 (Fig. 2, g-i).

The exact cellular location of syntaxin 7 was determined by electron microscopy. NIH3T3 and H-4-II-E (rat hepatoma cell line) cells were processed for immunogold labeling with anti-syntaxin 7 antibodies. Syntaxin 7 was mainly localized to the limiting membrane of multivesicular compartments in the perinuclear region (about 75% of gold particles in NIH3T3) in both NIH3T3 (Fig. 3, a and b) and H-4-II-E (Fig. 3c) cells. A small portion of syntaxin 7 (about 20% in NIH3T3 cells) was also present on lucent endosomal compartments. Double labeling revealed a high degree of co-localization of syntaxin 7 and lamp-2 (Fig. 3d). These immunohistochemical studies indicated that syntaxin 7 is localized to the late endosomes but not to the early endosomes.

Properties of Cells Expressing the Cytoplasmic Domain of Syntaxin 7—The cytoplasmic domains of syntaxin-related proteins are known to interact with molecules involved in membrane docking/fusion, SNARE components and regulatory proteins (24, 38). Therefore, expression of mutant syntaxin 7 lacking the transmembrane domain (ΔTMD) could cause dominant interfering effects on organelle biogenesis. We established a stable NIH3T3 cell line expressing mutant syntaxin 7 under the control of the ecdysone-responsive promoter. Upon induction with muristerone A, the mutant syntaxin 7 was highly expressed, and the approximate ratio of mutant (ΔTMD) to endogenous (wild-type) syntaxin 7 reached 3:1 at the fully induced stage (Fig. 4a, ΔTMD, 60 h). About 40% of the mutant syntaxin 7 (ΔTMD) was found in the membrane fraction (Fig. 4b), possibly having bound with syntaxin 7-interacting membrane proteins. Thus, analysis of cells expressing the mutant protein (ΔTMD) may reveal the roles of syntaxin 7 in membrane trafficking.

Expression of Mutant Syntaxin 7 Alters the Morphology and Distribution of Endosomes—We examined the subcellular localization of lamp-2 and the morphology of the lamp-2-containing compartments in the established cell lines. The perinuclear location of the lamp-2 was evident in normal (Fig. 2e) and...
Expression of the Mutant Syntaxin 7 Blocks Endocytic Transport to Late Endosomes—We analyzed endocytic transport in the ΔTMD mutant cells using a fluid-phase endocytic tracer, FITC-dextran. After internalization for 1 h, FITC-dextran was found in punctate structures dispersed at the cell periphery (Fig. 6A, a and b), which is a typical distribution of early endosomes (Fig. 2h). In the control cells, the internalized FITC-dextran was co-localized with lamp-2 in the perinuclear region, indicating the arrival of FITC-dextran in late endosomes and lysosomes (Fig. 6B, d–f). FITC-dextran was not localized to lamp-2-containing compartments in the ΔTMD mutant cells and distributed throughout the cells even after a 6-h chase period (Fig. 6B, a–c), suggesting that FITC-dextran was not transported to the late endosomes and lysosomes. Interestingly, the close proximity of FITC-dextran-containing compartments and lamp-2-positive compartments was frequently observed.

Next, FITC-dextran was preloaded on late endosomes and lysosomes, and mutant syntaxin 7 was expressed for 60 h. lamp-2 was co-localized with FITC-dextran in the ΔTMD mutant and control cells (Fig. 6C). These results suggest that mutant syntaxin 7 did not affect the localization of lamp-2 to late endosomes and lysosomes.

The processing of cathepsin D was examined to assess the effect of mutant syntaxin 7 on the biosynthetic transport of lysosomal enzymes (Fig. 7). Cathepsin D is synthesized as inactive procathepsin D (51 kDa) in the endoplasmic reticulum, converted into the intermediate form (45 kDa) mainly in late endosomes, and finally converted into the lysosomal mature forms (30 kDa and 15 kDa) in lysosomes (39–42). Thus, the processing of cathepsin D can be a useful marker for examining the intracellular trafficking of lysosomal proteins. Procathepsin D was processed to the mature form with normal kinetics in the ΔTMD mutant cells (Fig. 7, compare ΔTMD and control cells). Thus, the cytoplasmic domain of syntaxin 7 did not block the transport of cathepsin D to late endosomes and lysosomes.

**DISCUSSION**

The present study clearly shows that syntaxin 7 is localized on late endosomes and mediates endocytic trafficking to this organelle. As observed using immunofluorescence microscopy,
FIG. 7. Processing of cathepsin D normally occurs in cells expressing mutant syntaxin 7. Stable transfectants harboring the control plasmid (Control) or expression plasmid (ΔTMD) were cultured in the presence of muristerone A for 60 h, and then the proteins were metabolically labeled with Tran[35S]-label for 1 h, followed by a 0-, 1.5-, or 3-h chase in the presence of unlabeled methionine and cysteine. Cathepsin D was immunoprecipitated from the cell lysates, resolved by polyacrylamide gel electrophoresis, and then visualized with a phosphorimaging analyzer. The positions of procathepsin D (p), the single chain intermediate form (i), and the heavy chain of the two-chain mature form (m) of cathepsin D are indicated.

FIG. 8. Model for the role of syntaxin 7 in membrane trafficking to lysosomes. Syntaxin 7 is localized on the late endosomal membrane and mediates endocytic trafficking from the early to the late endosomes. The transport of lamp-2 and cathepsin D does not occur via the early endosomes. This transport to the late endosomes is independent of syntaxin 7; therefore, the transport may utilize a different molecular machinery from that for the endocytic transport to the late endosomes. Alternatively, the transport of lamp-2 and cathepsin D may bypass the syntaxin 7-mediated system in cells expressing mutant syntaxin 7. There is a slight possibility, which we could not rule out, that mutant syntaxin 7 may interfere with some of the active machinery required for the transport of lamp-2 and cathepsin D, although syntaxin 7 is essential for their transport to late endosomes and lysosomes.

Syntaxin 7 is co-localized with rab7, a marker protein of late endosomes, but not with an early endosomal protein, EEA1. Furthermore, it was confirmed by immunoelectron microscopy that syntaxin 7 is present on multivesicular compartments and is co-localized with lamp-2. These results functionally distinguish syntaxin 7 from other syntaxin-related proteins found in early and recycling endosomes (43).

We have established a stable NIH3T3 cell line that inducibly expresses the cytoplasmic domain of syntaxin 7 (ΔTMD). A significant portion of mutant syntaxin 7 was found in the membrane fraction, suggesting that the mutant protein binds to syntaxin 7-interacting proteins and thereby interferes with the normal interaction of endogenous (wild-type) syntaxin 7. This mutant syntaxin 7 did not affect the morphology and transport in early stages of the secretory pathway (cathepsin D maturation) and endocytic internalization of FITC-dextran; consistently, syntaxin 7 was not localized in these organelles.

Internalized FITC-dextran was not transported to the lamp-2-containing late endosomes and lysosomes in cells expressing mutant syntaxin 7 (ΔTMD). This result clearly suggests that syntaxin 7 is required for trafficking from early to late endosomes (Fig. 8). On the other hand, lamp-2 was localized in the mutant late endosomes and lysosomes preloaded with FITC-dextran, even after incubation for 60 h in the presence of muristerone A. The half-life of lamp-2 was previously shown to be 60 h in mouse fibroblasts (44). The co-localization of lamp-2 with FITC-dextran suggests that overproduction of mutant syntaxin 7 did not affect the transport of newly synthesized lamp-2 to late endosomes and lysosomes. Pulse-labeled procathepsin D was normally processed to the lysosomal mature form in the ΔTMD mutant cells. These results imply that the transport of lamp-2 and cathepsin D to lysosomes does not occur through the early endosomes and that syntaxin 7 is not involved in the biosynthetic (lamp-2 and cathepsin D) transport to the late endosomes. The biosynthetic transport may utilize a molecular machinery different from that used for endocytic transport.

Finally, it is noteworthy that the present study was extended from the finding that syntaxin 7 could complement a yeast vam3 mutant. This mutant lacks normal vacuoles and, instead, accumulates small vacuolar structures (16). Other mutants with an altered vacuolar morphology (20) may allow further identification of the mammalian proteins essential for the assembly of lysosomes and endosomes.

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