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PMR1, a Ca^{2+}-ATPase in Yeast Golgi, Has Properties Distinct from Sarco/endoplasmic Reticulum and Plasma Membrane Calcium Pumps*

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PMR1, a P-type ATPase cloned from the yeast *Saccharomyces cerevisiae*, was previously localized to the Golgi, and shown to be required for normal secretory processes (Antebi, A., and Fink, G.R. (1992) *Mol. Biol. Cell* 3, 633–654). We provide biochemical evidence that PMR1 is a Ca^{2+}-transporting ATPase in the Golgi, a hitherto unusual location for a Ca^{2+} pump. As a starting point for structure-function analysis using a mutagenic approach, we used the strong and inducible heat shock promoter to direct high level expression of PMR1 from a multicopy plasmid. Yeast lysates were separated on sucrose density gradients, and fractions assayed for organellar markers. PMR1 is found in fractions containing the Golgi marker guanosine diphosphatase, and is associated with an ATP-dependent, protonophore-insensitive $^{45}Ca^{2+}$ uptake activity. This activity is virtually abolished in the absence of the expression plasmid. Furthermore, replacement of the active site aspartate within the phosphorylation domain had the expected effect of abolishing Ca^{2+} transport activity entirely. Interestingly, the mutant enzymes (Asp-371 → Glu and Asp-371 → Asn) demonstrated proper targeting to the Golgi, unlike analogous mutations in the related yeast H^+-ATPase. Detailed characterization of calcium transport by PMR1 showed that sensitivity to inhibitors (vanadate, thapsigargin, and cyclopiazonic acid) and affinity for substrates (MgATP and Ca^{2+}) were different from the previously characterized sarco/endoplasmic reticulum and plasma membrane Ca^{2+}-ATPases. PMR1 therefore represents a new and distinct P-type Ca^{2+}-ATPase. Because close homologs of PMR1 have been cloned from rat and other organisms, we suggest that Ca^{2+}-ATPases in the Golgi will form a discrete subgroup that are important for functioning of the secretory pathway.

In eukaryotic cells, the vast bulk of cellular calcium is sequestered within intracellular calcium stores, which maintain cytoplasmic calcium ion concentrations at submicromolar levels and release calcium in response to physiological signals. A major intracellular calcium pool is the endoplasmic reticulum, which is well known for its prominent role in inositol 1,4,5-trisphosphate- and caffeine-mediated calcium release (2). Filling of this store is accomplished by a thapsigargin-sensitive Ca^{2+}-ATPase, a member of the ubiquitous family of P-type ion pumps, and best characterized by the isoform in skeletal muscle sarcoplasmic reticulum (SERCA1[1]; Refs. 3 and 4). There are data supporting the existence of another ionomycin-sensitive pool of intracellular calcium that is unresponsive to both inositol 1,4,5-trisphosphate and caffeine, is non-mitochondrial, and appears to be loaded by a thapsigargin-insensitive Ca^{2+}-ATPase (5). One candidate organelle that may correspond to this pool is the Golgi; subcellular imaging of calcium using the techniques of electron probe x-ray analysis (6) and ion microscopy (7) has revealed that the Golgi apparatus sequesters calcium at concentrations of 1–2 mM, accounting for about 5% of the total intracellular calcium stores. This finding is consistent with numerous reports of an ATP-dependent Ca^{2+} transport activity in isolated Golgi-rich membrane fractions from tissues such as rat liver (8) and mammary gland (9), and from yeast (10).

Evidence for the role of the Golgi apparatus in regulation of cytoplasmic Ca^{2+} is scarce; uptake of Ca^{2+} into the Golgi following a mitogen-induced Ca^{2+} transient has been demonstrated in NIH3T3 fibroblasts (11), and in LLC-PK_1 porcine kidney cells, there is a loss of Golgi calcium following treatment with arginine-vasopressin (12). There is considerable evidence, however, for the importance of Golgi calcium in the exocytic or secretory pathway. For example, calcium depletion inhibits proteolytic processing of precursors in the Golgi/TGN by inhibiting Ca^{2+} dependent endoproteases (13). In fact, many resident Golgi enzymes require concentrations of Ca^{2+} in the millimolar range for activity, including KEX2 endoprotease and guanosine diphosphatase (14, 15) in yeast. In mammalian cells, Ca^{2+}-dependent aggregation of chromogranins and secretogranins in the low pH and Ca^{2+}-rich environment of the TGN plays a major role in formation of secretory granules (16). Finally, calcium accumulation in the Golgi has been shown to be of particular importance in tissues involved in calcium secretion, such as lactating mammary gland (17), intestine (18–20), and tooth-forming odontoblasts (21).

The isolation of a gene encoding the putative Golgi Ca^{2+}-ATPase, *PMR1*, in the yeast *Saccharomyces cerevisiae* (22) allowed a genetic approach toward understanding the cellular role of Golgi calcium; strains carrying a null allele of *PMR1* manifest a glycosylation defect, secrete unprocessed α factor, and suppress various *sec* mutants blocked in endoplasmic reticulum/Golgi and post-Golgi transport, consistent with disruptions of Golgi function (1). These phenotypes can be reversed by

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[1] The abbreviations used are: SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; TGN, trans-Golgi network; PMCA, plasma membrane; Ca^{2+}-ATPase.

addition of high Ca^{2+} (10 mM) to the medium, implicating a direct role for calcium in Golgi function. An epitope-tagged version of PMR1 was localized to Golgi both by subcellular fractionation and immunofluorescence staining of cells (1).

The deduced amino acid sequence of PMR1 carries all the hallmarks of the P-type ion pumps. There are 10 conserved stretches of sequence, including the region around the aspartate residue that forms the phosphorylated reaction intermediate, and regions involved in ATP binding and hydrolysis that are common to all members of this family. The predicted topology of PMR1 is that of the major P2 subgroup of P-ATPases (23), with 8–10 transmembrane spans, cytoplasmic N and C termini, and two defined cytosolic domains linking transmembrane segments 2 and 3, and 4 and 5. Notably, the full-length sequence of PMR1 does not bear a striking homology to either the sarco/endoplasmic reticulum (SERCA) or the plasma membrane (PMCA) Ca^{2+}-ATPases, both well characterized and distinctly different members of the P-ATPase family. However, a closer examination of PMR1 in the region of transmembrane segments 5 and 6, which have been implicated in calcium transport by mutagenesis studies on SERCA and PMCA (24, 25), reveals a significant similarity to Ca^{2+}-ATPases (22). A close homolog of PMR1 from rat has been cloned (26), and named SPCA (secretory pathway Ca^{2+}-ATPase) for its putative functional equivalence to PMR1. The sequence similarity suggests that these two new genes may form a distinct subgroup important in the functioning of the secretory pathway.

In this work, we provide the first biochemical demonstration that PMR1 is indeed a Golgi-specific Ca^{2+} pump, and show that its kinetic properties and inhibitor-sensitivities are distinct from both SERCA and PMCA, justifying its inclusion in a new subgroup of Ca^{2+}-ATPases. Furthermore, we describe a high level expression system, with low background Ca^{2+} transport activity, for future structure-function analysis of PMR1. This expression system is also suitable for the study of heterologous Ca^{2+} pumps.

EXPERIMENTAL PROCEDURES

Media, Strains, Plasmids, and Mutagenesis—Culture media contained yeast nitrogen base (Difco; 6.7 g/liter), dextrose (2%), and supplements as needed. All yeast strains used in this study were a generous gift from Kyle Cunningham (Johns Hopkins University) and have been described previously (27). YEpHR1 is a yeast 2μ plasmid carrying the PMR1 coding sequence under control of a tandem repeat of a yeast heat shock element (28), which was derived from plasmid YCp2HSE (29) as follows. The *Sal*I to *Sac*I fragment containing the heat shock elements and CYC1 upstream sequences from plasmid YCp2HSE was cloned into plasmid YEplac195 (30) from which the *Hin*dIII site had been destroyed by filling in. An internal *Hin*dIII site in the PMR1 coding sequence was removed by site-directed mutagenesis (see below), without change in amino acid sequence; this allowed cloning of the intact 3.6-kb *Hin*dIII fragment encoding PMR1 into the corresponding site within the *Sal*I-*Sac*I fragment in YEplac195 to give the expression plasmid YEpHR1.

Oligonucleotide-directed mutagenesis was by the polymerase chain reaction, using the megaprimer method (31) as modified (32). The following oligonucleotides were used (5′ → 3′, base substitutions underlined): *Hin*dIII site (GATAAAACGGACAGCTTTGGATC), Asp-371 → Glu (CTGCTCCGAAAAAACAGGTAC), Asp-371 → Asn (GTTATCTGCTCCAACAAAACA). In each case, the subcloned PCR fragment was entirely sequenced to rule out unwanted mutations. Reconstruction of the intact *PMR1* gene in YEpHR1 was by standard cloning techniques.

Isolation of Total Membranes—Yeast cells were resuspended in Bead buffer (10 mM Tris/HCl, pH 7.4, 0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$) containing protease inhibitors (1 mM diisopropyl fluorophosphate, 2 μg/ml chymostatin, and 1 μg/ml each leupeptin, pepstatin, and aprotinin; all from Sigma) at a density of 200 A_{600} units/ml. Acid-washed glass beads (Sigma) were added to the meniscus and cells were lysed on a vortex in three 1-min pulses, with intermittent chilling on ice. Lysates were centrifuged in a table-top centrifuge (Sorvall, RT6000B) at 800 rpm for 5 min, the supernatants carefully withdrawn and centrifuged for 1 h at 45,000 rpm (Beckman, Ti50). Membrane pellets were resuspended in 20 mM K-Hepes, pH 7.4, with protease inhibitors.

Subcellular Fractionation and Isolation of Golgi—Fractionation of yeast lysates by sucrose gradient centrifugation was performed essentially as described by Antebi and Fink (1), with some modifications. Strain K616 transformed with plasmid YEpHR1 (K616/YEpHR1) was grown in 600-ml cultures at 25 °C to A_{600} of 0.5–0.8 prior to a 2-h shift to 37 °C for heat-shock induced expression of PMR1. Before harvest, NaN_3 was added to 10 mM and the culture was chilled on ice. Cells were converted to spheroplasts using yeast lytic enzyme (10 mg/1600 OD units; ICN) for 45 min at 37 °C. Lysis buffer contained 0.3 M sorbitol, 20 mM triethanolamine acetate, pH 7.2, 1 mM EDTA, and protease inhibitors (above). The spheroplasts were suspended in 3–6 ml of lysis buffer and homogenized using 30 strokes of a Wheaton A Dounce homogenizer. Following two short centrifugations (3 min at 2700 rpm in a Sorvall SS34 rotor) to remove unlysed cells, the lysate (3 ml) was layered on top of a 10-step gradient containing 3 ml each of sucrose (w/w; 18–54% in 4% increments) in 10 mM Hepes, pH 7.5, 1 mM $MgCl_2$. Gradients were spun for 2 h at 27,000 rpm in a Beckman SW28 rotor at 4 °C. Gradients were fractionated from top to bottom, manually, in 3-ml aliquots (fractions 1–11, respectively). For collection of Golgi membranes, fractions containing 26, 30, and 34% sucrose were pooled, diluted to 0.8 M sucrose by addition of 10 mM Hepes, pH 7.5, 1 mM $MgCl_2$, and centrifuged for 1 h as before. The pellet was resuspended in 0.5 M sucrose, 10 mM Mes/NaOH, pH 6.0, 0.15 M KCl containing protease inhibitors (above) and stored at −70 °C in aliquots.

Protein Methods and Enzyme Assays—Protein concentration was determined by a modified Lowry assay (33) following precipitation of samples containing sucrose by 10% trichloroacetic acid, and using bovine serum albumin as a standard. Guanosine diphosphatase was assayed according to Abeijon *et al.* (15); free P_i was measured by the method of Fiske and Subbarow (34). α-Mannosidase activity was measured using 4-methylumbelliferyl-α-D-mannopyranoside (Sigma) as a substrate (35). NADPH-cytochrome *c* reductase was assayed as described by Feldman *et al.* (36).

$^{45}Ca^{2+}$ Transport Assays—Calcium transport in sucrose gradient fractions was assayed in O-Buffer containing 10 mM Hepes/NaOH, pH 6.7, 0.15 M KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 5 mM NaN_3, and 10 μM CCCP. $^{45}CaCl_2$ (10 Ci/g; ICN) was added to 0.4 μCi/ml. Fractions (0.3 ml) were incubated for 12 min in 1 ml of assay mix at room temperature. Samples were collected by rapid filtration through Millipore 0.45-μm HAWP filters and washed twice with 5 ml of 10 mM Hepes, pH 7.5, 0.15 M KCl. Filters were dissolved in 1 ml of dimethylformamide and radioactivity determined by liquid scintillation counting with 10 ml of Aquasol (DuPont). Pooled Golgi membranes were incubated for 1–2 min at room temperature, in a volume of 100 μl and at a final protein concentration of 100 μg/ml. In studies involving inhibitors (vanadate, lanthanum, and cyclopiazonic acid), the "O-Buffer" mix was used. In other assays, the mix contained in addition, 10 μM non-radioactive $CaCl_2$ and 10 μM EGTA (to give a final free Ca^{2+} concentration of 4.9 μM, as determined by the computer program of Fabiato and Fabiato; (37)), and $^{45}CaCl_2$ was at 2 μCi/ml. Concanamycin A (Sigma; 10 nM) and CCCP (25 μM) were included in titrations of calcium and ATP, to completely eliminate activity of the vacuolar H^+/Ca^{2+} exchanger. In experiments involving variations of pH, calcium concentration was held constant at 10 μM and EGTA was omitted due to the marked effect of pH on the buffering capacity of EGTA.

SDS-PAGE, Antibodies, and Western Blotting Methods—SDS-PAGE gels and Western blotting was performed as described previously (29). Samples (5–100 μg of protein) were precipitated by addition of trichloroacetic acid to 10% (v/v) and centrifuged for 20 min in an Eppendorf microcentrifuge (4 °C). Pellets were resuspended in sample buffer prior to loading on the gels. A C-terminal fusion of PMR1 to *E. coli* TrpE (pL144; Ref. 1) was used to generate polyclonal antisera from rabbit (Pocono Rabbit Farms, PA). A partial purification of IgG from the antiserum was obtained by $(NH_4)_2SO_4$ precipitation, followed by dialysis against phosphate-buffered saline (38). Antibodies were used at a dilution of 1:200. Antibodies against the plasma membrane H^+-ATPase were obtained from Carolyn Slayman (Yale University).

RESULTS

High Level Expression of PMR1 by Heat Shock Induction from Multicopy Plasmids in Yeast

Polyclonal antibodies were raised in rabbit against a fusion protein containing the C-terminal third of PMR1 expressed in-frame with the bacterial TrpE protein (see "Experimental Procedures" and Ref. 1). Immunoblotting of a total membrane

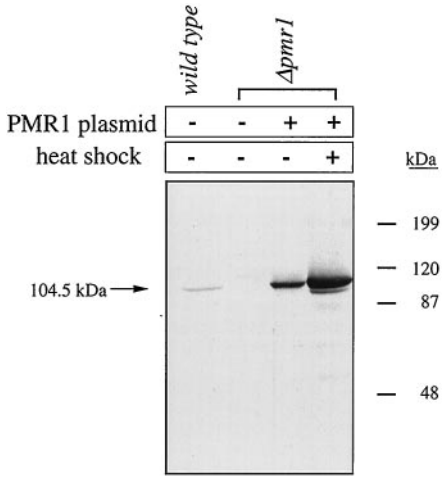

FIG. 1. **Western blot of yeast total membranes with anti-PMR1 antibody.** Membranes (100 μg), isolated from wild-type (*leftmost lane*) or *pmr1* null mutants (*right three lanes*), were probed with anti-PMR1 antibody as described under "Experimental Procedures." Expression of PMR1 from plasmid YEpHR1 was assessed in the Δ*pmr1* host strain K616 (*right two lanes*), in the presence or absence of heat shock, as indicated.

fraction from wild type yeast with anti-PMR1 antibody revealed a single, faint band of 104.5 kDa, which was missing from the isogenic strain carrying a chromosomal disruption of the *PMR1* gene (Fig. 1, *lanes 1* and *2*). The mobility of this band matches the molecular weight of PMR1 predicted from its amino acid sequence (22). The low levels of chromosomal expression of PMR1 necessitated a strategy of overexpression to facilitate biochemical characterization of the pump. Initially, the strong and constitutive *PMA1* promoter (39) was used to direct expression of PMR1 from a yeast multicopy (2μ) plasmid. However, introduction of this construct into yeast was lethal, although the equivalent single-copy construct (CEN) was tolerated. This suggested that constitutive, high level expression of PMR1 is toxic to yeast. We therefore chose to use the transient and inducible heat shock promoter described in earlier work with the *PMA1* gene (29). In addition, the heat shock element is a weaker promoter relative to that of *PMA1*, based on the activity of β-galactosidase fusions (28, 64). A construct of *PMR1* under control of a tandem repeat of the yeast heat shock promoter on a multicopy plasmid (YEpHR1; see "Experimental Procedures") gave some "leak-through" expression of PMR1 even in the absence of heat shock (Fig. 1, *lane 3*), however, no adverse effect on cell growth was apparent. A further, large amplification of PMR1 expression was observed following a 2-h heat shock of the cells at 37 °C (Fig. 1, *lane 4*).

To unambiguously demonstrate Ca^{2+} pump activity associated with PMR1 expression, and to pave the way for future analysis of recombinant pumps, plasmid YEpHR1 was introduced into the yeast strain K616 which carries a deletion of the chromosomal *PMR1* gene (27). Additionally, K616 carries a deletion of *PMC1*, encoding a second Ca^{2+}-ATPase (27), resulting in virtual elimination of background levels of Ca^{2+} pump activity in all fractions of the yeast lysate (see below and Fig. 2). Finally, it was necessary to disrupt *CNB1*, the gene encoding the regulatory subunit of calcineurin, a Ca^{2+}-, calmodulin-activated protein phosphatase (40), to maintain viability of the double deletion of *PMR1* and *PMC1* (27).

Golgi Localization and Ca^{2+} Transport Activity of PMR1

Following induction of PMR1 expression by a 2-h heat shock of strain K616 transformed with plasmid YEpHR1 (K616/YEpHR1), yeast lysates were fractionated on sucrose density gradients, and individual fractions were assayed for the distribution of various organellar markers as described under "Experimental Procedures." The data, summarized in Fig. 2, show an optimal separation of Golgi membranes from vacuoles, endoplasmic reticulum, and plasma membranes. The bulk of the total protein, representing components of the cytosol, remained in the loading fraction on top of the gradient, while discrete bands of membranes were recovered from the interfaces of the sucrose steps within the gradient. Nearly all (76%) of the total units of activity of the vacuolar marker, α-mannosidase, was recovered from the top four fractions of the gradient; in contrast, markers for the endoplasmic reticulum (NADPH-cytochrome *c* reductase) and plasma membrane (PMA1) were distributed in the denser half of the gradient, peaking near the bottom.

Both ^{45}Ca transport activity and PMR1 expression coincided with guanosine diphosphatase activity, a marker for the Golgi membranes (15). This confirms earlier immunological data on the localization of an hemagglutinin epitope-tagged PMR1 by Antebi and Fink (1), and demonstrates ATP-dependent Ca^{2+} transport into yeast Golgi vesicles. Finally, in the absence of the PMR1 expression plasmid, ATP-dependent ^{45}Ca uptake was virtually eliminated from strain K616 (Fig. 2*B*), providing direct evidence for calcium transport by PMR1.

Fractions showing maximal ^{45}Ca transport activity (fractions 4–6; corresponding to 26–34% sucrose) were pooled and the membranes collected by centrifugation. The pooled membranes were considerably enriched for PMR1 (Fig. 2*C*, *lanes 1* and *2*) and were used for further characterization of Ca^{2+} transport activity. Yields of pooled Golgi membranes averaged 1 mg from a small-scale culture of 600 ml (480–600 OD_{600} units).

Calcium Transport by PMR1 into Isolated Golgi Vesicles Is ATP-dependent and Protonophore-insensitive

^{45}Ca transport by the pooled Golgi membranes (100 μg of protein/ml) was time-dependent and saturable, with half-maximal uptake occurring at 1 min (Fig. 3). Initial rates of uptake under the conditions of this assay were >1 nmol/min/mg with maximal levels approaching 2 nmol/mg in 20 min. This suggests that uptake is rapidly limited by a build up of free Ca^{2+} within the vesicles, since most of this calcium (90%) is readily released by addition of the calcium ionophore A23187 (10 μM). Unlike vesicles derived from sarcoplasmic reticulum, no enhancement of uptake was observed following addition of either oxalate or phosphate (10 mM), possibly reflecting a lack of an anion transporter in these membranes. In the absence of ATP, an apparent uptake of 10–15% of maximal levels was observed (Fig. 3); however, there was no effect of the enzyme apyrase (which would degrade endogenous ATP), and no effect of the protonophore CCCP (which would collapse a pH gradient and abolish H^+/Ca^{2+} exchange). Furthermore, addition of the calcium ionophore elicited only a small (15% after 5 min) decrease in ^{45}Ca associated with the membranes. These results suggest that in the absence of ATP, there is a largely nonspecific association of ^{45}Ca with the membrane surface.

Inclusion of the protonophore CCCP (10 μM) in the transport buffer eliminated up to 20% of the total ATP-dependent Ca^{2+} transport activity. CCCP-sensitive $^{45}Ca^{2+}$ uptake was likely due to VCX1, a vacuolar H^+/Ca^{2+} exchanger (41), as it was also abolished by bafilomycin A (100 nM), a specific inhibitor of the vacuolar H^+-ATPase. In the absence of the PMR1 expression plasmid, all ^{45}Ca transport activity observed in the K616 mutant strain was CCCP-sensitive (not shown); the protonophore was therefore included in all the transport assays reported in this study.

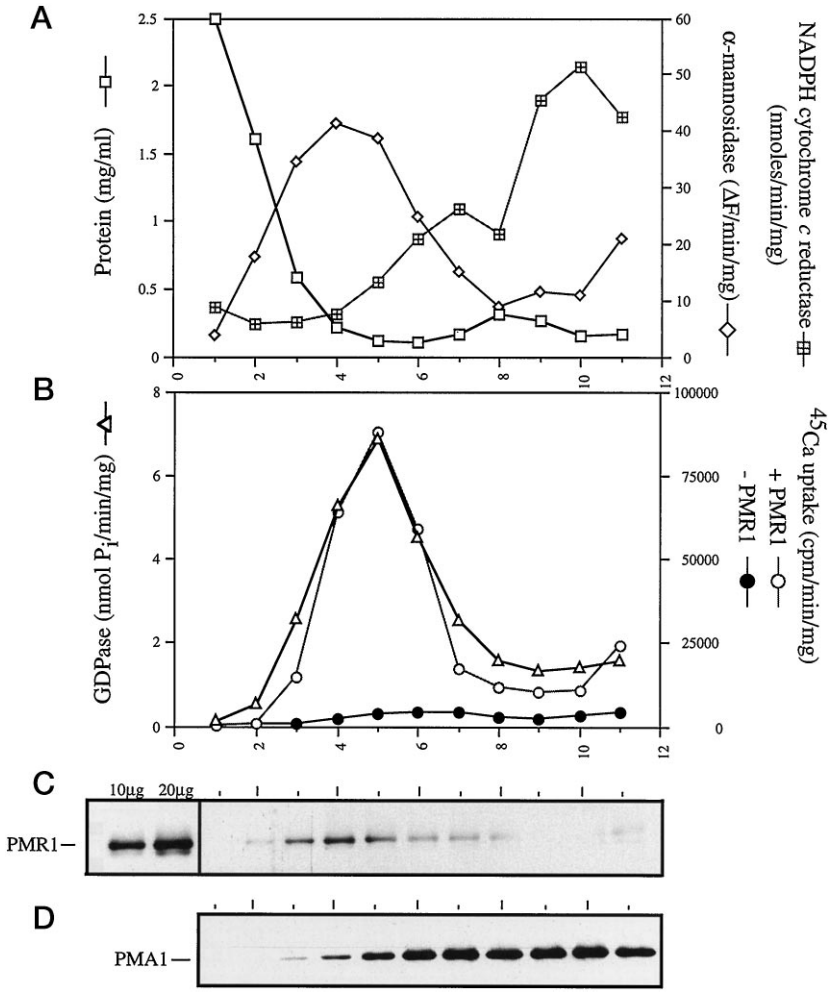

FIG. 2. **Fractionation of yeast lysates by sucrose density gradient centrifugation.** Yeast lysates prepared from strain K616/YEpHR1 were fractionated and assayed as described under "Experimental Procedures." *Panel A*, distribution of total protein, and markers for the vacuole (α-mannosidase) and endoplasmic reticulum (NADPH-cytochrome *c* reductase) from the top (fraction 1) to the bottom (fraction 11) of the gradient. Δ*F*, fluorescence in arbitrary units. *Panel B*, distribution of the Golgi marker (guanosine diphosphatase, *GDPase*), and ^{45}Ca uptake in the presence or absence of the PMR1 expression plasmid, as indicated. *Panel C*, Western blot probed with anti-PMR1 antibody showing (from *left* to *right*) 10 and 20 μg of the pooled Golgi membranes, as described in the text, followed by 10 μg of gradient fractions 1–11, as above. *Panel D*, Western blot probed with anti-PMA1 antibody, of 20 μg of gradient fractions 1 through 11, showing distribution of the plasma membrane H^+-ATPase.

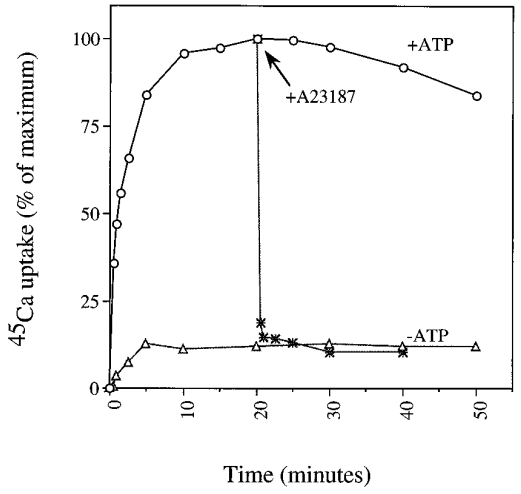

FIG. 3. **Time course of ^{45}Ca uptake in pooled Golgi vesicles.** Assay of ^{45}Ca uptake in the presence or absence of ATP, is described under "Experimental Procedures." Single data points are shown. Where indicated, calcium ionophore A23187 (10 μM) was added to an aliquot of the assay, and uptake monitored for an additional 20 min. Maximal uptake in the presence of ATP was 2 nmol/mg, and the initial rate was estimated to be ≥1 nmol/min/mg.

Inhibitor Sensitivity of PMR1 Is Distinct from That of Plasma Membrane or Endoplasmic Reticulum Ca^{2+}-ATPases

Vanadate is a characteristic inhibitor of P-type ATPases and is believed to mimic the transitional state of inorganic phosphate in the E2 conformation of the enzyme. However, sensitivity to vanadate varies between different P-ATPases, with K_i ranging from 0.6 μM for the plasma membrane Ca^{2+}-ATPase from erythrocytes (42) to about 50–300 μM for different isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPase (43, 44). Sensitivity of PMR1 to vanadate more closely resembled the intracellular Ca^{2+} pumps, with a K_i of 130 μM (Fig. 4*A*). Lanthanum, a calcium channel blocker, inhibited Ca^{2+} transport with half-maximal inhibition occurring at 55 μM (not shown). The loss of 80% activity within one order of magnitude (10–100 μM) suggests cooperative binding of the lanthanum ion to the ATPase.

Thapsigargin, a plant sesquiterpene lactone, which has been characterized as a potent and specific inhibitor of the SERCA family of Ca^{2+} pumps (45), had no effect on PMR1 activity at concentrations of up to 5 μM. Similarly, the mycotoxin cyclopiazonic acid inhibited ^{45}Ca transport with poor affinity (K_i of 200 μM) relative to its potent effect on SERCA pumps (K_i of 10–20 nM; Ref. 46).

Characterization of ^{45}Ca Transport by PMR1

pH Dependence—Golgi vesicles, resuspended in 10 mM Mes/Tris buffer at pH 6.0 (containing 0.5 M sucrose, 0.15 M KCl for osmotic stability), were assayed for ^{45}Ca uptake in transport buffer ranging from pH 5 to 9, as described under "Experimental Procedures." Fig. 4*B* shows a distinct pH optimum of 6.7, similar to the pH optimum of the SERCA1 and SERCA2 isoforms (44).

Affinity for Calcium—Free calcium was adjusted by buffering with EGTA as described by Fabiato and Fabiato (37), and

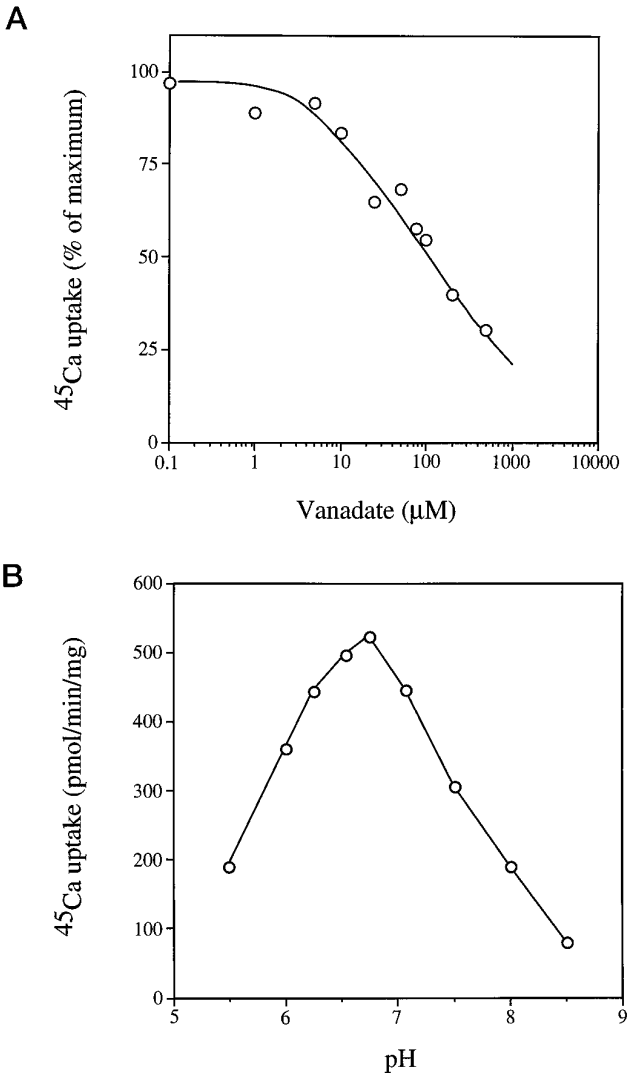

FIG. 4. **Characteristics of ^{45}Ca transport activity by PMR1.** *A*, Inhibition of PMR1 by vanadate. *B*, pH optimum of ^{45}Ca transport into Golgi vesicles. ^{45}Ca uptake in pooled Golgi vesicles (0.1 mg/ml) was assayed for 2 min as described under "Experimental Procedures." Average of duplicates is shown, variance was less than 5%. Control uptake, in the absence of vanadate, was 702 pmol/2 min/mg. pH was varied in Mes/Tris buffers containing a fixed concentration of total calcium (10 μM), and EGTA was omitted.

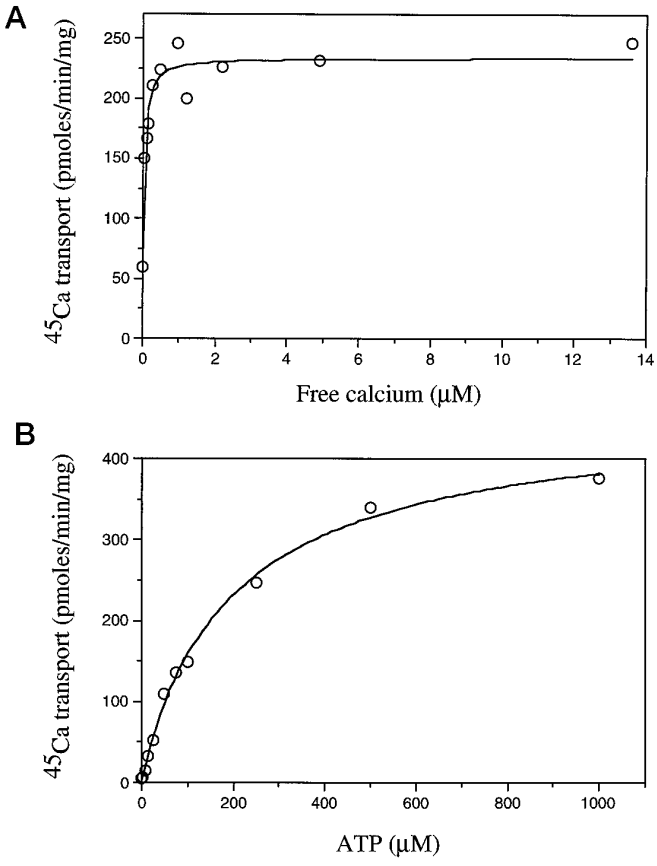

FIG. 5. **Kinetics of ^{45}Ca transport into Golgi vesicles by PMR1.** *A* and *B*, calcium dependence (*A*) and ATP dependence (*B*) of ^{45}Ca transport into Golgi vesicles. Calcium-EGTA mixtures were used to buffer free calcium concentration, according to the program described by Fabiato and Fabiato (37). MgATP (1:1) was varied in the transport buffer described in the text, which contained 5 mM $MgCl_2$. Average of duplicates is shown, data represent one of three similar experiments. The data in *A* were fit to the equation $v = V_{max} \times S/(K_m + S)$, and in *B*, to the equation $v = [V_{max1} \times S/(K_M1 + S)] + [V_{max2} \times S/(K_M2 + S)]$ using Kaleidagraph (version 3).

the corresponding initial velocity of ^{45}Ca transport was fitted as a function of a single site, with an affinity of ≤0.1 μM, shown in Fig. 5*A*. In this respect, PMR1 resembles the plasma membrane Ca^{2+}-ATPase of sarcolemma and erythrocyte membranes which has a single, high affinity calcium binding site (47). In contrast, the SERCA pump has two calcium binding sites which display strong positive cooperativity, with a Hill coefficient of 2, and an apparent K_D of 2 μM (48).

Affinity for ATP—Assay of calcium transport as a function of ATP concentration revealed two separate affinities of 73 and 225 μM (Fig. 5*B*). Based on evidence from chemical modification studies and secondary structure predictions on other P-type ATPases (reviewed in Refs. 49 and 50), a single nucleotide binding domain occurs on each Ca^{2+}-ATPase monomer. Therefore, the two separate affinities are likely to correspond to the E1 and E2 conformations of the ATPase. By analogy with more detailed analysis of nucleotide binding sites on the SERCA pump (51), it is postulated that the formation of the phosphoenzyme intermediate is coupled to a high affinity site on the E1 conformation of the enzyme. Further velocity increase by higher ATP concentrations occurs without concurrent increase in phosphoenzyme formation of the SERCA pump and is likely to correspond to a low affinity site, also referred to as the "regulatory site," on the E2 conformation.

Substitution of Asp-371 with Glu or Asn Leads to Complete Loss of ^{45}Ca Transport Activity

In every P-ATPase tested thus far, modification of the invariant aspartate in the highly conserved phosphorylation motif (CSDKTGTLT) results in complete loss of function, consistent with its essential role as acceptor of the terminal phosphate of ATP during ATP hydrolysis and energy transduction (52–54). In the case of the yeast plasma membrane H^+-ATPase, the mutant enzymes also display a dominant lethal phenotype when expressed constitutively, and are retained in the endoplasmic reticulum (55, 56). It was therefore of particular interest to analyze the effects of substitutions of the equivalent aspartate in PMR1. Not surprisingly, ^{45}Ca transport activity in the mutants Asp-371 → Glu and Asp-371 → Asn was abolished to background levels (Fig. 6*A*; also compare with Fig. 2*B*). However, there was no inhibition of the growth of yeast strains expressing the mutant PMR1 proteins (measured at 23 °C, not shown), indicating an absence of toxic effects. In addition, expression levels of the mutant proteins in the Golgi fractions were nearly normal (Fig. 6, *B* and *C*, *lanes 3* and *4*). However, in the case of the Asp-371 → Asn mutant, there was significant

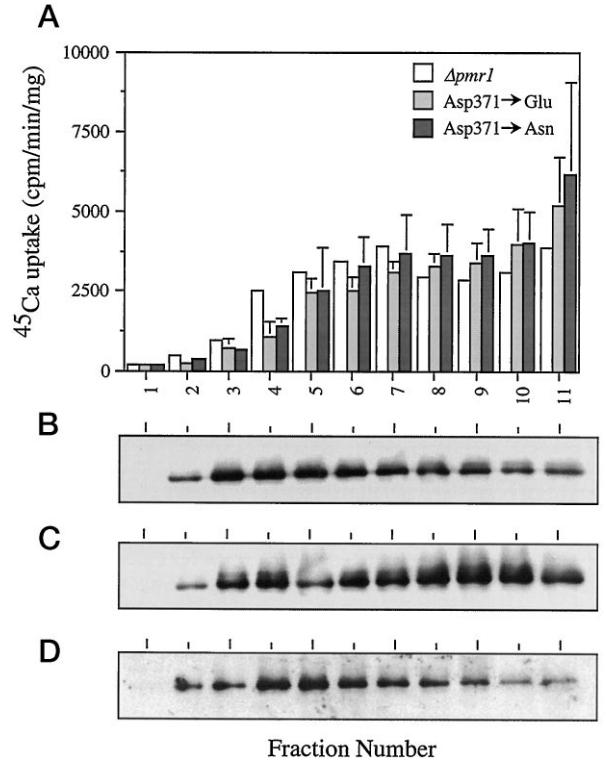

FIG. 6. **Effect of substitutions at Aspartate 371 on ^{45}Ca transport and Golgi localization.** Strain K616, transformed with plasmid YEpHR1 carrying wild-type PMR1 or, substitutions Asp-371 → Glu and Asp-371 → Asn in PMR1, or vector alone (no PMR1), was fractionated as described in the text. *Panel A*, fractions (300 μl) were assayed for ^{45}Ca uptake activity as described in the text, average of three experiments are shown for the mutants; the vector control is shown for comparison. *Panels B–D*, expression of PMR1 polypeptides on Western blots probed with anti-PMR1 antibody. Lanes correspond to 10 μg each, of gradient fractions 1–11, as described. *Panel B*, Asp-371 → Glu mutant; *panel C*, Asp-371 → Asn mutant; *panel D*, wild-type PMR1.

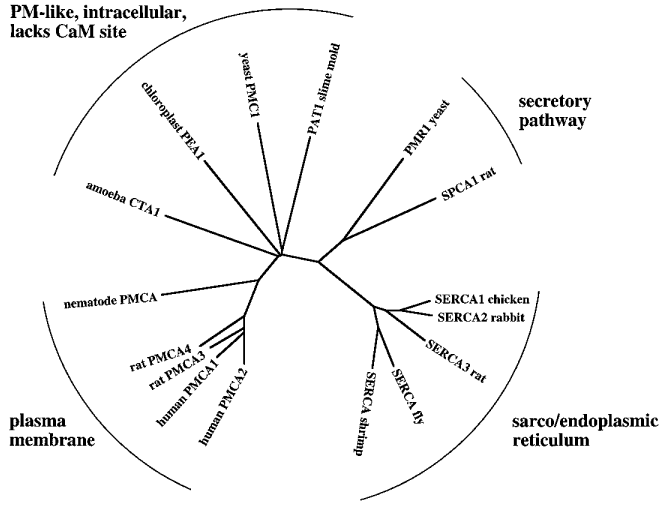

FIG. 7. **Phylogenetic tree of Ca^{2+}-ATPases.** A sampling of Ca^{2+}-ATPases were aligned and organized into a phylogenetic tree using CLUSTAL W (version 1.5). Graphic display of the output was generated by the DRAWTREE program in the PHYLIP package (version 3.572). Sequences were derived from GenBank, Protein Identification Resource (PIR), and European Molecular Biology Laboratory (EMBL, Swiss Prot) using the FASTA program. Accession numbers in clockwise direction, beginning with the human PMCA2 isoform, are: A38871, P20020, A34308, S54356, Z68221, U20321, L08469, P38929, X89369, P13586, A42764, P13585, S04269, A34307, A36691, and P35316. Grouping of Ca^{2+}-ATPases, as indicated, was based on phylogenetic similarity as well as criteria described more completely in the text.

retention of mutant polypeptide in the denser fractions of the gradient, corresponding to endoplasmic reticulum (Fig. 6*C*, *lanes 8–10*).

DISCUSSION

We describe, in this work, the use of a reverse genetics approach to demonstrate the properties of a novel Ca^{2+}-ATPase in yeast. With the recent completion of the yeast genome sequence, and ongoing efforts to sequence other genomes, the functional analysis of many such newly identified putative transporters will be of increasing importance. One of the first obstacles we faced was low expression of PMR1 in wild type yeast, which made its overproduction critical for subsequent biochemical characterization. However, we also found that there were limitations to overproduction, because constitutive expression of PMR1 from the powerful *PMA1* promoter was toxic to yeast growth. As a workable compromise, we used the inducible heat shock promoter described in earlier work (29), on a multicopy plasmid. A second aspect of the design of this expression system was engineering of the host strain. We observed that chromosomal deletion of the *PMR1* gene led to dramatic compensatory increases in expression of other Ca^{2+} transporters,[2] making additional deletions (in *PMC1* and *CNB1*) necessary to obtain low background levels of Ca^{2+} transport activity. This host strain should be of general utility for the heterologous expression of other Ca^{2+}-ATPases.

Until now, two classes of Ca^{2+}-ATPases have been described in the literature, each with multiple isoforms and splice variants that show tissue-specific distribution: the SERCA and PMCA pumps differ from one another in their subcellular distribution, mode of regulation, kinetic characteristics, and inhibitor sensitivities (reviewed by Refs. 57 and 58). Furthermore, sequence comparisons between these two classes of enzymes reveal a divergence similar to that observed between unrelated members of the P-ATPase family, suggesting a very early divergence from one another, or possibly even a convergence in evolution of Ca^{2+} selectivity. PMR1, and its putative homolog from rat, SPCA, fall into a third group on the basis of sequence homology, somewhat more closely related to the SERCA class (Fig. 7). In this work, we show that the properties of PMR1 are clearly distinct from both SERCA and PMCA pumps. Thus, PMR1 differs from SERCA in the number and affinity of the calcium binding sites, and in the lack of sensitivity to inhibition by thapsigargin and cyclopiazonic acid. PMR1 also differs from PMCA in the relative insensitivity to vanadate and lanthanum. These data, together with the sequence relations, justify the inclusion of PMR1 in a separate class, termed the secretory pathway Ca^{2+}-ATPases. The identification and characterization of additional members of this family should assist delineation of this group. There is emerging evidence for a widespread distribution of PMR1 homologs: partial clones have been reported from the parasite *Schistosoma mansoni* (59) and an algal coccolithophore.[3] In addition, the prokaryotic Ca^{2+}-ATPases reported from *Synechococcus* (60, 61) and *Synechocystis* (62) resemble PMR1 and SPCA more closely (38% identity) than any other eukaryotic Ca^{2+}-ATPase sequence. Taken together with an *absence* of a gene encoding a SERCA-type Ca^{2+}-ATPase in yeast, these observations raise the intriguing possibility that the secretory pathway Ca^{2+}-ATPases may represent the most ancient and wide-spread class of Ca^{2+} pumps, despite being the least recognized. Our analysis (Fig. 7) has also revealed a fourth cluster of newly

[2] V. Marchi, A. Sorin, and R. Rao, unpublished observations.

[3] Y. Song, personal communication.

identified Ca^{2+}-ATPases that may be loosely grouped together on the basis of sequence relations; each member of this subgroup shares significant identity (40–50%) to the PMCA class, although there is no additional homology between members within the subgroup. All are intracellular in localization, typically in acidic compartments such as vacuoles, and lack the well known calmodulin-binding regulatory domain found in the C terminus of the plasma membrane Ca^{2+}-ATPases. The evolutionary relationship between this "vacuolar" subgroup and PMCA possibly reflects a topological and functional continuity between vacuolar and plasma membranes; for example, in *Dictyostelium*, the contractile vacuoles discharge their contents to the exterior by fusing with the plasma membrane, and in *Entamoeba*, invaginations of the plasma membrane develop into pinocytotic or phagocytotic vacuoles. Biochemical characterization of individual members in this cluster will be required to define the properties of the group further.

The yeast *S. cerevisiae* is a genetically defined system, eminently suitable for structure-function studies on Ca^{2+} pumps using a mutagenic approach. As a prelude to these studies, we report on the effects of substitutions in the catalytic site aspartate within the phosphorylation domain of PMR1. As expected, the Asp-371 → Glu and Asp-371 → Asn mutations led to complete loss of $^{45}Ca^{2+}$ transport activity, consistent with similar functional effects of analogous mutations in the sarcoplasmic reticulum Ca^{2+}-ATPase (52), the Na^+/K^+-ATPase (53) and the yeast H^+-ATPase (54). Additionally, the yeast H^+-ATPase mutants were misfolded (as judged by hypersensitivity to proteases),[4] exclusively retained in the endoplasmic reticulum, and had a dominant lethal phenotype when expressed constitutively (55, 56). These results indicated that substitutions at the phosphorylation site have large, global effects on protein conformation, and that the defective proteins are retained in the endoplasmic reticulum as part of a "quality control" mechanism. In contrast to these dramatic effects, phosphorylation site mutants in the Na^+/K^+-ATPase appeared to be correctly folded, as judged by their ability to bind cations, nucleotides, and ouabain, although there was no evidence for localization to the plasma membrane (63). In this work we show that phosphorylation site mutants in PMR1 lack deleterious effects on growth, and are correctly targeted to the Golgi membranes, as determined by subcellular fractionation on sucrose gradients. However, localization appeared to be significantly retarded relative to wild-type, since there was substantial retention of mutant polypeptide in fractions corresponding to the endoplasmic reticulum. Studies are currently under way to determine whether the mutant proteins retain native conformation and partial function, such as ability to bind calcium. In the future, PMR1 may serve as a model for structure-function studies on the Golgi subgroup of Ca^{2+}-ATPases.

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