Docking of a vesicle at the appropriate target membrane involves an interaction between integral membrane proteins located on the vesicle (v-SNAREs) and those located on the target membrane (t-SNAREs). GATE-16 (Golgi-associated ATPase enhancer of 16 kDa) was shown to modulate the activity of SNAREs in the Golgi apparatus and is therefore an essential component of intra-Golgi transport and post-mitotic Golgi re-assembly. GATE-16 contains a ubiquitin fold subdomain, which is terminated at the carboxyl end by an additional amino acid after a conserved glycine residue. In the present study we tested whether the COOH terminus of GATE-16 undergoes post-translational cleavage by a protease which exposes the glycine 116 residue. We describe the isolation and characterization of HsApg4A as a human protease of GATE-16. We show that GATE-16 undergoes COOH-terminal cleavage both in vivo and in vitro, only when the conserved glycine 116 is present. We then utilize an in vitro assay to show that pure HsApg4A is sufficient to cleave GATE-16. The characterization of this protease may give new insights into the mechanism of action of GATE-16 and its other family members.

Vesicular transport of proteins is mediated by coated vesicles that bud from membrane-bound compartments and fuse with a target acceptor organelle. This process is highly conserved from yeast to man. Docking of a vesicle at the appropriate target membrane involves an interaction between integral membrane proteins located on the vesicle, the v-SNAREs, and the t-SNAREs at the target membrane (1–3). After fusion of the membranes, the tightly bound v-t-SNARE complex binds two soluble factors: N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment protein (SNAP). NSF catalyzes SNARE complex disassembly via its ATPase activity, thus allowing a new round of fusion to take place (4–6).

GATE-16 (Golgi-associated ATPase enhancer of 16 kDa) is an essential component of intra-Golgi transport (7) and post-mitotic Golgi re-assembly (8). GATE-16 is recruited to unpaired GOS-28, a Golgi-specific SNARE, in an NSF- and SNAP-dependent manner (7, 8), thus preventing GOS-28 from interacting with its cognate t-SNARE syntaxin 5. As was determined by its crystal structure (9), GATE-16 contains a ubiquitin fold decorated by two additional NH2-terminal helices. GATE-16 belongs to the ubiquitin-like protein family and shares a high level of sequence identity with an expanding family of proteins that have been implicated in a variety of cellular processes. Members of this family include light chain 3, a subunit of the neuronal microtubule-associated protein complex (10) recently implicated in autophagocytosis (11, 12) and the GABA receptor-associated protein (GABARAP), which was implicated in GABA receptor trafficking and post-synaptic localization (13–15). In Saccharomyces cerevisiae there is only one known homologue of GATE-16, Aut7/Apg8, which was found to function in membrane dynamics during autophagy (16, 17). This process constitutes a major pathway for delivery of proteins and organelles to the lysosome or vacuole, where they are degraded and recycled. Autophagy is particularly important during development as well as under certain environmental stress conditions such as nitrogen starvation. Aut7p is essential for autophagosome biogenesis, playing a key role in the formation (12) and elongation of autophagosomes (18). Despite its hydrophilic nature, considerable amounts of Aut7p are covalently bound to membranes, specifically conjugated to phosphatidylethanolamine (PE) (19, 20). For this conjugation to occur, Aut7p must undergo a series of modifications. First, newly synthesized Aut7p is cleaved downstream of glycine 116 (out of 117 AA) by Apg4p/Aut2p, a cytosolic cysteine protease. Next, Aut7p becomes conjugated to PE through the exposed COOH-terminal glycine by a ubiquitination-like mechanism, involving Apg7p as an E1-like enzyme and Apg3p as an E2-like enzyme (20). Finally, conjugated Aut7p is cleaved again by Apg4p downstream of glycine 116 and thereby released from the membrane (19).

We have previously found that GATE-16 isolated from bovine brain lacks its phenylalanine residue at position 117 (7). We now describe the isolation and characterization of HsApg4A as a human protease of GATE-16. We show that GATE-16 is cleaved downstream of a conserved and essential glycine 116, both in vivo and in vitro. We then utilize a cell-free assay to show that HsApg4A is sufficient to cleave GATE-16. These findings, together with the identification of the human Apg7 (21) and Apg3 (22), indicate that GATE-16 undergoes a similar modification to that of its yeast homologue, Aut7. The isolation of HsApg4A provides a new tool for further investigation and...
Characterization of the role of GATE-16 in intracellular membrane trafficking.

EXPERIMENTAL PROCEDURES

Cloning of the Human Apg4 Homologue—Based on the DNA sequence of HsApg4A (GenBank™ accession number AB066214), we cloned an open reading frame of the HsApg4A cDNA by PCR, using an expressed sequence tag clone (number 309851 from ResGen, Invitrogen) as a template. This cDNA fragment was sequenced and found to correspond to the authentic HsApg4A sequence. The fragment was then subcloned into a pQE-30 expression vector (Qiagen), upstream to a sequence encoding for a hemagglutinin (HA) tag, to produce a protein tagged by HA at the COOH terminus. This construct, designated pDNA3GATE-16-HA, was used for the in vivo studies. Two mutant genes were constructed based on the WT construct: G116A, in which glycine 116 was replaced with alanine, and F115A, in which phenylalanine 115 was replaced with alanine. Mutagenesis was carried out by the QuickChange™ site-directed mutagenesis protocol.

Expression and Purification of Recombinant Proteins—The pQE-30 plasmid containing GATE-16-WT plasmid was transformed into the Escherichia coli M15 (pREP4) strain. Cells were grown to an OD600 of 0.3–0.6 and induced with 1 mM isopropyl-1-thio-D-galactopyranosidase for 3–4 h at 37 °C. Following centrifugation in a Sorvall SS-34 rotor at 8500 rpm for 15 min, the pellet was resuspended in a breaking buffer (10 mM Hepes, pH 7.4, 200 mM KCl, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin A) and lysed by high pressure (8000 p.s.i.). The lysates were cleared by 30-min centrifugation at 40,000 rpm in a Beckman Ti60 rotor and then loaded onto a nickel beads column (nickel-nitritotriacetic acid (Ni²⁺-NTA), Qiagen). The column was washed with washing buffer (10 mM Hepes, pH 7.4, 50 mM KCl, 10 mM β-mercaptoethanol) containing 20 mM imidazole, and elution was carried out in a gradient of 20–500 mM imidazole in the same buffer. Samples were detected on SDS-PAGE, and the protein concentration was determined by the Bradford assay (Bio-Rad). Fractions containing pure GATE-16-WT and HA at the COOH terminus. Similarly, pQE-30 vectors containing the mutants G116A and F115A were constructed.

Expression of GATE-16 in Chinese Hamster Ovary (CHO) Cells—The pDNA3GATE-16-HA plasmid was transfected into CHO cells via electroporation (Bio-Rad kit) using 40 μg of DNA per 5 × 10⁶ cells, according to the manufacturer’s protocol.

Isolation of Cytosol from CHO Cells—Cells were washed twice with phosphate-buffered saline, then twice in a homogenization buffer containing 0.25 M sucrose, 25 mM Tris-HCl, pH 7.4, and 50 mM KCl. The cells were then homogenized in a Dounce homogenizer in the presence of 1 mM DTT for 1 h. Mass spectra were acquired on API Q-STAR Pulser’ electrospray-quadrupole time-of-flight mass spectrometer (MDS-Sciex and Applied Biosystems, Toronto, Canada) equipped with nano-electrospray ion source for sample introduction.

RESULTS

GATE-16 Undergoes COOH-terminal Cleavage in Vivo—GATE-16, a ubiquitin-like protein, was implicated in intra-Golgi transport and post-mitotic Golgi re-assembly. GATE-16 cDNA encodes for a 117-amino acid-long protein that is terminated by a VAYSSENTGFGF sequence. Amino acid sequencing of five different tryptic peptides, obtained from endogenous GATE-16 isolated from bovine brain, revealed that all but one were terminated by lysine or arginine residues. The peptide corresponding to the COOH terminus of the protein, however, was found to be VAYSSENTFGF. This finding alluded to the possibility that cytosolic GATE-16 lacks its last amino acid as a result of a specific protease activity. Consistently, it has been recently reported that Apg4p, a novel cytosolic cysteine protease, specifically removes a single amino acid from the COOH terminus of Atu7p, the yeast homologue of GATE-16 (19).

To investigate whether GATE-16 undergoes COOH-terminal cleavage by yet an unknown protease, we expressed GATE-16 with a HA epitope attached to its carboxyl terminus (GATE-16-HA) in CHO cells. Cells were harvested after 48 h, and the cytosols were analyzed by Western blot using anti-HA and anti-GATE-16 polyclonal antibodies. In addition, two mutants in which glycine 116 or phenylalanine 115 were replaced with alanine (G116A-HA and F115A-HA, respectively) were used to determine whether these residues were essential for cleavage of the COOH terminus. The cleavage of the HA tag from GATE-16-HA is displayed in Fig. 1. Notably, the mutant G116A-HA remained intact, while F115A-HA was processed as the wild type protein, indicating that the highly conserved glycine 116, but not phenylalanine 115, is essential for the cleavage of GATE-16.

Characterization of the Cleavage Process—A cell-free cleavage assay was used to further characterize this COOH-terminal processing. For that purpose, we produced recombinant GATE-16, tagged with six histidine residues (His6), at the NH₂ terminus and HA at the COOH terminus. Similarly, a mutant protein, in which glycine 116 was replaced by alanine, was produced. These proteins were incubated at 30 °C with crude cytosols prepared from CHO cells or rat brains for different durations of up to 1 h and subsequently analyzed by Western blot, using anti-His monoclonal or anti-HA polyclonal antibodies (Fig. 2). Incubation of recombinant GATE-16-HA with CHO cytosol (or crude rat brain cytosol, data not shown) for a period of up to 1 h revealed an increased proteolytic digestion of the HA tag.
Neither was the process affected by Ca$^{2+}$-calmodulin, or in the presence of cytosol treated with the thiol alkylating agent NEM. As a control, incubation was carried out at 30 °C for 1 h, in the presence of 1 mM NEM. Thus, it can be concluded that HsApg4A specifically removes a single glycine residue from the COOH terminus of GATE-16.

To further characterize this enzymatic reaction, we incubated a constant amount of GATE-16 with increasing amounts of HsApg4A (Fig. 4A) or crude rat brain cytosol (Fig. 4B) for 1 h and then analyzed the proteins by SDS-PAGE. Efficient cleavage of 1 µg (62 pmol) GATE-16 required at least 0.5 µg (11 pmol) of HsApg4A. GATE-16 was not fully cleaved by HsApg4A, even when larger amounts of HsApg4A were tested (up to 5 µg, data not shown). Under the same conditions, cleavage of GATE-16 by cytosol required 10 µg of rat brain cytosol. These results suggest that either HsApg4A is an extremely abundant protein or more likely that an additional cytosolic factor(s) is involved in the cleavage process.

**DISCUSSION**

GATE-16 has been recently implicated in fusion processes that take place in the Golgi apparatus (7, 8, 23). Although it lacks a significant amino acid sequence similarity to ubiquitin, GATE-16 was found to contain a single ubiquitin fold subdomain decorated by two helices at its NH$_2$ terminus (9). Like ubiquitin, GATE-16 is synthesized as a precursor protein. GATE-16 has a single, COOH-terminal phenylalanine residue following the conserved phenylalanine and glycine residues, and it stands to reason that this terminal phenylalanine residue must be removed for a conjugation process to take place. Here we showed that HsApg4A, a specific cysteine protease, cleaves GATE-16 at its COOH terminus. We presented evidence that this cleavage takes place in vivo and in vitro using either crude cytosol or purified HsApg4A. Our results indicate that HsApg4A specifically removes a single amino acid from the COOH terminus of GATE-16, thus exposing glycine 116. Furthermore, glycine 116 appears essential for this process inasmuch as replacing it with alanine abolishes the ability of GATE-16 to be cleaved. It appears that GATE-16 is a substrate for HsApg4A, which in turn renders it into a potential substrate for a ubiquitin-like conjugase. In accord with this suggestion, GATE-16, as well as its two other mammalian homologues, GABARAP and LC-3, were recently shown to be covalently attached to human.
A homologues of E1 (Apg7)- and E2 (Apg3)-conjugating enzymes (Refs. 21 and 22, respectively).

HsApg4A exhibits significant amino acid sequence homology to the yeast autophagic factor Apg4p, a novel cysteine protease that specifically cleaves the carboxyl terminus of Aut7p downstream of Gly-116 (19). Apg4p has been shown to cleave the newly synthesized Aut7p, a reaction that precedes a ubiquitination-like process, mediated by E1 and E2 ligases, which specifically conjugates Aut7p to PE (20). Apg4p then releases Aut7p to the cytosol by cleaving the Aut7p-PE bond. These cleavage processes mediated by Apg4p are essential for both autophagy and cytosol to vacuole (Cvt) pathways (19).

Interestingly, while in S. cerevisiae there is only a single Aut7p-like protein and a single Apg4-like protease, mammals possess at least 10 different GATE-16-like proteins and at least 2 Apg4-like proteases. A possible explanation for this phenomenon is that HsApg4A cleaves the newly synthesized GATE-16 as well as the other members of the family.
its other mammalian homologue, HsApg4B, cleaves these proteins from their targets (possibly PE). Alternatively, it is feasible that different mammalian Apg4 proteins are specific to different subsets of the GATE-16 family members. This may imply that the two Apg4s have a different tissue specificity or subcellular localization or that they may be expressed at different developmental stages. Indeed, it has been recently reported that loss of one of the Apg4 homologues in Drosophila melanogaster resulted in significant defects in cut and Notch signaling pathways (24).

HsApg4a activity is similar to that of the deubiquitination enzymes and of SUMO-specific proteases. On the basis of their amino acid sequence homology, deubiquitinating enzymes can be broadly divided into two classes: the ubiquitin-processing protease (UBP) family and the ubiquitin carboxy-terminal hydrolase (UCH) family (25–27). Both UBP's and UCHs are cysteine proteases containing in their active site cysteine, aspartate, and histidine residues. While UCH amino acid sequences are well conserved, UBP's share very limited homology (28). Recently, a distinct family of cysteine proteases acting on SUMO-1-conjugated substrates has been identified (28). The Apg4 family may be considered as a novel subgroup of these cysteine proteases that specifically cleaves the glycine-X bond at the COOH terminus of different GATE-16-like family members. Little is known about the precise cellular function of UBP's and UCHs and the cellular mechanism that regulates their activity. Here we showed that the cleavage activity of recombinant HsApg4a is significantly lower than that found in crude cytosol, suggesting that other cellular factors are involved in the cleavage process. For example, HsApg4B may be required for reconstitution of the full cleavage activity implying that the two mammalian Apg4s act as a complex. Alternatively, the activity of Apg4a may be regulated by other yet unidentified protein(s). Such regulation of Apg4a activity may influence the function and possibly the subcellular localization of GATE-16 and its other family members.

GATE-16 is the closest mammalian homologue of Aut7. We have previously shown that Aut7p, similarly to GATE-16, interacts genetically and physically with several v-SNAREs, which are involved in ER to Golgi trafficking, and in vacuolar inheritance (29). GATE-16 has been found to act at late stages of membrane fusion, coincidentally with the Golgi SNARE GOS-28 (7, 8). The precise mechanism by which GATE-16 promotes fusion is yet unclear. It has been shown that GATE-16 binds preferentially to unpaired GOS-28 and inhibits GOS-28 binding to its cognate t-SNARE syntaxin 5 (8). This activity of GATE-16 is similar to the inhibition of syntaxin/VAMP binding by Munc18 (30) and the Sec5p/Bet1p binding by Sly1p (31). In the case of GATE-16, it may act as a SNARE protector that needs to be removed from GOS-28 prior to fusion (2). However, GATE-16 may actively promote fusion. Accordingly, the interaction with GOS-28 specifically localizes GATE-16 in a close proximity to the sites of fusion. Hence, the potential of GATE-16 to be transiently lipidated, similarly to its yeast homologue Aut7p (20), may serve to stimulate the formation a fusion pore during SNAREpin formation and bilayer mixing. The activity of HsApg4a described in the present work would be essential for such a process to occur.

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