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A Yeast Golgi E-type ATPase with an Unusual Membrane Topology*

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E-type ATPases are involved in many biological processes such as modulation of neural cell activity, prevention of intravascular thrombosis, and protein glycosylation. In this study, we show that a gene of Saccharomyces cerevisiae, identified by similarity to that of animal ectoapapyrase CD39, codes for a new member of the E-type ATPase family (Apy1p). Overexpression of Apy1p in yeast cells causes an increase in intracellular membrane-bound nucleoside di- and triphosphate hydrolase activity. The activity is highest with ADP as substrate and is stimulated similarly by Ca2+, Mg2+, and Mn2+. The results also indicate that Apy1p is an integral membrane protein located predominantly in the Golgi compartment. Sequence analysis reveals that Apy1p contains one large NH2-terminal hydrophilic apyrase domain, one COOH-terminal hydrophilic domain, and two hydrophobic stretches in the central region of the polypeptide. Although no signal sequence is found at the NH2-terminal portion of the protein and no NH2-terminal cleavage of the protein is observed, demonstrated by the detection of NH2-terminal tagged Apy1p, the NH2-terminal domain of Apy1p is on the luminal side of the Golgi apparatus, and the COOH-terminal hydrophilic domain binds to the cytoplasmic face of the Golgi membrane. The second hydrophobic stretch of Apy1p is the transmembrane domain. These results indicate that Apy1p is a type III transmembrane protein; however, the size of the Apy1p extracytoplasmic NH2 terminus is much larger than those of other type III transmembrane proteins, suggesting that a novel translocation mechanism is utilized.

E-type ATPases (E-ATPases) are found in most eukaryotic cells and hydrolyze nucleotide tri- and/or diphosphates in the presence of Ca2+ or Mg2+ (1). They play important roles in many biological processes including the modulation of neural cell activities (2), prevention of intravascular thrombosis (3, 4), and regulation of immune responses (5). The molecular identity of the E-ATPases was revealed recently by the purification and cloning of a soluble apyrase from potato tubers (Solanum tuberosum) (6). The deduced amino acid sequence for potato apyrase conserved region; ER, endoplasmic reticulum; PCR, polymerase chain reaction; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole.

plasma gondii NTPase (9). All of these proteins contain four highly conserved sequences called apyrase conserved regions (ACR1–4). The sequences of ACR1 and ACR4 are similar to those of the actin-hsp70-hexokinase β and γ-phosphate binding motifs, respectively, suggesting a possible role in nucleotide binding (6). CD39 was subsequently shown to exhibit E-ATPase activity (5), which confirmed the existence of a novel protein family for E-ATPases. Since then, additional members of the E-ATPase family have been identified (10–16).

Most E-ATPases are transmembrane proteins (5, 10–12, 14, 17, 18). Transmembrane proteins of the endoplasmic reticulum (ER) and ER-derived cell organelles are inserted cotranslationally into the ER membrane in a signal recognition particle-dependent manner (19–21). During the insertion process, membrane topology is determined by interaction between topogenic signals within the nascent protein and the translocation machinery; these signals are only partially understood (22). Four types of single-spanning membrane proteins can be distinguished based on the topogenic sequence involved in the insertion (23, 24). Type I membrane proteins have a cleaved NH2-terminal signal sequence followed by a transmembrane anchor segment, and their mature NH2 terminus is extracytoplasmic (Nexo). Type II proteins contain an uncleaved signal/anchor sequence resulting in the cytoplasmic localization of the NH2 terminus (Ncyt). In type III proteins, the NH2 terminus is translocated to the lumen of the ER (Nexo), and the signal sequence is not cleaved (23). Type IV proteins have a Ncyt/Nexo topology like type II proteins but have the transmembrane segment very close to the COOH terminus and are inserted into the ER membrane by uncharacterized machinery (24, 25). All reported membrane-bound E-ATPases have a type II-like orientation.

In the lumen of the Golgi apparatus, proteins and lipids become glycosylated. Nucleotide sugars are synthesized in the cytosol and transported into the Golgi lumen via specific carrier proteins (26, 27). After transfer of sugar residues to proteins and lipids by glycosyltransferases, the resulting nucleoside diphosphates are converted to nucleoside monophosphates by nucleotide diphosphatases. In this way, nucleoside diphosphates that are inhibitors of glycosyltransferases do not accumulate in the lumen of the Golgi apparatus. The nucleoside monophosphates exit the lumen of Golgi in exchange with cytosolic sugar nucleotides. It has been shown previously that a Saccharomyces cerevisiae GDPase (GDA1) is required for protein and lipid mannosylation (17, 28). Gda1p was recognized as an E-ATPase because of its similarity to potato apyrase (6) and to animal ectoapapyrase CD39 (5). Deletion of the gene has a minor effect on the growth of yeast but does result in decreased mannosylation of membrane proteins (28). A sequence homology search in the GenBank data base revealed another gene from S. cerevisiae (GenBank accession number P40009) with high similarity in the ACR1–4 motifs to members of the E-ATPase family. This gene is on chromosome V, encodes a hypothetical 71.9-kDa protein, and was proposed recently to be the second E-ATPase found in yeast (14). In this study, we...
FIG. 1. Nucleotide sequence of chromosomal DNA encoding the yeast 71.9-kDa protein together with the deduced amino acid sequence. Nucleotides and amino acid residues are numbered. Upstream and downstream sequences of the gene are shown. Before the ATG initiation codon, four stop codons are shadowed. The four highly conserved ATG initiation codon, four stop codons are shadowed.

The putative tyrosine kinase phosphorylation site is in **bold**. Two potential glycosylation sites are in *italics* and **bold**. The putative tyrosine kinase phosphorylation site is in **bold**.
tional glycosylation site (D619N) in the N371 mutant, the PCR product of primer XZ105 and primer XZ122 (5'-ACCGGTGTCGACTCTCAAAAT-TTGAGAAAATTTAGCATTAGAAAAAGGC-3'), containing an SalI site and a sequence complementary to nucleotides 2113–2145 with the mismatched nucleotide underlined) was digested with SalI and used to replace the corresponding SalI fragment of pGZ114 to form pGZ120. To delete amino acids 552–630 from Apy1p, the PCR product of primer XZ111 and primer XZ124 (5'-CCCGGATCTCTAATCTGATCTCCTTAGAAUUCTCAA-3', containing a BamHI site and a sequence complementary to nucleotides 1906–1929) was digested with BamHI and subcloned into pG1 to form pGZ121. All the constructs were verified by DNA sequencing.

Membrane Preparation and Cell Fractionation—Cells were grown to A600 ~ 4.0 in the proper yeast drop-out medium containing 2% glucose at 30 °C. NaN3 was added to 10 mM, and the culture was harvested by centrifugation and washed once in 10 mM NaN3. Cells were resuspended at 0.25 g/ml in homogenization buffer (50 mM-Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 1 mM EGTA, 5 mg/ml bovine serum albumin, 2 mM dithiothreitol, 2.5 mg/ml chymostatin, 1 mM phenylmethylsulfonyl fluoride) and lysed by passage through a French pressure cell (SLM-Amino, Urbana, IL) at 20,000 p.s.i. The lysate was centrifuged at 10,000 × g for 20 min; the supernatant fraction was then centrifuged at 120,000 × g for 1 h. The membrane pellet was resuspended in 10 mM-Tris-HCl, pH 7.0, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride.

Isolation of Golgi membranes by differential centrifugation was done as described (36). Briefly, spheroplasts (300 A600 units) were lysed by dilution in hyposomic buffer. The lysate was centrifuged at 1,000 × g for 10 min to generate the P1 (pellet) and the S1 (supernatant) fractions, and the latter was centrifuged at 13,000 × g (P13) and 120,000 × g (P120) for 20 and 60 min, respectively. The P13 and P120 pellets were resuspended in 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA.

Protease Protection Assay—To examine the protease accessibility of Apy1p, 25 μl of the P120 pellet containing Golgi-enriched vesicles in 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA were added to the test solutions: (a) 25 μl of 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA; (b) 25 μl of proteinase K (800 μg/ml) in 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA; (c) 25 μl of 2% Triton X-100 in 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA; (d) 25 μl of 2% Triton X-100, 800 μg/ml proteinase K, 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA. All samples were incubated on ice for 45 min, and trichloroacetic acid was added to a final concentration of 10%. The samples were pelletted and washed with ice-cold acetone. The dried pellets were resuspended in sample buffer and subjected to 10% SDS-PAGE.

Measurement of Nucleoside Di- and Triphosphatase Activity—To measure apyrase activity, crude membranes of yeast cells (15 μg) were suspended in 90 μl of buffer A (20 mM HEPES-Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, and 1 mM EGTA) with or without 10 mM CaCl2 and preincubated for 5 min at 37 °C. The nucleotidase reactions were initiated by the addition of 10 μM of the same buffer containing 20 mM nucleotide. The divalent cation-stimulated apyrase activity was determined by measuring the inorganic phosphate released as described by Ames (37) and by subtracting values obtained with EGTA alone from those with 10 mM CaCl2 plus chelator (14).

Deglycosylation of Apy1p—Flavobacterium meningosepticum glycopeptidase F (New England Biolabs, Beverly MA) was used to deglycosylate asparagine-linked glycans (38). Yeast crude membrane (50 μg) was incubated on ice for 30 min with 10 μl of the enzyme, and the reaction was terminated by the addition of 10 μl of 8 M mercaptoethanol and 0.1% SDS. The denatured protein mixture was then incubated with 1% Nonidet P-40, 10 mM sodium phosphate, pH 8.2, and 2 units of glycopeptidase F at 37 °C for 20 h.

Immunoblotting and Immunofluorescence Staining—Anti-HA monoclonal antibody (12C5A, 1:2,000 dilution for immunoblotting) was purchased from Berkeley Antibody Corp. (Berkeley, CA). Anti-myc monoclonal antibody (9E10, 1:500 for immunoblotting) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated goat anti-mouse antibody (1:2,000) was purchased from Sigma. Immunoblotting was done as described previously (5), except that the incubation with the first antibody was for 5 h. Indirect immunofluorescence of the Apy1p-HA protein was performed by standard methods (39) using the anti-HA antibody 12C5A at 1:150 dilution and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:128, Sigma). Samples were observed and photographed with a Nikon microphot 5A epifluorescence microscope.

FIG. 2. Expression and membrane association of Apy1p. Panel A, expression of Apy1p. Crude membrane proteins isolated from BCY123/pG1 (lane 1) and BCY123/pGZ103 (lane 2) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. HA-tagged Apy1p protein was detected by immunoblotting with anti-HA monoclonal antibody. Panel B, deglycosylation of Apy1p. Crude membrane proteins from BCY123/pGZ103 were deglycosylated as described under "Materials and Methods." Protein samples were resolved by 8% SDS-PAGE, and Apy1p was detected by anti-HA. Panel C, membrane association of Apy1p. Crude membranes from BCY123/pGZ103 were incubated with 0.1 mM sodium carbonate, pH 11.0 (lanes 1 and 2), 1% Triton X-100 (lanes 3 and 4) on ice for 30 min, then centrifuged at 100,000 × g for 20 min in a TLA100.3 rotor. Both pellets and supernatant fractions were analyzed by 10% SDS-PAGE, and Apy1p was detected by immunoblotting with anti-HA.

RESULTS

Cloning of the APY1 Gene from Yeast Chromosomal DNA—The full-length DNA sequence encoding the yeast 71.9-kDa protein (Apy1p) was cloned by PCR using yeast DNA as a template. Fig. 1 shows the sequence of the clone which has a few differences compared with the yeast genomic data base sequence. The ATG initiation codon at nucleotides 273–275 is the most likely translational initiation site because there are four stop codons, shown shadowed, preceding the ATG in the same open reading frame. The apyrase conserved regions (ACR1–4) are indicated in boldface. There are two potential N-glycosylation sites, shown in bold and italics, at amino acid residues 371–373 and 592–594.

Expression of Apy1p—To determine whether this gene can be expressed in yeast cells, the sequence for a HA tag was added in-frame to the 3'-end of the open reading frame, and the resulting cDNA was expressed behind a constitutive promoter in the yeast vector pG1. As shown in Fig. 2A, yeast cells transformed with the APY1-HA plasmid expressed 73-kDa and 72-kDa proteins recognized by anti-HA monoclonal antibody (lane 2). No bands were detected in the immunoblot of yeast cells with the control vector (lane 1). When crude membranes of yeast cells expressing Apy1p-HA were treated with glycopepti-
stimulated similarly by Ca2+ ADP as the substrate. The activity of Apy1p ADPase was highest with nucleoside phosphatase activity of Apy1p was highest with scatters throughout the cytoplasm but excluded from the vacuolar membrane, and plasma membrane are found in the Golgi enzymes. (P120) is a Golgi-enriched fraction, containing the majority of Golgi enzymes (Kex2p (43). We routinely found that about 70% of Apy1p-HA was in the P120 fraction, suggesting that Apy1p is localized in the Golgi fraction. To confirm further the Golgi localization of Apy1p (Fig. 2C), the membrane fraction was extracted with 0.1 M sodium carbonate, pH 11.0 (lanes 1 and 2), and 1% Triton X-100 (lanes 3 and 4). The treated samples were centrifuged at 100,000 × g to produce pellet and supernatant. Nearly all Apy1p sedimented in the membrane pellet after the treatment with alkaline carbonate buffer but was extracted from the membrane into supernatant fraction with Triton X-100. These data indicate that Apy1p is an integral membrane protein. A 15-kDa band, present in lanes 3 and 4, is probably a degradation product of Apy1p. Moreover, a considerable amount of Apy1p was Triton X-100-insoluble and remained in the pellet after extraction.

**Function of Apy1p**—It was proposed that Apy1p might be a new member of the E-ATPase family because Apy1p contains four ACR motifs (14). To study the function of Apy1p, crude membrane fractions isolated from yeast cells overexpressing Apy1p and control yeast cells were assayed for their nucleotidase activities. As shown in Fig. 3A, expression of HA-tagged Apy1p increased the membrane-associated nucleotidase activity on various nucleoside di- and triphosphates. Both ADPase and TDPase activities are more than 20-fold higher than those of membranes from control yeast cells. Neither 1 mM azide (inhibitor of F-type ATPases) nor 0.5 mM vanadate (inhibitor of P-type ATPases) inhibited the activities. It is known that E-ATPases are resistant to these inhibitors (1), so Apy1p appears to be a new E-ATPase.

Fig. 3B shows the ADP and TDP concentration dependence of nucleotidase activity. These activities were determined under conditions where the activities were linear with respect to time, and the substrate concentration did not change during the assay. ADPase and TDPase activities reached maximum values at concentrations of 4 mM and 2 mM, respectively. The nucleoside phosphatase activity of Apy1p was highest with ADP as the substrate. The activity of Apy1p ADPase was stimulated similarly by Ca2+, Mn2+, and Mg2+ (Fig. 3C). Maximum activation was obtained at 5 mM divalent cation. The effect of cations on Apy1p activity is different from that on yeast GDPase (17) and human UDPase (14), in which Ca2+ is more effective than Mn2+ or Mg2+.

**Golgi Localization of Apy1p**—To study the cellular localization of this yeast E-ATPase, yeast cells expressing Apy1p-HA were subjected to subcellular fractionation by differential centrifugation. Spheroplasts were lysed in a hypo-osmotic buffer, and the lysate was subjected to high speed centrifugation to separate the membrane fraction (pellet) and cytosol fraction (supernatant). Apy1p-HA was detected only in the membrane fractions, not in the cytosol (data not shown). To address further the membrane association of Apy1p (Fig. 2C), the membrane fraction was extracted with 0.1 M sodium carbonate, pH 11.0 (lanes 1 and 2), and 1% Triton X-100 (lanes 3 and 4). The treated samples were centrifuged at 100,000 × g to produce pellet and supernatant. Nearly all Apy1p sedimented in the membrane pellet after the treatment with alkaline carbonate buffer but was extracted from the membrane into supernatant fraction with Triton X-100. These data indicate that Apy1p is an integral membrane protein. A 15-kDa band, present in lanes 3 and 4, is probably a degradation product of Apy1p. Moreover, a considerable amount of Apy1p was Triton X-100-insoluble and remained in the pellet after extraction.

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To determine whether the NH₂ terminus might have an irregular cleavable signal sequence, Apy1p was tagged with a NH₂-terminal myc-tagged and COOH-terminal HA-tagged Apy1p were immunoblotted with anti-HA and anti-myc monoclonal antibodies, indicating that the NH₂ terminus of Apy1p is protected from protease digestion, suggesting that it is in the Golgi lumen and that the COOH-terminal end of Apy1p is not protected and is in the cytoplasm. The finding that protease digestion only removes 2 kDa from the COOH-terminal end of the protein suggests that most of the COOH-terminal hydrophilic domain is not available to the protease.

To demonstrate further that the NH₂-terminal domain of Apy1p is in the lumen and the COOH-terminal end is in the cytosol, two glycosylation sites (Asn-371 and Asn-532) of Apy1p located on NH₂- and COOH-terminal sides of the putative transmembrane domains were mutated to see if glycosylation of Apy1p would be affected. As is shown in Fig. 6A, protease K digestion reduced the size of Apy1p by 2 kDa, detected by anti-myc antibody (lane 2). However, no band was visible in the protease-treated sample examined with the anti-HA antibody (lane 6). These data indicate that the NH₂ terminus of Apy1p is protected from protease digestion, suggesting that it is in the Golgi lumen and that the COOH-terminal end of Apy1p is not protected and is in the cytoplasm. The finding that protease digestion only removes 2 kDa from the COOH-terminal end of the protein suggests that most of the COOH-terminal hydrophilic domain is not available to the protease.

The localization of the apyrase activity of Apy1p was investigated by measuring the ADPase activity of crude membranes. In the absence of additions, the nucleotidase activity is low; however, in the presence of digitonin, a nonionic detergent, and of alamethicin, a pore-forming antibiotic that allows ADP to traverse the membrane, the ADPase activity increases 2- and 4-fold, respectively (Fig. 7). This result also suggests that the active site of the enzyme is in the lumen of the Golgi. Triton X-100 appears to inhibit the enzymatic activity.

Because the NH₂- and COOH-terminal domains of Apy1p are not on the same side of the membrane, these results also indicate that Apy1p has an odd number of transmembrane domains, probably one, whereas there are two potential hydrophobic stretches in Apy1p. To determine which hydrophobic
stretch is the real transmembrane domain, two COOH-terminal truncated versions of Apy1p were constructed. The \( \Delta 491-630 \) mutant lacked the COOH-terminal hydrophilic domain and the second hydrophobic stretch, and it had a HA tag at the end of the 33-amino acid hydrophilic loop connecting the two hydrophobic stretches. The \( \Delta 552-630 \) mutant lacked the COOH-terminal 79 amino acids following the second hydrophobic segment. As is shown in Fig. 8A, \( \Delta 491-630 \) Apy1p was not glycosylated (lanes 1 and 2), indicating that the NH\(_2\)-terminal domain was not in the lumen. Fig. 8B shows that \( \Delta 552-630 \) Apy1p was glycosylated (lanes 1 and 2), indicating that the NH\(_2\)-terminal domain was in the lumen. These data support the view that the second hydrophobic stretch of Apy1p is the transmembrane domain.

Proposed Membrane Topology Model of Apy1p—Based on the results obtained above, the membrane topology of Apy1p is shown in Fig. 9. The intact NH\(_2\)-terminal apyrase domain (amino acids 1–500) is located in the Golgi lumen with glycosylation of residue Asn-371. The COOH-terminal 113-amino acid hydrophilic domain is in the cytoplasm; since most of this domain is protected from protease digestion, it may bind to the membrane through its many positive charges. Furthermore, the second hydrophobic stretch (amino acids 501–517) of Apy1p is the likely transmembrane domain.

FIG. 6. The NH\(_2\)-terminal domain of Apy1p is in the Golgi lumen, and its COOH-terminal domain binds to cytoplasmic face of the Golgi membranes. Panel A, accessibility of Apy1p domains to proteinase K digestion. As described under “Materials and Methods,” Golgi membrane fractions were digested by proteinase K. The samples were immunoblotted with both anti-HA and anti-myc antibodies. Panel B, the N371I mutation not N532I eliminates the glycosylation of Apy1p. Crude membranes isolated from BCY123/pGZ114 (Apy1p N371I mutant), BCY123/pGZ113 (Apy1p N532I mutant), and wild type Apy1p expressing strains were treated or not treated with glycopeptidase F (GNFase). Samples were blotted with anti-HA antibody.

FIG. 7. Nucleotidase activity of membranes before and after the addition of detergents and alamethicin to permeabilize the membrane. The NDPase activity of crude membranes of BCY123/pGZ105 (APY1-HA-expressing strain) and BCY123/pG1 (strain with the empty vector) was measured in the absence or presence of Triton X-100 (0.1% (v/v)) or digitonin (0.1% (v/v)) or alamethicin (0.2 mg/ml). Assays were carried out at a substrate concentration of 2 mM as described under “Materials and Methods.” All values are means \( \pm S.D. \) (\( n = 4 \)).

FIG. 8. The second hydrophobic stretch of Apy1p is the transmembrane domain. Crude membranes isolated from BCY123/pGZ118, expressing Apy1p with a deletion of the second hydrophobic segment and the COOH-terminal hydrophilic domain (panel A), and from BCY123/pGZ121, expressing Apy1p with a deletion of the COOH-terminal 79 amino acids (panel B), were treated or not treated with glycopeptidase F (GNFase). The proteins were separated by 10% SDS-PAGE and identified by immunoblotting with anti-HA antibodies (panel A) and anti-myc antibodies (panel B).

DISCUSSION

In this study, we have demonstrated that a putative yeast 71.9-kDa protein (Apy1p) is a new member of the E-ATPase family. Apy1p hydrolizes various nucleotides, and its activity is highest with ADP. By membrane fractionation and immunofluorescence staining, Apy1p was found to be localized in the Golgi apparatus. Based on the results of protease protection assays and site-directed mutagenesis experiments, we have shown that Apy1p has a large NH\(_2\)-terminal extracellular domain, a transmembrane domain, and a smaller COOH-terminal cytoplasmic domain; the protein does not have an explicit signal sequence.

It is interesting that there are two E-ATPases in yeast, and both are located in the Golgi. Yeast GDPase is involved in protein and lipid mannosylation. Its null mutant (\( gda1 \)) is viable, but it has a partial block in O- and N-glycosylation of...
acid long NH₂ terminus, with the glycosylation site at Asn-371, is in the NH₂-terminal apyrase domain is located in the Golgi lumen produced by examination of the primary structures of the for the substrate specificity is not known and cannot be de- nucleoside diphosphates (17). At present, the structural basis family of E-type ATPases embraces enzymes that are specific cation preference of these enzymes is also different (17). The GDPase (Gdp1p) is quite specific for GDP (17). The divalent phosphates in the Golgi.

plant ectoapyrase may be involved in phosphate transport; it is appa- ratus and that the translocation of ATP into the Golgi translocation of ATP into the Golgi. It has been reported that Gda1p and Apy1p are not essential for transport into the Golgi. We also dem- 

secreted proteins and a decrease in the transport of GDP- mannose into the Golgi lumen (17, 28). The APY1 null mutant (apy1) is viable and grows at approximately the same rate as the wild type at 30 °C; the double deletion mutant (gda apy1) is also viable and grows approximately four times more slowly than the wild type strain. Because the yeast GDP-mannose transporter null mutation is lethal, suggesting that glycosyla- tion of protein and lipid in the Golgi is essential (52), it appears that the activities of Gda1p and Apy1p are not essential for protein and lipid glycosylation, probably because there is yet another way to hydrolyze nucleotide diphosphates. As the enz- ymatic properties of these two enzymes are different, further experiments are required to determine whether they have different biological functions.

Another possible function of Apy1p is in the conversion of ADP to AMP, which is then used as an antiporter in the translocation of ATP into the Golgi. It has been reported that an ATP transport activity is required for phosphorylation of proteoglycans and secretory proteins in the lumen of the Golgi apparatus and that the translocation of ATP into the Golgi lumen is coupled to the exit of AMP (27, 53). This hypothesis is consistent with the view that the antiporotein for sugar nucleotides transport into the Golgi are nucleoside monophosphates (27). In a recent paper (54) it has been suggested that a plant ectoapyrase may be involved in phosphate transport; it is possible that Gda1p and Apy1p are a link to transport of phosphates in the Golgi.

Apy1p has a broad substrate specificity, whereas yeast GDPase (Gdp1p) is quite specific for GDP (17). The divalent cation preference of these enzymes is also different (17). The family of E-type ATPases embraces enzymes that are specific for ATP (55, 56), for nucleotide di- and triphosphates (1), and nucleoside diphosphates (17). At present, the structural basis for the substrate specificity is not known and cannot be de- 

The most surprising finding in this study is that a large NH₂-terminal apyrase domain is located in the Golgi lumen although it lacks a signal sequence for translocation through the membrane. Only a few membrane proteins with a long extracellular NH₂ terminus lacking a signal sequence are known. Some G protein-coupled receptors belong in this class, but their NH₂-terminal segment is less than 100 residues; above this size limit, an NH₂-terminal signal sequence is em- ployed (51). It has been reported that Neu differentiation factor has a 240-amino acid extracellular NH₂ terminus and lacks a typical signal sequence, but the NH₂-terminal 13 amino acids are cleaved, suggesting the existence of some kind of NH₂- terminal signal (57). To our knowledge, Apy1p has the longest translocated NH₂ terminus reported so far. If translocation of the NH₂ terminus of Apy1p takes place by a new mechanism, Apy1p may be classified as a type V transmembrane protein. We predict that hypothetical protein C3H15.14 of Caenorhabditis elegans, a candidate E-ATPase member (14), also belongs to this new subgroup because the primary sequence suggests that it has a large hydrophilic NH₂-terminal apyrase domain lacking a signal sequence and a single hydrophobic segment.

The mechanism for the translocation of the NH₂-terminal domain of Apy1p across the membrane should be different from those of type I and III membrane proteins. It has been shown that the orientation of the insertion of the transmembrane segments of membrane proteins correlates best with Δ(C-N), the difference in charges within an arbitrary window of 15 residues flanking the transmembrane segment on either side (58). Because the end of the transmembrane segment with the most positive charges is retained in the cytoplasm, the value of Δ(C-N) can be used to predict the orientation of the transmembrane segment. Similar to type III proteins, the value of Δ(C-N) for Apy1p is positive in accordance with our localization of the NH₂ terminus and COOH terminus in the lumen of the Golgi and in the cytoplasm, respectively. Compared with other E-ATPases, Apy1p has a long COOH- terminal domain. Most of it may bind to the cytoplasmic face of the Golgi membrane, probably because of its highly positive charge. The functional significance of this binding is unknown; however, deletion of two-thirds of the COOH-terminal domain does not affect the membrane topology of Apy1p. It is worthy of note, however, that a single tyrosine kinase phosphorylation site is present at the end of the COOH-terminal domain (622–629 KFKDSRLY). It will be interesting to find out whether this site can really be phosphorylated and whether there is any activity change when this site is mutated or deleted.

In summary, we have shown that a yeast hypothetical 71.9- kDa protein, Apy1p, possesses a cation-stimulated nucleotidase activity and is located mainly in the Golgi. We also demon- 

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REFERENCES

1. Plesner, L. (1995) Int. Rev. Cytol. 158, 141–214
2. Zimmermann, H. (1994) Trends Neurosci. 17, 420–426
3. Kaczmarek, E., Koritzik, K., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach, F. H., and Robson, S. C. (1996) J. Biol. Chem. 271, 33116–33122
4. Marcus, A. J., Broekman, M. J., Drosopoulos, J. H., Islam, N., Alyonycheva, T. N., Safier, L. B., Hajjar, K. A., Posnett, D. N., Schoenborn, M. A., Schooley, K. A., Gayle, R. B., and Maliszewski, C. R. (1997) J. Clin. Invest. 99, 1351–1360
5. Wang, T. F., and Guidotti, G. (1996) J. Biol. Chem. 271, 9688–9691
6. Handa, M., and Guidotti, G. (1996) Biochem. Biophys. Res. Commun. 218, 916–923
7. Maliszewski, C. R., Delespesse, G. J., Schoenborn, M. A., Armitage, R. J., Fanslow, W. C., Nakajima, T., Baker, E., Sutherland, G. R., Poindexter, K.
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