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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 ectoapyrase 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 Ca^{2+}, Mg^{2+}, and Mn^{2+}. 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 NH_2-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 NH_2-terminal portion of the protein and no NH_2-terminal cleavage of the protein is observed, demonstrated by the detection of NH_2-terminal tagged Apy1p, the NH_2-terminal domain of Apylp 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 Apylp is a type III transmembrane protein; however, the size of the Apy1p extracytoplasmic NH_2 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)[1] are found in most eukaryotic cells and hydrolyze nucleotide tri- and/or diphosphates in the presence of Ca^{2+} or Mg^{2+} (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 had sequence similarities with various other polypeptides in the data base, including CD39, a human and mouse lymphoid cell antigen (7), garden pea NTPase (8), and *Toxoplasma 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 NH_2-terminal signal sequence followed by a transmembrane anchor segment, and their mature NH_2 terminus is extracytoplasmic (N_{exo}). Type II proteins contain an uncleaved signal/anchor sequence resulting in the cytoplasmic localization of the NH_2 terminus (N_{cyto}). In type III proteins, the NH_2 terminus is translocated to the lumen of the ER (N_{exo}), and the signal sequence is not cleaved (23). Type IV proteins have a N_{cyto}/C_{exo} 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 ectoapyrase 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

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[1] The abbreviations used are: E-ATPase(s), E-type ATPase(s); APR, apyrase conserved region; ER, endoplasmic reticulum; PCR, polymerase chain reaction; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole.

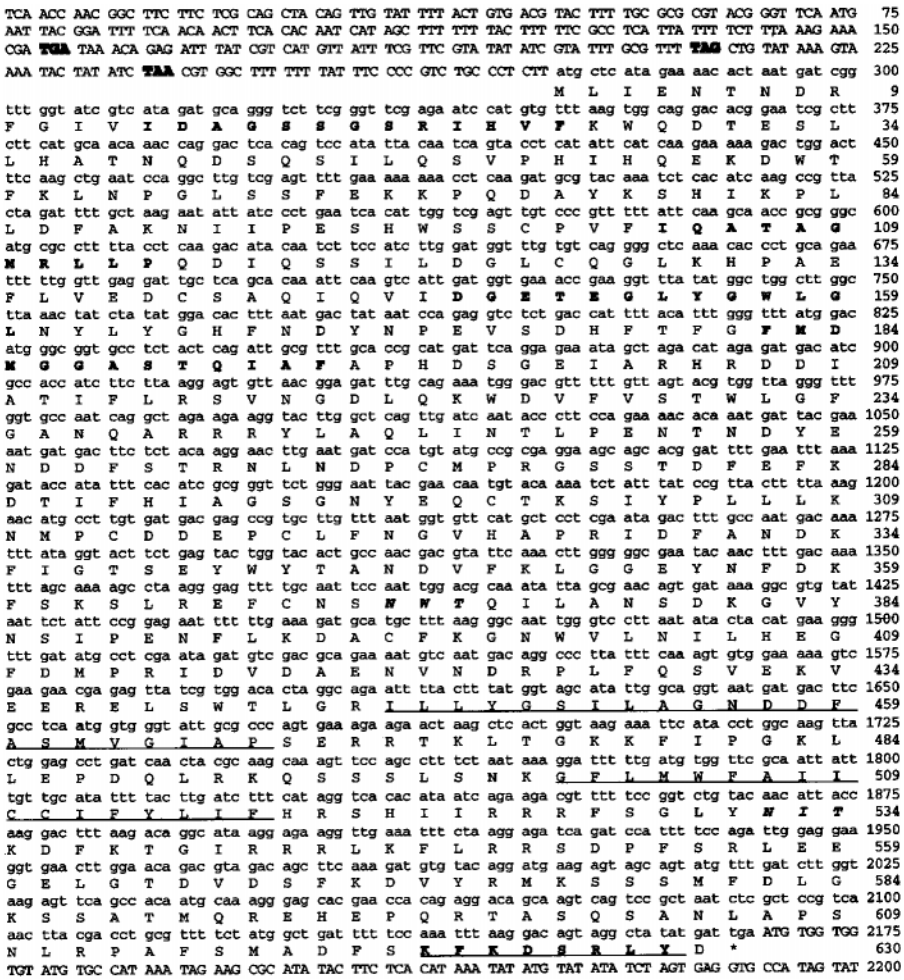

FIG. 1. **Nucleotide sequence of chromosomal DNA encoding the yeast 71.9-kDa protein together with the deduced amino acid sequence.** Nucleotides and amino acid residues are *numbered*. Upstream and downstream sequences of the gene are shown. Before the ATG initiation codon, four stop codons are *shadowed*. The four highly conserved apyrase regions (ACR1–4) are in *bold*. Two hydrophobic stretches determined with the use of the algorithm of Kyte and Doolittle are *underlined*. The putative tyrosine kinase phosphorylation site is in *bold* and *underlined.* Two potential glycosylation sites are in *italics* and *bold.*

demonstrate that this 71.9-kDa protein is indeed a novel E-ATPase. Interestingly, it is located in the Golgi and has an unusual membrane topology.

MATERIALS AND METHODS

Strains, Media, and Reagents—All DNA manipulations were performed using the *Escherichia coli* strain DH5α (*supE44D lacU169 (80lacZDM15) hsdr17 recA1 endA1 gyrA96 thi-1 relA1*). All Apy1p constructs and its derivatives were expressed in *S. cerevisiae* strain BCY123 (*MATa pep4::HIS3 prb1:: LEU2 bar1::HISG lys2 ::GAL1/10-GAL4 can1 ade2 trp1 ura3 his3 leu2–3, 112*, originally obtained from the laboratory of R. Kornberg, Stanford University). An isogenic derivative of strain SK1 (29) was used for chromosomal DNA isolation. Standard rich (YPD) and complete minimal tryptophan dropout media were used (30). Standard rich medium for *E. coli* was used (31). Nucleoside phosphates were purchased from Sigma. Zymolyase 20T was purchased from ICN (Irvine, CA).

DNA Constructs—DNA manipulations were carried out according to standard protocols (32). To clone a full-length *APY1* gene, two primers were designed to amplify the DNA fragment encoding the putative 71.9-kDa protein from yeast chromosomal DNA by PCR. Yeast SK1 chromosomal DNA was isolated by standard protocol (33). The sense primer XZ101 (5′-CCCAAGCTTATCAACCAACGGCTTCTTCTCGCA-3′), with a *Hin*dIII site at the 5′-end, contained a sequence of the open reading frame (nucleotides 1–23 in Fig. 1). The antisense primer XZ102 (5′-CGCGGATCCTCAGGCGTAGTCCGGGACGTCATATGGGTAATC-ATATAGCCTACTGTCCTTAAATTTGGA-3′) contained a *Bam*HI site at the 5′-end, an antisense sequence encoding an influenza hemagglutinin epitope (HA) recognized by monoclonal antibody 12CA5, and a sequence complementary to the *APY1* gene (nucleotides 2134–2163 in Fig. 1). A stop codon was introduced between the *Bam*HI site and the HA-tagged sequence in the antisense primer. The resulting PCR fragment was cloned into the 2-μm vector pRS316 (34; kindly provided by Dr. Neil Hunter, Harvard University); the resulting construct was named pGZ98. To express *APY1-HA* behind the glycerol dehydrogenase promoter, a PCR fragment was generated using pGZ98 as a template, primer XZ102 and primer XZ105 (5′-CGCGGATCCGTAACCATGCTC-ATAGAAAACACTAAT-3′, which contained a Kozak sequence right in front of the start codon, a *Bam*HI site at the 5′-end, and nucleotide sequences 274–294 (Fig. 1)). The resulting PCR product was subcloned into the expression vector pG1 (35; kindly provided by Sean Burgess, Harvard University); the resulting construct was named as pGZ103.

Two primers were used to amplify a DNA fragment encoding an NH_2-terminal myc-tagged Apy1p-HA. The sense primer XZ111 (5′-CG-CGGATCCGTAACCATGGAGCAAAAGCTGATATCTGAAGAGGACT-TGCTCATGAAAACACTAAT-3′) contained a *Bam*HI site, a Kozak sequence, a start codon, a sense sequence encoding the myc epitope (EQKLISEEDL), and nucleotide sequence 277–294 in Fig. 1. The antisense primer was XZ102. The resulting PCR product was subcloned into pG1 (pGZ105).

To introduce point mutations N532I and N371I by PCR, primers XZ102 and XZ105 were used as two outsider primers, and pGZ103 was used as the template. Sense primer XZ118 (5′-TCCGGTCTGTACAT-CATTACCAAGGAC-3′, mismatched nucleotide underlined) and antisense primer XZ119 (5′-GTCCTTGGTAATGATGTACAGACCGGA-3′, mismatched nucleotide underlined) were used for the N532I mutation. The resulting PCR product was digested with *Bam*HI and subcloned into pG1 (pGZ113). To introduce point mutation N371I, sense primer XZ116 (5′-TTTTGCAATTCCATTTGGACGCAAATA-3′, mismatched nucleotide underlined) and antisense primer XZ117 (5′-TATTTGCGTC-CAAATGGAATTGCAAAA-3′, mismatched nucleotide underlined) were used. The resulting PCR product was digested with *Sac*II and *Bsp*EI, and the corresponding fragment in pGZ105 was replaced with this *Sac*II/*Bsp*EI PCR fragment (pGZ114).

To delete the segment between amino acid residues 500 and 630 of Apy1p, the PCR product of primer XZ105 and primer XZ123 (5′-CGC-GGATCCTCAGGCGTAGTCC-GGGACGTCATATGGGTATTTATTAG-AAAGGCTGGA-3′, containing a *Bam*HI site, an antisense HA sequence and an sequence complementary to nucleotide sequence 1756–1773) was subcloned into pG1 with *Bam*HI (pGZ118). To introduce an addi-

tional glycosylation site (D619N) in the N371I mutant, the PCR product of primer XZ105 and primer XZ122 (5′-ACGCGTCGACTCACTTAAATTTGGAAAAATTAGCCATAGAAAACGC-3′, containing an *Sal*I site and a sequence complementary to nucleotides 2113–2145 with the mismatched nucleotide underlined) was digested with *Sal*I and used to replace the corresponding *Sal*I fragment of pGZ114 to form pGZ120. To delete amino acids 552–630 from Apy1p, the PCR product of primer XZ111 and primer XZ124 (5′-CGCGGATCCT- CAATCTGATCTCCTTAGAAATTTCAA-3′, containing a *Bam*HI site and a sequence complementary to nucleotides 1906–1929) was digested with *Bam*HI and subcloned into pG1 to form pGZ121. All the constructs were verified by DNA sequencing.

Membrane Preparation and Cell Fractionation—Cells were grown to $A_{600} \sim 4.0$ in the proper yeast drop-out medium containing 2% glucose at 30 °C. NaN_3 was added to 10 mM, and the culture was harvested by centrifugation and washed once in 10 mM NaN_3. 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 EGTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride.

Isolation of Golgi membranes by differential centrifugation was done as described (36). Briefly, spheroplasts (300 A_{600} units) were lysed by dilution in hypo-osmotic 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 following 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 pelleted 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 $CaCl_2$ and preincubated for 5 min at 37 °C. The nucleotidase reactions were initiated by the addition of 10 μl 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 $CaCl_2$ 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 boiled for 5 min in 50 μl of a solution with 10 mM β-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 (12CA5, 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 12CA5 at 1:150 dilution and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:128, Sigma). Samples were observed and photographed with a Nikon microphot SA epifluorescence microscope.

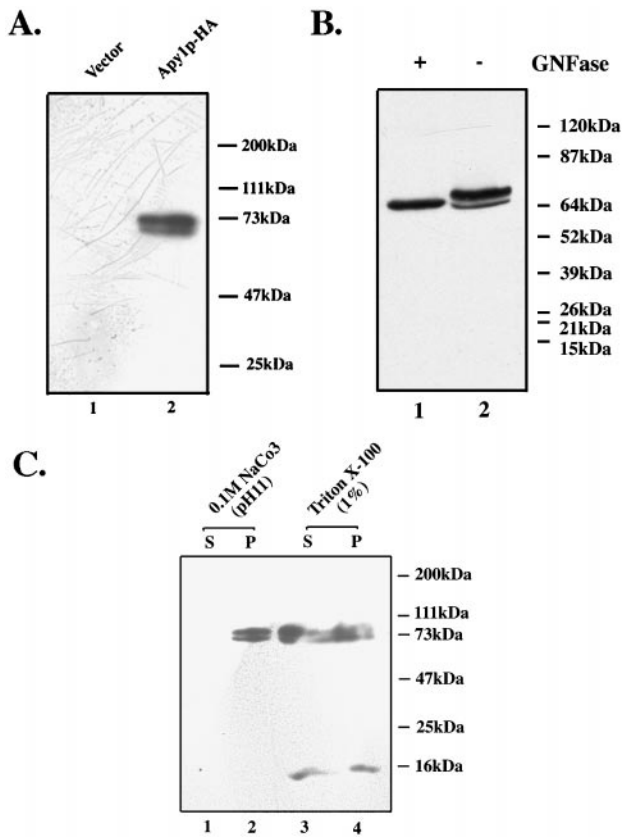

FIG. 2. **Expression and membrane association of Apy1p.** *Panel A*, expression of Apy1p. Crude membrane proteins isolated from BCY123/pG1 (*lane 1*) and BCY123/pGZ103 (*lane 2*) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. HA-tagged Apy1p protein was detected by immunoblotting 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 M 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 532–534.

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. 2*A*, 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-

dase F (Fig. 2*B*), a single band at 72 kDa (*lane 1*) was observed, showing that the 73-kDa protein band is the glycosylated form of Apy1p.

After the cells expressing *APY1-HA* were mechanically lysed as described under "Materials and Methods," 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. 2*C*), 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. 3*A*, 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. 3*B* 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 Ca^{2+}, Mn^{2+}, and Mg^{2+} (Fig. 3*C*). 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 Ca^{2+} is more effective than Mn^{2+} or Mg^{2+}.

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 sequential centrifugation at 1,000 × *g*, 13,000 × *g*, and 120,000 × *g*. Most of the ER, vacuolar membrane, and plasma membrane are found in the 13,000 × *g* pellet (40, 41), whereas the 120,000 × *g* pellet (P120) is a Golgi-enriched fraction, containing the majority of the Golgi enzymes α-1,3-mannosyltransferase (Mnn1p) (36, 42), guanosine diphosphatase (Gda1p) (17), and endopeptidase 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-HA, immunofluorescence experiments with monoclonal anti-HA antibody were done. Yeast cells containing *APY1-HA* on a 2-μm plasmid displayed a punctuated staining pattern scattered throughout the cytoplasm but excluded from the vacuole and nucleus, as judged by Nomarski optics and in double staining experiments with DAPI (Fig. 4, *D–F*). This signal was absent in control cells (Fig. 4, *A–C*). The staining pattern is typical of the yeast Golgi apparatus (44); it is also seem for endopeptidase Kex1p, dipeptidyl aminotransferase A, Ca^{+2}-ATPase Pmr1p, Mnn1p, Gda1p, and Kex2p (36, 45–49). Because the copy number of the 2-μm plasmid can vary between 10 and 40 copies per cell within a population (50), the number of distinct fluorescent spots corresponding to Apy1p-HA also varies from cell to cell, ranging from a weak signal to a very strong signal. All of the cells expressing Apy1p-HA displayed a Golgi staining pattern (Fig. 4*E*); however, cells exhibiting a large amount of the protein, presumably because of a high

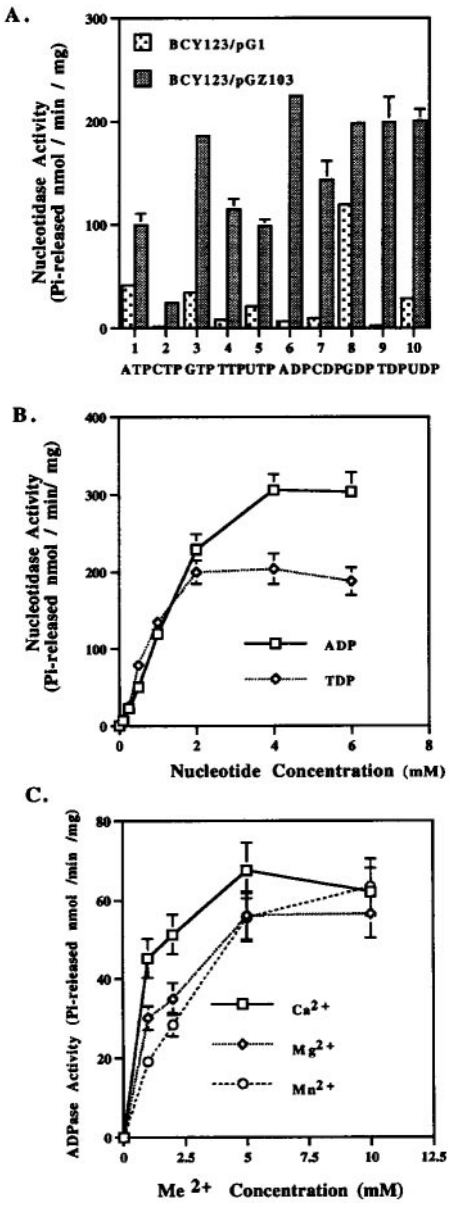

FIG. 3. **Enzymatic activity of Apy1p.** *Panel A*, Ca^{2+}-stimulated nucleotidase activities in the crude membranes of BCY123/pG1 (strain with the empty vector) or BCY123/pGZ103 (APY1-HA-expressing strain). Substrate specificity was determined with different nucleoside phosphates at a substrate concentration of 2 mM as described under "Materials and Methods." *Panel B*, nucleotidase activities with different concentrations of ADP and TDP (0.2–6 mM). Assays were carried out for 10 min. *Panel C*, effects of divalent cations on ADPase activity. ADPase activities in the presence of various concentrations of divalent ions ($CaCl_2$, $MgCl_2$, $MnCl_2$) were determined. All values are means ± S.D. ($n = 4$).

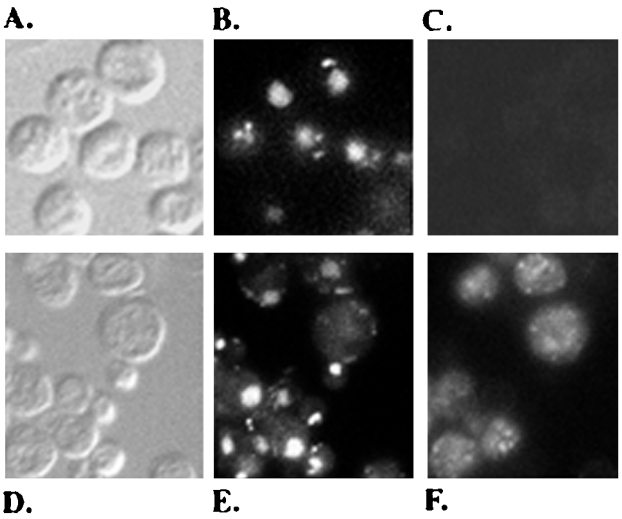

FIG. 4. **Subcellular localization of Apy1p by indirect immunofluorescence gives a punctate staining pattern typical of the yeast Golgi complex.** As described under "Materials and Methods," transformants with pG_1 (*panels A–C*) or *APY1-HA* expressing plasmid pGZ103 (*panels D–F*) were fixed, solubilized, and treated with affinity-purified anti-HA monoclonal antibodies. The cells were then reacted with fluorescein isothiocyanate-coupled anti-mouse antibody; in addition, cells were treated with DAPI. The appearance of cells as detected by differential interference contrast microscopy (DIC, *panels A* and *D*), DAPI fluorescence (*panels B* and *E*), and indirect immunofluorescence for HA (*panels C* and *F*) is shown.

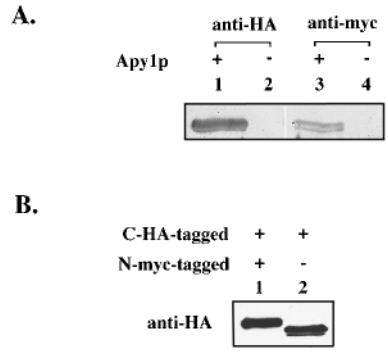

FIG. 5. **The NH_2 terminus of Apy1p is not cleaved during translocation.** *Panel A*, crude membranes from BCY123/pGZ105 (expressing NH_2-terminal myc-tagged and COOH-terminal HA-tagged Apy1p) were immunoblotted with anti-HA and anti-myc monoclonal antibodies, respectively. *Panel B*, comparison of the size of NH_2-terminal and COOH-terminal tagged Apy1p with that of COOH-terminal tagged Apy1p. Crude membranes of BCY123/pGZ103 (expressing COOH-terminal HA-tagged Apy1p) and BCY123/pGZ105 (expressing NH_2-terminal myc-tagged and COOH-terminal HA-tagged Apy1p) were immunoblotted with anti-HA antibodies.

dosage of the *APY1-HA* gene, also had a brightly stained vacuole (5–10% of cells of a *pep4-3* strain, data not shown). Thus, cells producing high levels of Apy1p-HA showed mislocalization of the protein to the vacuole, which has also been reported for other yeast Golgi enzymes (36, 48).

Membrane Arrangement of Apy1p—Hydrophobicity analysis using the Kyte and Doolittle algorithm (59) predicts two adjacent hydrophobic stretches in the polypeptide (amino acid residues 446–467 and 501–517), which can potentially serve as transmembrane domains. The NH_2-terminal 445-amino acid hydrophilic segment of Apy1p contains the apyrase domain. The apyrase domains of other E-ATPases are all located extracellularly, through either a cleaved (6, 8, 9) or a noncleaved signal sequence (5, 14, 17). Surprisingly, there is no signal sequence in the NH_2-terminal portion of Apy1p; in addition, this domain (445 amino acids) is much larger than the extracellular domain of known type III membrane proteins (<100 amino acids) (51). This finding raises the question as to whether the apyrase domain is located in the cytoplasm or in the lumen of the Golgi. Both possibilities are problematic: the apyrase domain would not be expected to be in the cytoplasm because of its nucleotidase activity; on the other hand, the presence of the large domain in the lumen of the Golgi in the absence of a signal sequence would be unprecedented. Accordingly, we set out to determine the membrane topology of Apy1p.

To determine whether the NH_2 terminus might have an irregular cleavable signal sequence, Apy1p was tagged with a myc epitope at the NH_2-terminal end and with a COOH-terminal HA tag. As is shown in Fig. 5*A*, Apy1p could be detected by both anti-HA and anti-myc antibodies, indicating that the NH_2-terminal domain of Apy1p is intact. The results in Fig. 5*B* further support the conclusion that no NH_2-terminal cleavage has occurred because addition of the myc tag composed of 10 amino acids to Apy1p-HA (myc-Apy1p-HA) increased the size of the protein by 1 kDa (Fig. 5*B*, *lane 1*).

To determine the location of the NH_2- and COOH-terminal ends of Apy1p, a protease digestion protection assay was used. Golgi membrane fractions containing myc-Apy1p-HA were isolated and subjected to proteinase K digestion as described under "Materials and Methods." As is shown in Fig. 6*A*, proteinase 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 NH_2 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 NH_2-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 NH_2- 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. 6*B*, the N371I mutation eliminated the formation of the 73-kDa glycosylated Apy1p (*lanes 1–4*), whereas the N532I mutation did not affect glycosylation (*lanes 5–8*). These results indicate that the NH_2-terminal domain is indeed in the lumen, that glycosylation of Asn-371 contributes to the formation of the 73-kDa form of Apy1p, and that Asn-532 is not utilized for glycosylation, which is consistent with the cytoplasmic location of the COOH-terminal domain. Introduction of a new glycosylation site (D619N) into the COOH-terminal end of the Apy1p N371I mutant did not result in a glycosylated form of Apy1p (data not shown), supporting the cytoplasmic location of this domain.

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 NH_2- 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

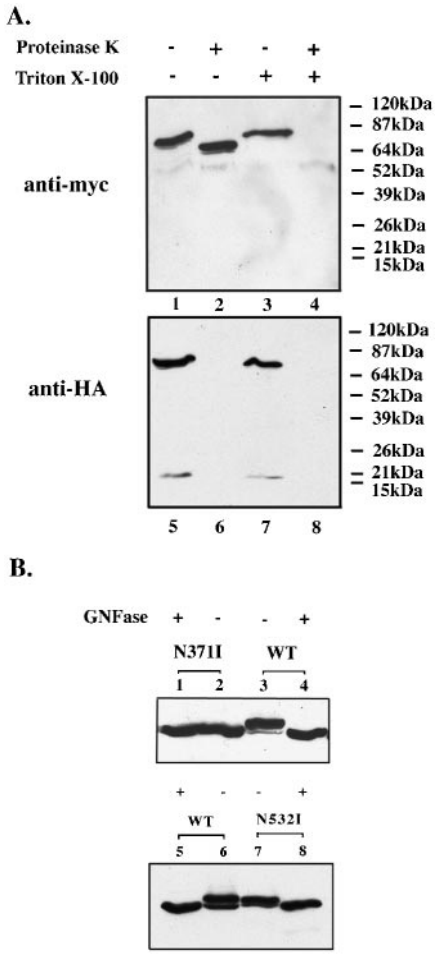

FIG. 6. **The NH_2-terminal domain of Apy1p is in the Golgi lumen, and its COOH-terminal domain binds to cytoplasmic face of the Golgi membranes.** *Panel A*, accessibility of Apy1p domains to proteinase K digestion. As described under "Materials and Methods," Golgi membrane fractions were digested by 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.

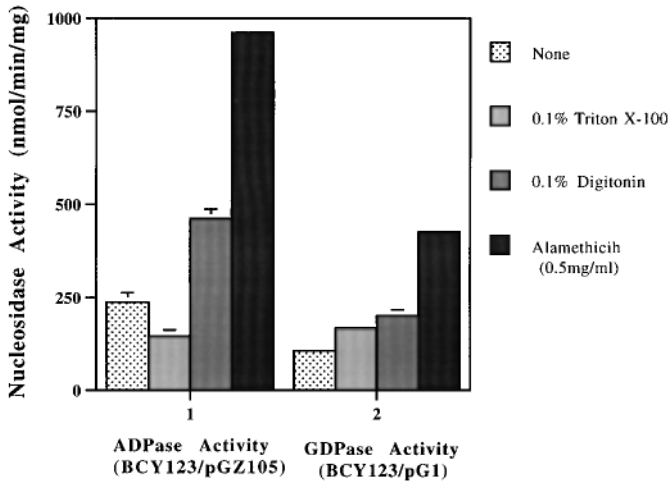

FIG. 7. **Nucleotidase activity of membranes before and after the addition of detergents and alamethicin to permeabilize the membrane.** The NDPase activity of crude membranes of BCY123/pGZ105 (APY1-HA-expressing strain) and BCY123/pG1 (strain with the empty vector) was measured in the absence or presence 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 ± S.D. (n = 4).

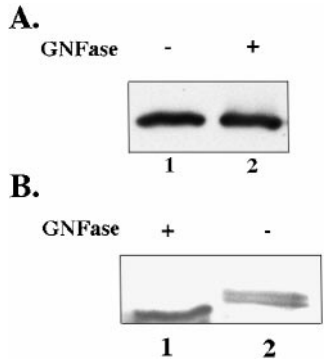

FIG. 8. **The second hydrophobic stretch of Apy1p is the transmembrane domain.** Crude membranes isolated from BCY123/pGZ118, expressing Apy1p with a deletion of the second hydrophobic segment and the COOH-terminal hydrophilic domain (*panel A*), and from BCY123/pGZ121, expressing Apy1p with a deletion 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*).

stretch is the real transmembrane domain, two COOH-terminal truncated versions of Apy1p were constructed. The Δ491–630 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 Δ552–630 mutant lacked the COOH-terminal 79 amino acids following the second hydrophobic segment. As is shown in Fig. 8*A*, Δ491–630 Apy1p was not glycosylated (*lanes 1* and *2*), indicating that the NH_2-terminal domain was not in the lumen. Fig. 8*B* shows that Δ552–630 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.

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 hydrolyzes 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

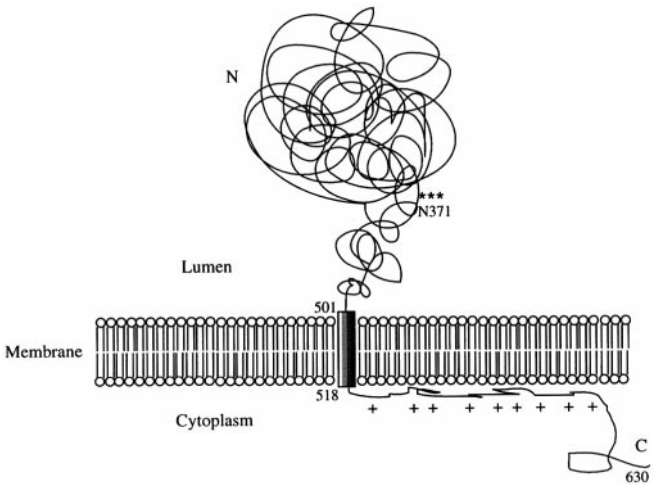

FIG. 9. **Proposed membrane topology of Apy1p.** The 500-amino acid long NH_2 terminus, with the glycosylation site at Asn-371, is in the lumen of the Golgi; the second hydrophobic segment is the transmembrane domain; and the COOH-terminal 113 amino acid residues are on the cytoplasmic surface of the Golgi membrane and appear to be attached to the membrane surface.

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.[2] Because the yeast GDP-mannose transporter null mutation is lethal, suggesting that glycosylation 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 enzymatic 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 antiport molecules for sugar nucleotide 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.

Apylp 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 deduced by examination of the primary structures of the enzymes.

The most surprising finding in this study is that a large NH_2-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 NH_2 terminus lacking a signal sequence are known. Some G protein-coupled receptors belong in this class, but their NH_2-terminal segment is less than 100 residues; above this size limit, an NH_2-terminal signal sequence is employed (51). It has been reported that Neu differentiation factor has a 240-amino acid extracellular NH_2 terminus and lacks a typical signal sequence, but the NH_2-terminal 13 amino acids are cleaved, suggesting the existence of some kind of NH_2-terminal signal (57). To our knowledge, Apy1p has the longest translocated NH_2 terminus reported so far. If translocation of the NH_2 terminus of Apy1p takes place by a new mechanism, Apy1p may be classified as a type V transmembrane protein. We predict that hypothetical protein C33H5.14 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 NH_2-terminal apyrase domain lacking a signal sequence and a single hydrophobic segment.

The mechanism for the translocation of the NH_2-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 NH_2 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 notice 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 demonstrate that Apy1p has a large translocated NH_2 terminus and an unusual membrane topology. Based on the distinct features of Apy1p, we propose that Apy1p and a hypothetical protein of *C. elegans* belong to new subgroup of transmembrane proteins.

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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., Koziak, 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,** 9898–9901
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.,

[2] X. Zhong and G. Guidotti, unpublished observations.

Birks, C., Aplert, A., Della, F., Gimple, S. D., and Gayle, R. B., III (1994) *J. Immunol.* **153,** 3574–3583
8. Hsieh, H. L., Tong, C. G., Thomas, C., and Roux, S. J. (1996) *Plant Mol. Biol.* **30,** 135–147
9. Bermudes, D., Peck, K. R., Afifi, M. A., Beckers, C. J., and Joiner, K. A. (1994) *J. Biol. Chem.* **269,** 29252–29260
10. Vasconcelos, E. G., Ferreira, S. T., de Carvalho, T., de Souza, W., Kettlun, A. M., Mancilla, M., Valenzuela, M. A., and Verjovski-Almeida, S. (1996) *J. Biol. Chem.* **271,** 22139- 22145
11. Wang, T. F., Rosenberg, P. A., and Guidotti, G. (1997) *Brain Res. Mol. Brain Res.* **47,** 295–302
12. Kirley, T. L. (1997) *J. Biol. Chem.* **272,** 1076–1081
13. Chadwick, B. P., and Frischauf, A. M. (1998) *Genomics* **50,** 357–367
14. Wang, T. F., and Guidotti, G. (1998) *J. Biol. Chem.* **273,** 11392–11399
15. Nagy, A. K., Knowles, A. F., and Nagami, G. T. (1998) *J. Biol. Chem.* **273,** 16043–16049
16. Smith, T. M., and Kirley, T. L. (1998) *Biochim. Biophys. Acta* **1386,** 65–78
17. Abeijon, C., Yanagisawa, K., Mandon, E. C., Hausler, A., Moremen, K., Hirschberg, C. B., and Robbins, P. W. (1993) *J. Cell Biol.* **122,** 307–323
18. Chadwick, B. P., and Frischauf, A. M. (1997) *Mamm. Genome* **8,** 668–672
19. Hegde, R. S., and Lingappa, V. R. (1997) *Cell* **91,** 575–582
20. Powers, T., and Walter, P. (1997) *Science* **278,** 2072–2073
21. Siegel, V. (1997) *Cell* **90,** 5–8
22. Harley, C. A., Holt, J. A., Turner, R., and Tipper, D. J. (1998) *J. Biol. Chem.* **273,** 24963–24971
23. von Heijne, G., and Gavel, Y. (1988) *Eur. J. Biochem.* **174,** 671–678
24. Kutay, U. H., E., and Rapoport, T. A. (1993) *Trends Cell Biol.* **3,** 72–75
25. Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B., and Rapoport, T. A. (1995) *EMBO J.* **14,** 217–223
26. Abeijon, C., Mandon, E. C., and Hirschberg, C. B. (1997) *Trends Biochem. Sci.* **22,** 203–207
27. Hirschberg, C. B., Robbins, P. W., and Abeijon, C. (1998) *Annu. Rev. Biochem.* **67,** 49–69
28. Berninsone, P., Miret, J. J., and Hirschberg, C. B. (1994) *J. Biol. Chem.* **269,** 207–211
29. Kane, S. M., and Roth, R. (1974) *J. Bacteriol.* **118,** 8–14
30. Sherman, F. (1991) *Methods Enzymol.* **194,** 3–21
31. Miller, J. H. (1992) *A Short Course in Bacterial Genetics*, pp. 439–440, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Piper, P. (1996) *Methods Mol.Biol.* **53,** 103–107
34. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
35. Schena, M., Picard, D., and Yamamoto, K. R. (1991) *Methods Enzymol.* **194,** 389–398
36. Graham, T. R., Seeger, M., Payne, G. S., MacKay, V. L., and Emr, S. D. (1994) *J. Cell Biol.* **127,** 667–678
37. Ames, B. N. (1966) *Method Enzymol.* **8,** 115–117
38. Tarentino, A. L., Gomez, C. M., and Plummer, T., Jr. (1985) *Biochemistry* **24,** 4665–4671
39. Pringle, J. R., Adams, A. E., Drubin, D. G., and Haarer, B. K. (1991) *Methods Enzymol.* **194,** 565–602
40. Herman, P. K., Stack, J. H., DeModena, J. A., and Emr, S. D. (1991) *Cell* **64,** 425–437
41. Hurt, E. C., McDowall, A., and Schimmang, T. (1988) *Eur. J. Cell Biol.* **46,** 554–563
42. Graham, T. R., and Krasnov, V. A. (1995) *Mol. Biol. Cell* **6,** 809–824
43. Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984) *Cell* **37,** 1075–1089
44. Chapman, R. E., and Munro, S. (1994) *EMBO J.* **13,** 4896–4907
45. Antebi, A., and Fink, G. R. (1992) *Mol. Biol. Cell* **3,** 633–654
46. Cooper, A., and Bussey, H. (1989) *Mol. Cell. Biol.* **9,** 2706–2714
47. Redding, K., Holcomb, C., and Fuller, R. S. (1991) *J. Cell Biol.* **113,** 527–538
48. Roberts, C. J., Nothwehr, S. F., and Stevens, T. H. (1992) *J. Cell Biol.* **119,** 69–83
49. Vowels, J. J., and Payne, G. S. (1998) *Mol. Biol. Cell* **9,** 1351–1365
50. Rose, M. D., and Broach, J. R. (1991) *Methods Enzymol.* **194,** 195–230
51. Wallin, E., and von Heijne, G. (1995) *Protein Eng.* **8,** 693–698
52. Dean, N., Zhang, Y. B., and Poster, J. B. (1997) *J. Biol. Chem.* **272,** 31908–31914
53. Capasso, J. M., Keenan, T. W., Abeijon, C., and Hirschberg, C. B. (1989) *J. Biol. Chem.* **264,** 5233–5240
54. Thomas, C., Sun, Y., Naus, K., Lloyd, A., and Roux, S. (1999) *Plant Physiol.* **119,** 543–552
55. Smith, T. M., and Kirley, T. L. (1999) *Biochemistry* **38,** 321–328
56. Heine, P., Braun, N., Heilbronn, A., and Zimmerman, H. (1999) *Eur. J. Biochem.* **262**, 102–107
57. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. (1992) *Cell* **69,** 559–572
58. Hartmann, E., Rapoport, T. A., and Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5786–5790
59. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132

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

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