Involvement of a Novel Q-SNARE, D12, in Quality Control of the Endomembrane System

Received for publication, September 2, 2005; in revised form, December 7, 2005; published, JBC Papers in Press, December 14, 2005; DOI 10.1074/jbc.M509715200

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The cellular endomembrane system requires the proper kinetic balance of synthesis and degradation of its individual components, which is maintained in part by a specific membrane fusion apparatus. In this study, we describe the molecular properties of D12, which was identified from a mouse expression library. This C-terminal anchored membrane protein has sequence similarity to both a yeast soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE), Use1p/Slt1p, and a recently identified human syntaxin 18-binding protein, p31. D12 formed a tight complex with syntaxin 18 as well as Sec22b and bound to α-SNAP, indicating that D12 is a SNARE protein. Although the majority of D12 is located in the endoplasmic reticulum and endoplasmic reticulum–Golgi intermediate compartments at steady state, overexpression or knockdown of D12 had no obvious effects on membrane trafficking in the early secretory pathway. However, suppression of D12 expression caused rapid appearance of lipofuscin granules, accompanied by apoptotic cell death without the apparent activation of the unfolded protein response. The typical cause of lipofuscin formation is the impaired degradation of mitochondria by lysosomal degradative enzymes, and, consistent with this, we found that proper post-Golgi maturation of cathepsin D was impaired in D12-deficient cells. This unexpected observation was supported by evidence that D12 associates with VAMP7, a SNARE in the endosomal-lysosomal pathway. Hence, we suggest that D12 participates in the degradative function of lysosomes.

Specific fusion of biological membranes is required for many cellular processes, including membrane trafficking between different organelles and within individual organelles, and is executed in eukaryotic cells by fusogenic soluble N-ethylmaleimide-sensitive factor (NSF)3 attachment protein (SNAP) receptors (SNAREs) (1). SNAREs are a group of membrane proteins that localize to and function in the diverse endomembrane system, where docking and fusion between membranes take place. All SNAREs are characterized by homologous stretches of 60–70 amino acids referred to as SNARE motifs, which are adjacent to the membrane anchor domains. SNAREs can be classified into subgroups: the syntaxin and SNAP-25 families contain a conserved glutamine at a central position called the “0” layer of the SNARE motif and are therefore called Q-SNAREs, and the VAMP (also called synaptobrevin) family contains a conserved arginine at this position and are therefore called R-SNAREs (2). SNARE motifs contribute to the formation of an extended parallel four-helix bundle, termed the SNARE core complex (3). Assembly of the SNARE core complex first leads to a tight connection between two membranes and then drives lipid mixing and subsequent opening of a fusion pore (4, 5). Although SNARE complexes are the critical machinery of membrane fusion, their role in determining the specific sites of fusion within the endomembrane system remains to be established.

The early secretory pathway, which comprises the ER, the Golgi apparatus, and the intermediate compartments, is highly dynamic and tightly regulated. Cargo proteins are efficiently exported from the ER, and the constituents of the ER are retrieved by retrograde transport from the Golgi. In yeast, a variety of SNAREs or SNARE-related proteins have been implicated in the processes of retrograde (Ufe1p, Sec22p, Sec20p, and Tip20p) and anterograde (Sed5p, Sec22p, Bos1p, and Bet1p) transport (6). Recently, Use1p/Slt1p was identified as a novel SNARE located in the ER. This is an essential protein and is thought to function in retrograde transport from the Golgi to the ER, because Ufe1p/Slt1p is associated with the retrograde SNAREs Ufe1p, Sec22p, and Sec20p but not with the anterograde SNAREs Bos1p and Bet1p (7, 8). However, it was also shown that Use1p/Slt1p interacts with the farnesylated SNARE Ykt6p (8). Because Ykt6p has been implicated in multiple intracellular transport pathways, particularly in homotypic fusion with vacuoles, an alternative possibility involving the later secretory pathway cannot be excluded. Recently, the mammalian homologues of yeast genes have been identified, including syntaxin 18, Sec22b, syntaxin 5, membrin, and rBet1, which in yeast correspond to Ufe1p, Sec22p, Sed5p, Bos1p, and Bet1p, respectively (9–15). More recently, BNI1 and p31, which have similarity to Sec20p and Use1p/Slt1p in yeast, were identified as syntaxin 18-associated proteins. BNI1 forms a SNARE complex with syntaxin 18, Sec22b, and p31 and participates in the formation of the ER network structure (16). Interestingly, BNI1 appears
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to be involved in an apoptosis pathway through the regulation of α-SNAP function (16). However, the role of p31 in membrane trafficking and apoptosis is unclear.

In this paper, we report the identification and characterization of a novel SNARE, D12, from murine hematopoietic stem cells using a modified FL-REX (fluorescence localization-based retrovirus-mediated expression cloning) method (17). D12 shows significant homology to Usel1p/Slit1p and p31. We show here that D12 is a Q-SNARE and, whereas it is mostly localized in the ER and ERGIC, it also binds to VAMP7, a SNARE involved in endosome-lysosome transport. Our analyses indicate that D12 plays no role in membrane trafficking in the early secretory pathway but rather is involved in the maintenance of proper lysosomal function.

MATERIALS AND METHODS

Cell Culture and Transfection—NIH3T3 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. PLAT-E cells, a packaging cell line (18), were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum containing blasticidin S (10 μg/ml) and puromycin (1 μg/ml). Cells were transfected by the calcium phosphate method with Cell Phect (Amersham Biosciences) or by the lipofection method using FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen). For retrovirus-mediated gene expression, NIH3T3 cells were infected with recombinant retroviruses produced by PLAT-E packaging cells as previously described (19).

Modified FL-REX to Search for Proteins in the Secretory Pathway—The murine hematopoietic stem cell cDNA was described previously (20). The 3’ region of the cDNA was deleted by ExoIII digestion and inserted into a pMX vector containing the enhanced green fluorescent protein (EGFP) expression sequence followed by a nuclear localization signal (NLS) and an ER retention signal (KDEL) (pMX-cDNA-EGFP-NLS-KDEL). In detail, three tandem repeats of the NLS from the SV virus large T-antigen were excised from pECPF-Nuc (BD Biosciences), and the EGFP coding sequence was excised from pEGFP-C1 (Clontech). Twenty-four pooled plasmid DNA clones were transiently expressed in HEK293 cells in a 96-well dish using the standard calcium phosphate method and were examined with immunofluorescence microscopy. The cDNA pool that gave ER- or cytosolic localization of EGFP was divided into individual clones, and HEK293 cells were similarly screened for independent clones. Positive clones were isolated, and the inserted cDNAs were examined by genomic sequencing.

Expression of Proteins and Imaging Analyses—The full-length cDNA of mouse D12 was obtained by PCR using the MC9 cDNA library as a template. Full-length or C-terminal-deleted mutant D12 (D12Δtmt) were subcloned into the multicloning site of the pMX (21) and pCMV10 (Sigma) vectors. Expression vectors for mRFP1-tagged full-length D12 or the first 80 amino acids of D12 (D12(1–80)) were also constructed by inserting the open reading frame into appropriate sites of pmRFP1-N1, in which the EGFP open reading frame of pEGFP-N1 (BD Biosciences) was replaced with the mRFP1 open reading frame (22). These plasmids were introduced into cells using either FuGENE6 (Roche Applied Science) or bead loading (fluorescence correlation spectroscopy analysis) (23). For immunostaining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin for 1 min on ice or fixed and permeabilized with methanol at −20 °C essentially as described (24). After staining, the specimens were observed with confocal microscopy using an LSM PASCAL or LSM510metaConfocor2 (Carl Zeiss). For counting the number of cells with a condensed nucleus, the specimens were observed with confocal microscopy using an LSM PASCAL or LSM510metaConfocor2 (Carl Zeiss) and the images were analyzed using MetaMorph (Universal Imaging). The diffusion time of D12(1–80)-mRFP1 in living cells was estimated by using fluorescence correlation spectroscopy as described previously (24).

Antibodies—The antibodies used in this study were α-FLAG M2 (Sigma), α-GM130 (BD Bioscience), α-phospho-Ser51-eIF2α (Cell Signaling Technology), α-eIF2α (Cell Signaling Technology), α-PERK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), α-ATF6 (IMAGEGENEX), α-XBP-1 (Santa Cruz), α-HSP70 (Stressgen), α-cleaved caspase-3 (Cell Signaling), α-active BAX (Upstate Biotechnology, Inc., Lake Placid, NY), and α-GAPDH (Ambion). Antibodies against D12, Sec22b, syntaxin 5, syntaxin 18, calcinein, ERGIC53, and GRP78 were obtained by immunizing rabbits with the bacterially expressed GST fusion of each protein with the transmembrane domain deleted.

Identification of D12-binding Proteins—For the proteomic screening of D12 binding proteins, 2.4 × 10^6 293T cells were transfected with the plasmid containing the transmembrane domain-deleted D12 (pCMV10-D12Δtmt) using calcium phosphate precipitation. Forty-eight hours after transfection, the cells were harvested and lysed in lysis buffer (40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA, and protease inhibitor mixture (Nacalai, Japan)). The lysates were incubated on ice for 1 h and centrifuged, and the supernatant was collected and incubated with anti-FLAG M2 Affinity Gel (Sigma) at 4 °C for 12 h. The resins were washed five times with washing buffer (20 mM Tris-HCl (pH 7.4) and 150 mM NaCl) and eluted with 3× FLAG peptide (Sigma). The protein sample was concentrated and lysed with SDS-PAGE loading buffer containing 280 mM 2-mercaptoethanol. The sample was subjected to SDS-PAGE in a 10% gel, and the proteins were visualized by silver staining. The major bands were excised and subjected to in-gel digestion with trypsin, and the eluted peptides were loaded on liquid chromatography electrospray ionization tandem mass spectrometry (FINNIGAN LC Q DECA; Finnigan). The spectrophotometry data were searched for known proteins using Sequest software (25).

Subcellular Fractionation of NIH3T3 Cells—NIH3T3 cells cultured in two 100-mm plates were washed twice with ice-cold phosphate-buffered saline. All subsequent steps were performed at 4 °C. Cells were harvested with a cell scraper in 0.6 ml of homogenization buffer (10 mM Tris-HCl (pH 7.5), 250 mM sucrose) and homogenized by passage 10 times through a 27-gauge needle on a 1-ml syringe. Unbroken cells and nuclei were removed by centrifugation at 1,200 × g for 5 min. The postnuclear supernatant was loaded on preformed Nycodenz (Sigma) gradients, which were prepared for the HITACHI S52ST rotor from initial discontinuous gradients (24, 19, 15, and 10% Nycodenz in 10 mM Tris-HCl (pH 7.5), 3 mM KCl, and 1 mM EDTA) that were allowed to diffuse in a horizontal position for 45 min at room temperature and then centrifuged for 4 h at 37,000 rpm in a Hitach CS100GXL (Hitachi) ultracentrifuge to generate a nonlinear density gradient profile. The postnuclear supernatant was loaded on top of the gradient and centrifuged for 4 h at 37,000 rpm. Fourteen fractions were collected from the top, and the proteins in aliquots of the fractions were resolved by SDS-PAGE. The distribution of D12, syntaxin 18, Sec22b, GM130 (Golgi marker), ERGIC53 (ERGIC marker), and calcinein (ER marker) in the gradients was determined by immunoblotting using ECL (Amersham Biosciences).

For fractionation using Percoll, NIH3T3 cells grown in a 100-mm dish were harvested and homogenized in homogenization buffer (5 mM HEPES/KOH, 0.25 M sucrose, pH 7.2) by passage 10 times through a
27-gauge needle on a 1-ml syringe. After centrifugation (1,200 × g, 10 min) to remove cell debris and nuclei, the postnuclear supernatant was combined with 90% Percoll solution containing 250 mM sucrose and 5 mM HEPES/KOH, pH 7.2, so that the final concentration of Percoll was 17%. This mixture was layered over a 2.5 mM sucrose cushion and centrifuged at 29,000 × g for 75 min in a SS25ST rotor (Hitachi) at 4 °C. Gradients were fractionated from the top into 16 fractions, and the proteins in aliquots of the fractions were resolved by SDS-PAGE. The distribution of cathepsin D, D12, and Sec22b in the gradients were analyzed by immunoblotting using ECL.

**RESULTS**

**Identification of D12 Using an FL-REX Method Modified for Signal Sequence Trapping**—The FL-REX method (fluorescence localization-based retrovirus-mediated expression cloning) (17) was developed to clone cDNAs based on the cellular localization of cDNAs from an EGFP-fused cDNA library. To search for novel secreted or membrane-bound proteins, we modified the FL-REX method to include a signal sequence trap. To facilitate identification of proteins actively retained in the cytoplasmic compartment, we added two targeting signals, a classical nuclear localization signal (NLS) and a C-terminal ER retention signal (KDEL). When this EGFP-NLS-KDEL vector alone was expressed in cultured cells, it was confined to the nucleus. Hence, simply by examining the intracellular localization of expressed proteins with epifluorescence microscopy, this method should permit the identification of genes containing either a signal sequence or a membrane-anchoring domain. In addition, this should allow the identification of proteins with an affinity for extranuclear structures. We screened a total of 9000 clones of a murine hematopoietic stem cell cDNA library and observed several clones expressing the EGFP signal in the ER. These included SEP15 (a 15-kDa selenoprotein) (26) and KDAP (kidney-derived aspartic protease-like protein, Napsin) (27), both of which contain signal sequences. We also obtained clones encoding the cytoplasmic proteins Gng10 (guanine nucleotide binding protein γ 10), whose signal was mostly in the cytosol, and SAP18 (Sin3-associated polypeptide), whose signal was found in the ER (data not shown). Interestingly, we found a clone whose signal was partially expressed in the cytoplasm (Fig. 1A). Because this clone encoded the first 80 amino acids of a protein of unknown function, D12, we focused on its characterization.

**Characterization of D12—**D12 was originally identified from a cDNA library of mouse cultured bone marrow mast cells (GenBank™ accession number AF353245). According to the report, this is a 270-amino acid protein with two domains predicted to form coiled-coils, including two putative coiled-coil heptads in regions 13–39 and 206–243 identified with a 28-residue window width (28), and a C-terminal transmembrane domain (region 241–263) (Fig. 1B). When we sequenced the full-length D12 isolated from the MC9 cDNA library, we noticed the presence of a Ser after Ala153 due to the insertion of CAG after Cys469 in AF353245. The same insertion was observed in some cDNAs in the EST database (BU841695 or BQ287737), whereas others (CB204034 or BY708461) had no such insertion. For alignment (Fig. 1C, see below), we used the sequence we obtained experimentally from the clone in the MC9 library, and we refer to this sequence, which contains the Ser insertion after Ala153, as D12. Using the SMART data base (available on the World Wide Web at smart. embl-heidelberg.de/), we predicted two regions with weak similarity to the syntaxin N-terminal domain (residues 64–189) and the t-SNARE motif (residues 166–237) (Fig. 1B). Mouse D12 shows a low level of homology to yeast Use1p/Slt1p (20.1% identity) and a high level of homology to the human uncharacterized protein MDS032 (GenBank™ accession number AF220052), except in its N-terminal extension of 11 amino acids and an internal region (residues 139–164) (overall identity 84.9%; Fig. 1B).

D12-(1–80), which we initially identified by modified FL-REX, contains the first coiled-coil but no obvious hydrophobic signal sequence. To confirm that the observed weak cytoplasmic localization indicates the presence of a positive retention signal in extranuclear structures, we used fluorescence correlation spectroscopy to analyze whether the diffusional mobility of the fragment in the cytoplasm is restricted. We constructed D12-(1–80) tagged with a monomeric red fluorescent protein (mRFP1) at its N terminus (mRFP-D12-(1–80)) and expressed the construct in NIH3T3 cells. The intracellular localization was indistinguishable from that of the original EGFP fusion. When the diffusion time of mRFP-D12-(1–80) expressed in the cytoplasm was compared with that of mRFP using the one-component diffusion model, mRFP-D12-(1–80) showed 3–5-fold slower diffusion (data not shown), confirming that the cytoplasmic retention of the N-terminal fragment was actively maintained.

**Subcellular Localization of D12—**Use1p/Slt1p was recently identified as a SNARE that functions in retrograde protein transport from the Golgi to ER in yeast (7, 8). We therefore reasoned that mouse D12 may also function as a SNARE between the ER and Golgi. As a first step to investigate the subcellular distribution of D12 in NIH3T3 cells, a polyclonal D12 antibody was raised against the bacterially expressed protein lacking the transmembrane domain (GST-D12Δtm). The postnuclear supernatant of NIH3T3 cells was separated by Nycodenz gradient centrifugation, and each fraction was subjected to immunoblotting using antibodies against either D12 or the specific marker proteins of the ER (calnexin), ERGIC (ERGIC53/p58), and Golgi (GM130). As shown in Fig. 1C, calnexin was enriched in fractions 1–6, and GM130 in fractions 7–11. ERGIC53/p58, a protein that cycles between the ER, ERGIC, and Golgi, appeared to be enriched in fractions 7 and 8. Distribution of endogenous D12 was similar to that of calnexin. In contrast, more than one-third of syntaxin 18 was recovered in the lighter fractions. Enrichment in the lighter fractions was also observed for Sec22b, which is known to associate with the vesicular structures of ERGIC (29).

Because we were unable to obtain high quality immunofluorescence images of endogenous D12 using our antibody, we elected to analyze the localization by another method. When Percoll gradient fractionation was used to separate dense vesicles, mature lysosomes (as indicated by cathepsin D) were well separated from the ER-Golgi-related vesicles (Sec22b) (Fig. 1D). However, D12 appeared to be absent in lysosomes, suggesting that the D12 protein is mostly located in the early secretory pathway that includes the ER and ERGIC.

**D12** is a Novel SNARE Protein—To obtain information regarding the function of D12 in cells, we attempted to identify D12-binding proteins using immunoprecipitation and proteomics. A lysate of 2.4 × 10^6 293T cells overexpressing FLAG-tagged D12Δtm was used for immunopre-
precipitation with an anti-FLAG M2-agarose affinity gel. The eluate with the FLAG peptide was resolved by SDS-PAGE (Fig. 2A), and the major bands were excised from the gel to determine their amino acid sequences by in-gel tryptic digestion followed by liquid chromatography electrospray ionization tandem mass spectrometry. As shown in Fig. 2A, D12-bound proteins appeared to be classified into two groups.

**FIGURE 1.** D12 is a novel Q-SNARE located in the early secretory pathway. A, D12-(1–80)-EGFP-NLS-KDEL is partially retained in the cytoplasm (right, arrows) when compared with a control fusion protein, EGFP-NLS-KDEL (left). B, D12 shows similarity to MDS032 (human) and Use1p/Slt1p (Saccharomyces cerevisiae). Amino acids encoding D12, MDS032, and Use1p were aligned using ClustalW1.8 (available on the World Wide Web at searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Identical residues are in white on a black background, and similar residues are shaded. The **black** line indicates the syntaxin N-terminal domain, the **gray** line shows a t-SNARE motif, and the **dotted** line shows a transmembrane domain. The **numbers** denote positions in helix-helix contacts. Details of the features are described under “Results.” C, subcellular fractionation using a Nycodenz gradient. A postnuclear supernatant of wild-type NIH3T3 cells was fractionated using a Nycodenz gradient, and the distribution of endogenous D12 and each marker protein was analyzed by Western blotting as described under “Materials and Methods.” The **star** indicates a nonspecific band. D, subcellular fractionation using Percoll. To separate post-Golgi fractions, a postnuclear supernatant of NIH3T3 cells was subfractionated using 17% Percoll, and the distribution of either the pro- (45 kDa) or mature (41 kDa) cathepsin D was compared with D12. The top fractions were enriched with vesicles of the ER and Golgi, whereas mature lysosomes were recovered in fractions 14 and 15, as revealed by the distribution of mature cathepsin D. At steady state, D12 was not detected in these bottom fractions.
FIGURE 2. Binding partners of D12. A, cell lysates of 293T cells overexpressing 3×-FLAG-D12Δtm protein or nonfused FLAG were immunoprecipitated with an anti-FLAG M2 antibody. The recovered proteins were resolved by SDS-PAGE and visualized by silver staining (left). Each band was excised and subjected to in-gel digestion and then analyzed by mass spectrometry as described under “Materials and Methods.” A summary of the analysis is shown in the table. The details are described under “Results.” B, an EGFP-α-SNAP expression plasmid was cotransfected into 293T cells with the plasmids for FLAG-tagged full-length D12 or D12Δtm, syntaxin 18, or p47 (a subunit of p97/VCP). At 24 h after transfection, the cells were lysed and immunoprecipitated with an anti-FLAG M2 monoclonal antibody with the aid of protein G-agarose beads. The proteins bound to the beads were examined by Western blotting with an anti-GFP antibody. To confirm the precipitation of FLAG-tagged proteins, the blot was also probed with an anti-FLAG M2 antibody. The left side shows the amount of α-SNAP and FLAG-tagged proteins in 5% of the supernatant. C, binding of endogenous D12 to post-Golgi SNARE proteins was examined by immunoprecipitating FLAG-tagged proteins from the lysates of 293T cells expressing either syntaxin 18, p47, Ykt6, Sec22b, VAMP3, or VAMP7. The immunoprecipitates or 5% of the total lysate volumes were separated by SDS-PAGE and analyzed by Western blotting using an anti-D12 antibody (top and middle) or anti-FLAG antibody (bottom). In the anti-D12 blot, in addition to the full-length D12, three other immunoreactive bands (18.2-, 17.8-, and 17.2-kDa forms) were detected. Because of an intense signal from the IgG light chain, which migrated just below the full-length D12 band, evaluation of the full-length D12 band was hampered, particularly at low levels. Therefore, only the upper region of the blot containing the full-length D12 band and the lower region of the same blot containing the trimmed D12 fragments is shown. An entire image of a D12 blot is shown in supplemental Fig. 1. Binding to syntaxin 18 and Sec22b was observed for all four bands, and the full-length, 18.2-kDa, and 17.8-kDa forms were found in association with VAMP7. Association of D12 with p47, Ykt6, and VAMP3 was negligible in this assay.
the ER chaperones, including calnexin and GRP78/BiP, and the ER-Golgi membrane trafficking proteins, including NSF, α-SNAP, SNAP-29, syntaxin 18, and ZW10. The specific binding between D12 and calnexin, however, was not confirmed by Western blotting with an anti-calnexin antibody in small scale immunoprecipitation experiments (data not shown), indicating that the binding of calnexin and GRP78/BiP to D12 in the large scale experiment may be artifacts caused by their protein-mediated chaperone function (30). ZW10 is a spindle checkpoint protein associated with kinetochores and has also recently been reported to be a syntaxin 18-binding protein involved in membrane trafficking between the ER and Golgi (31).

This binding profile as well as the sequence homology of D12 to Use1p/Slt1p prompted us to examine the possibility that D12 is a novel SNARE. We prepared detergent extracts of 293T cells co-expressing FLAG-D12 with N-terminally EGFP-tagged α-SNAP (EGFP-α-SNAP) and immunoprecipitated the FLAG-D12 using an anti-FLAG antibody. We used FLAG-syntaxin 18 and FLAG-p47 (32, 33) as positive and negative controls, respectively. The immunoblots revealed that EGFP-α-SNAP co-precipitated with FLAG-D12 as well as with FLAG-syntaxin 18 but not with FLAG-p47 (Fig. 2B). In addition, even FLAG-D12tm lacking the transmembrane domain co-precipitated with EGFP-α-SNAP.

It has been reported that the yeast D12 homologue Use1p/Slt1p interacts with ER-located SNAREs, such as Ufe1p and Sec22p (7). We therefore tested whether D12 interacts with SNAREs located in the ER. Lysates of 293T cells expressing FLAG-SNAREs were incubated with an anti-FLAG antibody, and the immunoprecipitates were analyzed by Western blotting with an anti-D12 antibody (Fig. 2C). In this experiment, we also examined Ykt6, an R-SNARE involved in multiple intracellular fusion reactions, because yeast Ykt6p interacts with Use1p/Slt1p (8). VAMP3 and VAMP7 are members of the VAMP family of R-SNAREs and are distributed in various post-Golgi structures, such as endosomes, lysosomes, and plasma membranes (34). As shown in Fig. 2C, endogenous D12 co-precipitated with FLAG-syntaxin 18, FLAG-Sec22b, and FLAG-VAMP7 but not with FLAG-Ykt6 or FLAG-VAMP3. Because full-length D12 (28.2 kDa) partially overlapped with
the intense signal of the IgG light chain, Fig. 1C shows only the regions of the blot for full-length D12 (top) and the D12 fragments (18.2, 17.8, and 17.2 kDa, middle). Syntaxin 18 and Sec22b bound to the full-length D12 and all three D12 fragments, and VAMP7 bound to all except the 17.2-kDa form. In contrast to the association of Use1p/Slt1p and Ykt6p in yeast, no interaction between D12 and Ykt6 was detected under this condition. Although the physiological meaning of the degradation of D12 is unclear, these data indicate that D12 interacts with SNAREs involved in both the early and post-Golgi secretory apparatus.

**Overexpression of D12 Has No Obvious Effects on the Structures of the Early Secretory Pathway**—Next, we examined whether D12 may be involved in protein transport between the ER and Golgi apparatus. Overexpression of SNAREs that localize in the ER and Golgi, such as syntaxin 18 (32), Sec22b (35), and membrin (12), disrupts the normal morphology of the ERGIC and Golgi. To define the function of D12 as a component of the membrane fusion machinery, we examined the effects of overexpressing FLAG-D12 on structures of the early secretory pathway. Overexpression of FLAG-D12 had no effect on the Golgi (Fig. 3A) or ERGIC-53/p58 (data not shown), whereas overexpression of FLAG-syntaxin 18 caused disintegration of the Golgi apparatus (Fig. 3A).

We next examined whether the reduction of D12 expression would cause any change in the ERGIC or Golgi. For this purpose, we designed an siRNA (D12 siRNA1) targeted to the sequence of mouse D12 and transfected it into NIH3T3 cells. Because the siRNA transfection efficiency was typically 60–80%, we used NIH3T3 cells stably expressing full-length mouse D12 (D12/3T3 cells) to monitor the effects of the siRNA transfection. Cells were fixed and stained with an anti-active BAX antibody.
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RNAi in order to confirm that the tested phenotype occurred in cells where D12 expression was suppressed. As a result of stable D12 expression, the expression level of D12 was 2–4-fold higher in the D12/3T3 cells than in the wild type cells. As shown in Fig. 3B, D12 staining was almost abolished in cells transfected with D12 siRNA1 when compared with cells transfected with control siRNA. To examine whether ER-Golgi transport was disturbed by the reduction in D12 expression, we monitored the localization of the KDEL receptor. This integral membrane protein is located mainly in the Golgi and the ER at steady state and cycles between the two compartments in a manner dependent on both COPI and COPII. As has been shown previously, slight defects in transport result in enhancement of either its ER or Golgi staining (23).

We recorded more than 50 D12-deficient cells stained with an anti-KDEL receptor antibody and compared their steady-state distribution with that in control cells. However, we observed no significant difference in the KDEL receptor distribution (Fig. 3B). Similar results were obtained in wild type NIH3T3 cells (supplemental Fig. 2). Taken together, these results indicate that D12 is not required for at least the conventional membrane trafficking pathway between the ER and Golgi.

Suppression of D12 Expression Induces Apoptotic Cell Death—During the experiments, we noticed that treatment with D12 siRNA1 rapidly reduced cell numbers (see Fig. 3B). We had initially dismissed this phenotype, because double-stranded RNA tends to cause cell death through nonspecific toxicity. However, since the progression of cell death was so striking, we decided to investigate whether the loss of D12 expression was triggering a specific process that eventually led to cell death. For this purpose, we synthesized another siRNA (D12 siRNA2) to knock down D12 expression.

First, we compared the growth rates of cells after control and D12 siRNA transfection. Cell growth was almost completely arrested in cells transfected with D12 siRNA1 when compared with cells transfected with control siRNA. To examine whether ER-Golgi transport was disturbed by the reduction in D12 expression, we monitored the localization of the KDEL receptor. This integral membrane protein is located mainly in the Golgi and the ER at steady state and cycles between the two compartments in a manner dependent on both COPI and COPII. As has been shown previously, slight defects in transport result in enhancement of either its ER or Golgi staining (23). We recorded more than 50 D12-deficient cells stained with an anti-KDEL receptor antibody and compared their steady-state distribution with that in control cells. However, we observed no significant difference in the KDEL receptor distribution (Fig. 3B). Similar results were obtained in wild type NIH3T3 cells (supplemental Fig. 2). Taken together, these results indicate that D12 is not required for at least the conventional membrane trafficking pathway between the ER and Golgi.

Suppression of D12 Expression Induces Apoptotic Cell Death—During the experiments, we noticed that treatment with D12 siRNA1 rapidly reduced cell numbers (see Fig. 3B). We had initially dismissed this phenotype, because double-stranded RNA tends to cause cell death through nonspecific toxicity. However, since the progression of cell death was so striking, we decided to investigate whether the loss of D12 expression was triggering a specific process that eventually led to cell death. For this purpose, we synthesized another siRNA (D12 siRNA2) to knock down D12 expression.

First, we compared the growth rates of cells after control and D12 siRNA transfection. Cell growth was almost completely arrested in cells transfected with either D12 siRNA (Fig. 4A). We next determined whether the growth inhibition caused by the D12 siRNAs was the result of active cell death or inhibition of a mitotic signal. For this, we performed standard morphological and biochemical tests for the presence of apoptosis. When expression of D12 in D12/3T3 cells was reduced by D12 siRNA1, ~40% of the cells showed condensed nuclear chromatin as determined by DNA staining with Hoechst (Fig. 4B). To study whether the observed nuclear condensation was associated with apoptosis, we quantified the activation of caspase-3 by siRNA treatments in both D12/3T3 and wild-type NIH3T3 cells. In both cell types, marked augmentation of caspase-3 was observed upon reduction of D12 (Fig. 4C). Further, we confirmed that chromatin condensation was occurring in cells where caspase-3 was cleaved. When cells were co-stained with an anti-cleaved caspase-3 antibody and propidium iodide, the majority of the cells that showed aggregation of propidium iodide staining were positive for caspase-3 cleavage, whereas cells without cleaved caspase-3 staining showed little abnormality in propidium iodide staining (Fig. 4D).

We next investigated the factors upstream of caspase-3. BAX is one of the central proapoptotic factors of the Bcl-2 family and is thought to permeabilize mitochondrial membranes by changing its conformation to form ion channels in the membrane in response to apoptotic signals (36). To monitor any translocation/conformational changes of BAX upon loss of D12 expression, we used NIH3T3 cells stably expressing mRFP1-tagged D12 (D12-mRFP) to facilitate detection of the D12 knockdown. Although BAX is localized in both the cytoplasm and mitochondrial membranes before apoptosis, the epitope of the antibody used in this study becomes accessible to the antibody only after its conformational change upon apoptosis (37). As shown in Fig. 4E, transfection of D12 siRNA1 caused augmentation of the BAX signal within the cytoplasmic structures.

Why does the reduction of D12 expression lead to apoptosis? Since D12 is a SNARE, it is likely that loss of D12 disturbs normal cargo maturation in the early secretory pathway. This may lead to accumulation of misfolded proteins in the ER and eventually trigger activation of the unfolded protein response (UPR), which is characterized by up-regulation of several molecular chaperones and folding enzymes by transcriptional activation (38). Prolonged activation of the UPR is known to cause apoptosis through various pathways (39). We therefore examined whether the UPR was induced in the cells transfected with D12 siRNAs. We transfected cells with the siRNAs and after 48 h monitored the expression levels of various UPR proteins by Western blot analysis. As shown in Fig. 5, expression of UPR-related transcription factors (active ATF6 (50 kDa), PERK, and eIF2α) was not significantly up-regulated by transfection with D12 siRNAs. In a separate experiment, we found no induction of XBP-1 (data not shown). Consistent with Fig. 4D, activation of caspase-3 was also confirmed by Western blotting. Although GRP78/BiP was induced in cells transfected with D12 siRNAs, this level of GRP78/BiP up-regulation by the UPR did not activate caspase-3 (supplementary Fig. 3). Interestingly, the level of phosphorylation of eIF2α at Ser51 in D12 siRNA-transfected cells was increased. Phosphorylation of eIF2α Ser51 is an initial step in the negative regulation of translation (40). Indeed, expression of short lived proteins such as exogenously expressed Fc receptor (data not shown) or EYFP-ER (see below) was reduced in cells transfected with D12 siRNA. Consistent with a report showing that chemically synthesized double-stranded short RNA does not induce interferon leading to PKR activation (41), levels of eIF2α Ser51 phosphorylation were barely affected by control siRNA transfection. Hence, we reasoned that apoptosis induced in the D12 knockdown cells may be associated with shutdown of protein synthesis.

Reduced Expression of D12 Induces Formation of Lipofuscin and Mistargeting of Lysosomal Proteins—In the course of microscopic analysis of the cells with D12 siRNAs (D12 siRNA cells), we noticed a remarkable accumulation of amorphous autofluorescent structures in the cytoplasm. These structures were characterized by a peculiar fluo-

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rescence property: the Stokes shift was unusually large and the emission maximum of \( \text{H11011}_600 \text{ nm} \) was obtained with excitation at 488 nm (Fig. 6A).

This feature is consistent with the presence of lipofuscin granules, which are manifested as heterogeneous complexes of fluorescent lipid-protein aggregates in the cytosol (42, 43). It is well known that lipofuscin occasionally appears in cells when the culture condition has deteriorated. We therefore modified the siRNA transfection protocol to replace the medium with fresh complete medium after 8 h of transfection and as controls included another two sets of siRNA that inhibit expression of the unrelated proteins, ERp72 and VIPL (data not shown). To examine whether siRNA transfection was associated with any non-specific toxicity, we used NIH3T3 cells stably expressing EYFP-ER, a modified EYFP with a calreticulin signal sequence and a KDEL C-terminal sequence, an ER retention motif. When observed 48 h after the transfection of the four different siRNAs, lipofuscin formation was detected selectively in the D12 siRNA1-transfected cells but not in cells transfected with control, ERp72, or VIPL siRNAs. To analyze the image data, we plotted the fluorescence signal of lipofuscin on the \( x \) axis and that of EYFP-ER on the \( y \) axis. The frequencies at which lipofuscin and EYFP-ER are expressed at the same spots in the plots are expressed with a rainbow scale.

Although it is unlikely that lipofuscin granules are formed in the ER, most of the granules were recorded in the same pixel (0.2 \( \times \) 0.2 \( \mu \text{m/pixel} \)) when using a \( \times 40 \) objective lens, because the ER network is closely associated with lysosomes. The results (Fig. 6B, bottom) clearly show that the predicted inverse proportion between lipofuscin and EYFP-ER signals was observed only when D12 expression was suppressed, indicating that the marked reduction of EYFP-ER accompanies the formation of lipofuscin.

It is thought that lipofuscin is induced by a variety of reactive oxygen species, including hydrogen peroxide, singlet oxygen, and superoxide radical anion, and its accumulation leads to apoptotic cell death (43, 44). To examine how rapid lipofuscin formation by D12 knockdown correlates with the progression of apoptosis, we investigated the effects of N-acetylcysteine (NAC), a scavenger of reactive oxygen species, on lipofuscin formation and BAX activation in D12 siRNA cells. Upon knockdown of D12 expression by D12 siRNA transfection, the activation of BAX was almost completely abolished by treatment of the cells with NAC (Fig. 6C). However, production of lipofuscin appeared to be unaffected by the treatment, suggesting that the progression of apoptosis is probably the result of lipofuscin formation through reactive oxygen species (Fig. 6C). The coincidence plots show that the efficiency of lipofuscin formation was mostly unaffected by NAC treatment.

There is a prevailing view that lipofuscin is generated through incomplete lysosomal degradation of various cellular components, such as mitochondrial membranes (45, 46). We therefore examined the possibility that lysosomal proteases may not be properly targeted to mature lysosomes at the very early stages of D12 knockdown before formation of lipofuscin. For this purpose, we used BODIPY FL- pepstatin A (FL-pepstatin) in order to avoid artifacts due to immunostaining after permeabilization. Previously, we showed that active tyrosinase is delivered to structures highly enriched in the fluorescent pepstatin (24). Lysosomes were marked by LysoTracker Red DND-99, a lysosome-specific...
Because reduction of D12 in cells was detected as early as 18 h post-transfection and lipofuscin appeared after ~40 h, we examined the cells 24 h after transfection to avoid crossover between the lipofuscin and LysoTracker signals. In control siRNA-treated cells, LysoTracker-stained structures are largely co-stained with FL-pepstatin in living cells, as expected (Fig. 7A, top). Surprisingly, in the cells where D12 expression was reduced, FL-pepstatin was not concentrated in the majority of the LysoTracker-stained vesicles (Fig. 7A, bottom). Instead, FL-pepstatin appeared to accumulate in heterogeneous compartments and, in some cells, structures proximal to the Golgi region (arrow), suggesting improper post-Golgi traffic of cathepsin D. To address this, we fractionated cell homogenates using Percoll and studied whether cathepsin D was properly integrated into mature lysosomes in D12 knockdown cells. As shown in Fig. 7B, dense lysosomes, which contained LIMP II/Lgp85, one of the major lysosomal membrane proteins (47), were well separated from lighter prelysosomal vesicles. In control siRNA-treated cells, only 26.9% of the pro-form of cathepsin D was found in the lighter fractions (fractions 1–12). In contrast, upon suppression of D12 expression, conversion of the pro-form to the mature form was impaired by 48.4%, and, in addition, the pro-form was heterogeneously distributed throughout the post-Golgi fractions (fractions 1–12). Because conversion of procathepsin D to the mature form occurs in endosomes by a papain-type cysteine protease (48), these results suggest that reduction of D12 expression disturbs proper delivery of cathepsin D to mature lysosomes, which may directly lead to rapid lipofuscin formation by disturbance of the proper turnover of cellular components.

**DISCUSSION**

All SNAREs are characterized by one or two conserved stretches of α-helices, referred to as SNARE motifs, that are located adjacent to the
membrane anchor domains. The ternary SNARE complex consists of four parallel α-helix bundles, each comprised of a SNARE motif from separate subfamilies. The SNARE subfamilies are classified into R-, Qa-, Qb-, and Qc-SNARE motifs. In yeast, the SNARE complex required for retrograde transport from the Golgi to the ER is thought to consist of Sec22p (R-SNARE), Ufe1p (Qa-SNARE), Sec20p (Qb), and Use1p/Stl1p (Qc). In mammalian cells, the known homologous proteins include Sec22h (R) and syntaxin 18 (Qa). More recently, BNIP1 (Qb) was identified as a mammalian Sec20p homolog (6, 16).

In the present study, we identified a novel SNARE, D12, using an FL-REX method modified for use as a signal sequence trap. D12 is a membrane protein containing a SNARE motif and a single C-terminal transmembrane domain and is mostly enriched in the early secretory pathway at steady state. The overall primary structure of D12 is similar to that of the syntaxin (Qa-SNARE) family. However, the amino acid in the putative "0" layer of the D12 SNARE motif is an aspartate residue (D) instead of the glutamate residue (Q) that is conserved in the Q-SNARE family. Also, Qa-SNAREs contain bulky amino acids such as phenylalanine and glutamine in the "−3" layer position, but D12 has a small amino acid (Thr) in this position. Furthermore, D12 has glutamine in the "−8" position, similar to syntaxin 8, which functions in the homotypic fusion of late endosomes as a Qb- or Qc-SNARE (49), whereas most Qa-SNAREs have positively charged amino acids such as arginine. We therefore think that D12, as well as Use1p/Stl1p (7), should be classified as a Qb- or Qc-SNARE rather than a Qa-SNARE.

The length of the transmembrane domain has been shown to determine the localization of SNAREs (50–52). Plasma membrane-located SNAREs such as syntaxin 1A and syntaxin 2 have a transmembrane domain consisting of ≥23 amino acids, and SNAREs located in post-Golgi compartments, such as syntaxin 13 and VAMP 3, also have a 23-amino acid transmembrane domain. In contrast, ER-Golgi SNAREs such as syntaxin 18 and syntaxin 5 possess a relatively short, 17-amino acid transmembrane domain, which typically includes 2–5 phenylalanine residues. The length of the D12 transmembrane domain appears to be 24 residues (Fig. 1B). This is rather unusual for a SNARE whose localization was mainly observed in the early secretory compartments.

Another unexpected feature of D12 is that its overexpression or knockdown had little effect on the ER-Golgi system (Fig. 3). Previous studies have shown that overexpression of SNAREs causes dominant-negative effects such as inhibition of membrane trafficking and/or morphological changes of the related compartments, suggesting that they are functional in those compartments (12, 16, 32, 35). To monitor the effects of the over- or underexpression of D12, we examined the distribution of the KDEL receptor (Fig. 3B and supplemental Fig. 2), because this protein rapidly cycles between the ER and the Golgi, and slight disturbances result in its accumulation in either compartment (23).

Overexpression (Fig. 3A) or knockdown (data not shown) of D12 had no obvious effects on the Golgi structure as revealed by GM130 staining. Similarly, surface expression of VSVG-EGFP was not affected by such treatment (data not shown). We occasionally observed a slight decrease in the number of three-way junctions of the ER network, but the results were not consistently observed. We think that such effects were secondary to the progression of apoptosis.

The most dramatic phenotype of the D12 knockdown was the formation of lipofuscin. We believe this to be a direct and specific effect, and the marked progression of apoptosis is likely to be secondary to lipofuscin formation, since NAC treatment prevented BAX activation but not the formation of lipofuscin (Fig. 6C). Lipofuscin, also called age pigment or residual bodies, is an undigested pigment found in lysosomes and is thought to be formed by incomplete degradation of mitochondria. We initially assumed that this apoptosis was the result of activation of the UPR. However, none of the markers of the UPR showed enough induction required for the onset of apoptosis. On the other hand, we observed a significant increase of eIF2α Ser51 phosphorylation, which attenuates translation by inhibiting the exchange of GDP in eIF2α. It is unlikely that this is the result of PERK activation, because we were unable to detect PERK phosphorylation (data not shown). Considering the results of the UPR analysis, we think it likely that eIF2α phosphorylation was caused by another process that eventually leads to apoptosis, such as lipofuscin formation, rather than the UPR. Considering that mitochondria are the major source of reactive oxygen species, the inhibition of normal turnover of mitochondria in D12 knockdown cells could explain the accumulation of lipofuscin leading to apoptotic cell death.

The formation of lipofuscin can be induced by various pathological conditions, such as oxidative stress and/or impaired activity of lysosomal proteolytic enzymes (43, 46, 53, 54). Although the presence of this pigment in postmitotic cells during senescence has been known for a long time, the precise mechanism of its development remains elusive. In NIH3T3 cells, lipofuscin is observed at very low levels unless proliferation is inhibited. Importantly, we found that post-Golgi maturation of cathepsin D was partially impaired in D12 knockdown cells (Fig. 7). Based on our results, we think that D12 is involved in the regulation of post-Golgi transport of lysosomal degradative enzymes to mature lysosomes, thereby facilitating proper turnover of mitochondria. If D12 is used beyond the Golgi, this hypothesis may explain the unusual length of the D12 transmembrane domain for an ER/ERGIC SNARE, and the apparent lack of a phenotype in the early secretory pathway following overexpression or reduction of D12. There is some supporting evidence in yeast that Use1p/Stl1p interacts with Ykt6p (8), which participates in the membrane fusion of vacuoles. Similarly, we observed an interaction between D12 and VAMP7, which is involved in phagocytosis or trafficking between endosomes and lysosomes (55–57). Interestingly, we observed that the 17.2-kDa fragment of D12 failed to bind to VAMP7, although it did bind syntaxin 18 and Sec22b, and fragments longer than 17.8 kDa bound to VAMP7 (Fig. 2C). It is thus reasonable to assume that the binding may be mediated by distinct domains within D12. In conclusion, we propose that the ER/ERGIC is the main reservoir of D12 and that D12 is cycled between the ER and post-Golgi compartments to function in regulating membrane traffic.

Acknowledgments—We thank Drs. H. Yoshida, M. Matsumoto, and H. Hashimoto for helpful suggestions; E. Fujimoto, M. Takeuchi, and H. Hashimoto for technical assistance; and Y. Nishi for manuscript preparation.

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