The Golgi PMR1 P-type ATPase of Caenorhabditis elegans

IDENTIFICATION OF THE GENE AND DEMONSTRATION OF CALCIUM AND MANGANESE TRANSPORT*

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In recent years, it has been well established that the Ca\(^{2+}\) concentration in the lumen of intracellular organelles is a key determinant of cell function. Despite the fact that essential functions of the Golgi apparatus depend on the Ca\(^{2+}\) and Mn\(^{2+}\) concentration in its lumen, little is known on the transport system responsible for ion accumulation. The Golgi ion pump PMR1 has been functionally studied only in yeast. In humans, mutants in the orthologous gene ATP2C1 cause Hailey-Hailey disease. We report here the identification of the PMR1 homologue in the model organism Caenorhabditis elegans and after ectopic expression the direct study of its ion transport in permeabilized COS-1 cells. The C. elegans genome is predicted to contain a single PMR1 orthologue on chromosome I. We found evidence for alternative splicing in the 5'-untranslated region, but no indication for the generation of different protein isoforms. C. elegans PMR1 overexpressed in COS-1 cells transports Ca\(^{2+}\) and Mn\(^{2+}\) with high affinity into the Golgi apparatus in a thapsigargin-insensitive manner. Part of the accumulated Ca\(^{2+}\) can be released by inositol 1,4,5-trisphosphate, in agreement with the idea that the Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) store.

In eukaryotic cells, cytosolic Ca\(^{2+}\) acts as a second messenger in a large variety of cell functions. The increase of the cytosolic free Ca\(^{2+}\) concentration in response to a stimulus results from the opening of Ca\(^{2+}\) channels present in the plasma membrane and in the membranes of intracellular Ca\(^{2+}\) stores, mainly the endoplasmic reticulum (ER). It has more recently also become clear that the Ca\(^{2+}\) stored in the lumen of many intracellular compartments not only serves as a reservoir of releasable Ca\(^{2+}\), but also plays a regulatory role in several important cell biological functions. Luminal Ca\(^{2+}\) in the ER as well as in the Golgi or other components of the secretory pathway is required for the proper translation, translocation, folding, and processing of secreted proteins (for review, see Ref. 1). A sufficiently high level of intraorganellar Ca\(^{2+}\) has been implicated also in intra-Golgi membrane transport (2), transport between the Golgi and the ER (3), and in endosome fusion (4). Besides these constitutive actions, luminal Ca\(^{2+}\) controls movements of the Ca\(^{2+}\) ion itself: its release from the intracellular stores, its permeability through the nuclear pore complexes, and capacitative Ca\(^{2+}\) entry at the plasma membrane (for review, see Ref. 5).

The pivotal role of the ER as a Ca\(^{2+}\) store is well established. There is a growing consensus that also the Golgi apparatus can function as an agonist-releasable Ca\(^{2+}\) store (6), the importance of which may, however, greatly differ among the different cell types and ranges from relatively unimportant in Drosophila melanogaster S2 cells (7) to very important in renal LLC-PK\(_1\) cells (8). The storage capacity for Ca\(^{2+}\) in the Golgi is greatly increased by specific proteins like Calnuc/nucleobindin (9, 10) and members of the CREC family of Ca\(^{2+}\)-binding proteins (reviewed in Ref. 11). In contrast to the growing unanimity on the role of Golgi as a Ca\(^{2+}\) store, there remains some confusion as to the type of Ca\(^{2+}\)-accumulation system responsible to replenish the store. Ca\(^{2+}\) pumps belonging to the class of P-type ion-motive ATPases have been proposed to take up this task: SERCA2 (6, 10), the plasma-membrane Ca\(^{2+}\) pump PMCA en route to the plasma membrane (12) and PMR1 (6, 13). The SERCA pumps, which are encoded by three different genes in vertebrates (human gene names: ATP2A1–3), but apparently only by a single gene in invertebrates like C. elegans, are the best characterized members of this class and are responsible for the accumulation of Ca\(^{2+}\) into the ER or into the sarcoplasmic reticulum. The PMCA Ca\(^{2+}\) pumps (corresponding human gene names: ATP2B1–4) responsible for the extrusion of Ca\(^{2+}\) out of the cell, also belong to the same class but are themselves not involved in Ca\(^{2+}\) transport into the stores, with as a possible exception the above mentioned Golgi-based PMCA pumps which are on their way to the plasma membrane. The PMR1-type of Ca\(^{2+}\)-transport ATPase was first identified in the yeast Saccharomyces cerevisiae (14) and localized to the Golgi or one of its subcompartments (15). Genes homologous to the S. cerevisiae PMR1 have been reported for a number of other fungi (see Ref. 16 and references therein). The PMR1 ion-motive ATPase supplies the secretory pathway with Ca\(^{2+}\) and Mn\(^{2+}\) ions required for glycosylation, sorting, and ER-associated protein degradation (17, 18). A recent study has demonstrated capacitative Ca\(^{2+}\) entry in S. cerevisiae, a mechanism that in higher eukaryotes is thought to be initiated by depletion of intracellular stores that are filled by the SERCA Ca\(^{2+}\) pump (19). The process was stimulated in pmr1 mutants, indicating that in yeast capacitative Ca\(^{2+}\) entry in combination with PMR1 activity supplies the secretory pathway with Ca\(^{2+}\). Yeast pmr1 mutants do not grow on a medium containing

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EBI Data Bank with accession number(s) AJ300851 and AJ300852.

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The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum calcium adenosine triphosphatase; PMCA, plasma membrane calcium adenosine triphosphatase; EST, expressed sequence tag; PCR, polymerase chain reaction; CePMR1, C. elegans orthologous gene of the PMR1 gene of S. cerevisiae; IP\(_a\), inositol 1,4,5-trisphosphate; UTR, untranslated region; bp, base pair(s); kb, kilobase pair(s).
submicromolar concentrations of Ca\(^{2+}\) and show defects in the maturation of secretory proteins which are suppressed by supplying millimolar Ca\(^{2+}\) or micromolar Mn\(^{2+}\) to the growth medium. Mutations in PMR1 were also reported to rescue yeast mutants, which as a result of the lack of superoxide dismutase, show impaired growth in aerobic conditions (20). This effect is ascribed to increased cytosolic levels of Mn\(^{2+}\) resulting from a lack of accumulation of the ion in the Golgi compartment. Mn\(^{2+}\) is known for its capacity to scavenge superoxide ions. Mandal et al. (21) pointed recently to the critical role of transmembrane segment M6 in yeast PMR1 for defining the calcium-binding sites in general and in particular of residue Gln\(^{783}\) in this segment for the Mn\(^{2+}\) selectivity.

Relatively less is known on the PMR1 homologues in animal cells. The cDNA of the putative rat form of the yeast PMR1 was already cloned in 1992 with a SERCA-derived probe (13), but the authors failed in their efforts to show that the corresponding protein, upon its expression in COS cells, was able to catalyze the uptake of Ca\(^{2+}\) into vesicles consisting of fragmented membranes. In the meanwhile homologous cDNAs or genes were reported for D. melanogaster,\(^2\) Bos taurus,\(^3\) and C. elegans.\(^1\) But again until now no direct indication that any of these was involved in Ca\(^{2+}\) uptake has been provided. However, indirect evidence comes from two recent reports which show that Hailey-Hailey disease (MIM 169960), which is manifested by the impaired intercellular adhesion of epidermal keratinocytes, results from mutations in one of the alleles of a gene that encodes the protein encoded by the PMR1-like gene. The symptoms of Hailey-Hailey disease strongly resemble those of Darier-White disease (MIM 124200) which is due to a mutation in one of the alleles of the SERCA2 gene ATP2A2. This, together with the observation that expression of the mammalian SERCA1a prevented the lethality of the pmr1-1 pmr1-2 double mutations in yeast (24), strongly suggests that the human PMR1 pump can act as a Ca\(^{2+}\) pump.

We now show for the first time that an animal PMR1 homologue can transport Ca\(^{2+}\) or Mn\(^{2+}\) into the Golgi apparatus of COS-1 cells with high affinity and in a thapsigargin-insensitive manner. The accumulated Ca\(^{2+}\) can be released by IP\(_3\), in line with the view that the Golgi apparatus is an IP\(_3\)-sensitive Ca\(^{2+}\) store.

**Experimental Procedures**

Materials—\(\gamma\)-32P ATP and \(\gamma\)-3Ca were obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands), \(50\) pmol from PerkinElmer Life Sciences (Boston, MA). Sequencing was done with the AutoRead\(^{TM}\) 300 Sequencing Kit from Amersham Pharmacia Biotech. COS-1 cells were transiently transfected with FuGEN\(_{\text{E}}\)\(^{\text{TM}}\) 6 transfection reagent (Roche Molecular Diagnostics, Brussels, Belgium) according to the manufacturer’s instructions. Fluorescein isothiocyanate-labeled goat anti-rabbit antibodies were obtained from Sigma (Sigma/Aldrich NV, Bornem, Belgium) and horseradish peroxidase-labeled awine antirabbit antibodies from Dako (Dako A/S, Glostrup, Denmark). The full-length rabbit SERCA1a clone was kindly provided by J. P. Andersen and B. Vilsen (University of Aarhus, Denmark).

Construcst for Expression—The EST clones yk218a11 and yk334d5 were obtained from Yuji Kohara’s database, and sequenced. Both clones contained the complete open reading frame of C. elegans PMR1. It should, however, be remarked that the open reading frame is 63 bp smaller than predicted in the database annotation (see “Results”). Clone yk334d5 was used to make an expression construct. The 3.2-kb PMR1-encoding EcoRI/XhoI fragment of yk334d5 was ligated into the dephosphorylated pcDNA3 expression vector (Invitrogen Co., British Biotechnology Products Ltd., Abingdon, United Kingdom) cut with the same restriction enzymes. The pig SERCA2a expression vector has been described by Verboom et al. (25). The full-length rabbit SERCA1a was cloned in the pMT2 expression vector (26).

**Cell Culture and DNA Transfection**—For microsome preparation, COS-1 cells were seeded in 100-mm culture dishes at a density of 2.5 × 10\(^6\) cells per plate. For immunocytochemistry 2.0 × 10\(^6\) cells were seeded on gelatin (1%)-coated coverslips. For \(4^{\text{Ca}}\) or \(8^{\text{Mn}}\) fluxes 2.0 × 10\(^6\) cells were seeded in gelatin-coated 12-well plates. For microsome preparation and immunocytochemistry the cells were perfomed the day after seeding. For 45Ca fluxes the period between seeding and transfection was extended to 5 days to allow better attachment of the cells to the plates. After transfection, the cells were incubated for 60 h at 37 °C and 5% CO\(_2\).

**Preparation of Antiserum to PMR1 Protein**—The immunogen was a recombinant protein corresponding to the putative large cytoplasmic loop between transmembrane segments 4 and 5 of C. elegans PMR1. The protein was expressed in Escherichia coli using the QIAexpress Type IV System (Qiagen, Hilden, Germany). In a first step the cDNA corresponding to the loop region was amplified by PCR with primers PMR1CYTF (5’-CAACCGTCTGCCTGGAAGAGGATCGCC-3’) and PMR1CYTR (5’-CTAGTGGACCTTTTTCCCTCTCATTCCGCC-3’) containing, respectively, a SalI and SfiI restriction site at their 5’ end. The PMR1CYTF primer corresponds to nucleotides 26812–26831 of cosmid CECC4 (accession number Z81490) and PMR1CYTR primer to the inverse complement of nucleotides 136–154 of cosmid CEKZ256 (accession number Z82088). PCR was carried out for 20 cycles using the Expand\(^{TM}\) Long Template PCR System from Roche Molecular Diagnostics (Roche Molecular Diagnostics, Lone, CA) according to the manufacturer’s instructions. The recombinant protein migrated with an apparent molecular mass of about 42 kDa in 12% SDS-polyacrylamide gels and reacted with the monoclonal anti-polylhistidine antibody (clone HIS-1, from Sigma, dilution 1:1000) on Western blots. In a last step the recombinant cytoplasmic loop was concentrated by Centricon\(^{TM}\) Plus-20 centrifugal filtering (Millipore, Bedford, MA).

Rabbits were immunized with 0.1 mg of recombinant protein in 0.5 ml of phosphate-buffered saline emulsified with 0.5 ml of complete Freund’s adjuvant. Booster injections of the same immunogen with incomplete Freund’s adjuvant were given at 4-week intervals. Preimmune serum and serum obtained after 4 boosters were used. The antiserum is designated as Celpmloop.

**Rabbit Anti-Transcriptase Primer PCR System**—Total RNA was prepared from C. elegans with TriPure\(^{TM}\) Isolation Reagent (Roche Molecular Diagnostics) according to the manufacturer’s instructions. First strand cDNA synthesis was performed with the Thermoscript\(^{TM}\) RT-PCR System (Life Technologies NV, Merelbeke, Belgium) using the modified oligo(dT) primer 5’-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGGCA-3’ corresponding to nucleotides 1955–1976 of cosmid CEZK256, primer 485 (5’-GAGGACTCGAGCTCAAGG-3’) which partially contains the sequence of the modified oligo(dT) primer, primer CePMR1For (5’-TGCTCTCGGCTGTTCCTOC-3’) corresponding to the inverse complement of nucleotides 25104–25123 of cosmid CECC4, and SL1 primer (5’-GGTTTAAATACGAGAGGAAACTCAG-3’) and SL2 primer (5’-GGTTTAAAACGAGAGGAAACTCAG-3’) which contain the sequence of splice leaders SL1 and SL2, respectively. PCR amplifications were carried out for 30–35 cycles of 1 min at 94 °C, 1 min at 50–58 °C, and 2 min at 72 °C. PCR fragments were gel-purified (QiAquick Gel Purification Kit, Qiagen) and subcloned into pGEM-T Easy vector (Promega, Madison, WI). Several individual clones were sequenced.

**Membrane Preparations and Immunoblotting Analysis**—Microsomes were isolated from COS-1 cells as described by Verboom et al. (25). Membranes from C. elegans were isolated as described by Baylis et al. (28). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL). Denaturing gel electrophoresis and Western blotting were done as described earlier (29).

\(4^{\text{Ca}}\) and \(8^{\text{Mn}}\) fluxes—COS-1 cells were grown on 12-well plates. Loading with \(4^{\text{Ca}}\) and efflux were done essentially as described earlier (30). Cells were treated for 10 min with 20 μg/ml saponin at 25 °C and loaded for the indicated lengths of time in 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 5 mM ATP, 10 mM Na\(_3\)PO\(_4\) 0.44 mM (for \(4^{\text{Ca}}\) influx) or 0.1 mM (for \(8^{\text{Mn}}\) fluxes) EGTA, MgCl\(_2\), CaCl\(_2\), and MnCl\(_2\).

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were added to obtain a calculated free Mg\(^{2+}\) concentration of 0.5 mM and the indicated concentrations of free Ca\(^{2+}\) and Mn\(^{2+}\). Free concentrations of Ca\(^{2+}\) and Mn\(^{2+}\) were calculated based on the stability constants for EGTA and ATP given by Fabiato and Fabiato (31) (for Ca\(^{2+}\)) and by Martell and Smith (32) (for Mn\(^{2+}\)). Thapsigargin (2 \(\mu\)M) was added if inhibition of pumping by the SERCA Ca\(^{2+}\) pump was needed. Efflux was performed in 120 mM KCl, 30 mM imidazole (pH 6.8), and 1 mM EGTA. All experiments on Mn\(^{2+}\) transporting activity or Mn\(^{2+}\) effects on Ca\(^{2+}\) uptake activity were performed with Chelex 100 (Bio-Rad, Eke, Belgium)-treated solutions. 32P-Phosphoenzyme Formation and Electrophoresis in Acid SDS Gels—The phosphorylation reaction was carried out on ice in 100 \(\mu\)l of solution containing 15 \(\mu\)g of microsomal protein, 80 mM K-Hepes (pH 7.0), 5 mM NaCl, 1 mM dithiothreitol, and the indicated concentrations of total EGTA and free Mg\(^{2+}\), Ca\(^{2+}\), and Mn\(^{2+}\). Thapsigargin was used at 0.1 \(\mu\)M. The reaction was started by adding 5 \(\mu\)l of [\(\gamma\)-32P]ATP of 2 \(\muCi/\mu\)l. After 20 s the reaction was stopped by adding 0.4 ml of ice-cold stop solution (6% trichloroacetic acid, 10 mM phosphoric acid, 1 mM ATP). The mixture was left on ice for 0.5 h and centrifuged in the cold to precipitate the protein. The pellet was washed two more times in stop solution and once in 0.2 M acetic acid-NaOH (pH 5.3). To test the sensitivity of the phosphoprotein to hydroxylamine, the samples were additionally incubated for 20 min at room temperature in 0.2 M acetic acid-NaOH with or without 0.2 M hydroxylamine. The samples were then dissolved in a modified SDS loading buffer and subjected to SDS-gel electrophoresis in acid gels as described by Sarkadi et al. (33). The gels were dried between gel drying sheets (Promega) and exposed to screens for quantification of the radioactive bands on a Storm840™ scanner in combination with the ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA).

Immunocytochemistry—Cells grown on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, washed in phosphate-buffered saline, and permeabilized in 0.2% Triton X-100. Primary and secondary antibodies were diluted in phosphate-buffered saline containing 3% bovine serum albumin at the indicated dilutions.

RESULTS

Gene Structure of C. elegans PMR1 and Alternative Transcript Processing—The C. elegans genome is predicted to contain a single PMR1 orthologue on chromosome I, which is annotated ZK2561.1 by the C. elegans sequencing consortium (34). The genomic sequence can be found on the two overlapping cosmids CECC4 (accession number Z81490) and CEZK256 (accession number Z82088). Two EST clones from Yuji Kohara’s database, yk218a11 and yk334d5, representing cDNA clones of the C. elegans PMR1 gene, were completely sequenced. The deduced C. elegans PMR1 protein would contain 901 amino acids instead of the 922 predicted in the database annotation (protein identification number CAB04015.1). This is because a sequence of 63 bp is wrongly assigned by the Genefinder program to the putative exon 8 (303 bp). We found this sequence to be absent from both cDNA clones (see Fig. 1A) and hence the actual protein should correspondingly be 21 amino acids shorter. Furthermore, it was clear from clone yk334d5 that the 5’ UTR of C. elegans PMR1 is at least 257 bp long and contains, besides exon 1, two extra exons, comprising only untranslated sequences and designated exon 1’ and exon 3 for reasons discussed below (Fig. 1B, second line). Exon 1 (399 bp) starts at position –87 relative to the ATG start codon, which corresponds to nucleotide 23653 on cosmid CECC4. Exon 1’ (105 bp) and exon 3 (65 bp) correspond, respectively, to nucleotides 23111–23215 and 15957–16021. This implies that exon 3 is located more than 7 kb upstream from exon 1. EST clone yk218a11 does obviously not contain the entire 5’ UTR, as only the last part of exon 1 is represented in this clone.

A comparison of the exon/intron layout of CePMR1 to that of the D. melanogaster (accession number AC014929) and human orthologues (gene ATP2C1, Refs. 22 and 23) shows that the
human gene contains the most elaborate exon/intron layout, *D. melanogaster* the simplest, whereas *C. elegans* takes an intermediate position (Fig. 1B). Apparently *C. elegans* and to a larger degree *D. melanogaster* have lost most of the introns during evolution. The worm and human have in total 5 conserved exon boundaries, the worm and the fly only one. The localization of the exon 5/6 boundary in *CePMR1* corresponds to the exon 1/2 boundary in *D. melanogaster* and to the exon 19/18 junction in the human gene. There are four extra conserved exon/intron positions between *C. elegans* and the human ATP2C1 gene (*asterisk* in Fig. 1B) while there is no other exon/intron junction at homologous positions between the *C. elegans* and *D. melanogaster* *PMR1* genes. One extra exon boundary is conserved between exons 2 and 3 in *D. melanogaster* and between exons 21 and 22 in the human ATP2C1 gene. Among the exon borders of the PMR1 family, only one (exon 1/2 in *CePMR1*, exon 5/6 in the human ATP2C1) is conserved in the mammalian SERCA genes (exon 4/5 in human ATP2A1–3). Compared with the mammalian PMCA family, none of the exon/intron borders is conserved.

Because in *C. elegans* the majority of the mRNAs are trans-spliced, we have investigated whether this is also the case for transcripts of *CePMR1* in the worm. 5’ Rapid amplification of cDNA ends experiments were performed on whole worm RNA. PCR reactions with primer pairs SL1/CePMR1Rev and SL2/CePMR1Rev resulted in the amplification of an 824- and 818-bp fragment, respectively. The 818-bp PCR product corresponded to a cDNA in which splice leader SL2 was trans-spliced to exon −3 and exon −3 was in turn spliced to exon −1, i.e. an exon layout as found in EST clone yk334d5. By sequencing the SL1/CePMR1Rev fragment of 824 bp, we found that SL1 was spliced to a novel exon (−2) in the 5’ UTR of the gene, which corresponds to nucleotides 22950–23020 on cosmid CECC4 (Fig. 1B, upper line). Thus it appears that the *C. elegans* transcripts are both alternatively spliced and trans-spliced at their 5’ end. Both types of splicing are coupled: a mRNA transcript containing exon −1 and exon −2 is trans-spliced to SL1, a mRNA containing exon −1 and exon −3 is trans-spliced to SL2.

We also performed 3’ rapid amplification of cDNA ends with primer pair CePMR1For/468 to check for the possibility of 3’ alternative processing at the 3’ end of the gene’s transcript. PCR amplification gave a product of about 600 bp. By subcloning and sequencing of several individual clones, it became clear that the gel band actually consisted of a mixture of three products with small differences in length and corresponding to three polyadenylation isoforms. The isoforms are the result of the use of three polyadenylation sites (pA1, pA2, and pA3) in the 3’ UTR of the gene (Fig. 1B). Both yk218a11 and yk334d5 apparently used pA2. However, no indication was found for 3’ alternative splicing, neither in our 3’ rapid amplification of cDNA ends experiments nor by database searching.

In summary, the *C. elegans PMR1* gene consists of 12 exons (−3 to 9) of moderate length and spans a region of more than 19 kb of genomic DNA. The transcripts are both alternatively and trans-spliced at their 5’ end. At their 3’ end, however, no alternative splicing could be documented but alternative polyadenylation occurs.

The *C. elegans PMR1 Protein*—Fig. 2 shows the predicted amino acid sequence of *C. elegans PMR1* and its major domains together with an alignment with the corresponding sequences of three distant species, *Homo sapiens*, *D. melanogaster*, and *S. cerevisiae*. In the sequences of all species 10 hydrophobic segments can be identified, which like in the SERCA Ca$^{2+}$ pumps, presumably form the transmembrane domain. The highest degree of sequence similarity occurs around the phosphorylation site, in regions demonstrated in SERCA to contribute to the ATP-binding site or to form structurally important loops, and in transmembrane segments M4, M6, and M8, which have been documented in SERCA to form the binding sites for Ca$^{2+}$ (35, 36). The overall amino acid sequence is 37% identical to rat SERCA2a. The percentage identity with the PMR1 sequences of human, *D. melanogaster*, and *S. cerevisiae* is, respectively, 59, 57, and 49%. The amino acids that form the binding site for one of the transported Ca$^{2+}$ ions in SERCA, more specifically the site II, are conserved in the PMR1 homologues in all species. The amino acids belonging to site I are not conserved in the PMR1 protein. In *S. cerevisiae*, Gin$^{83}$ has been demonstrated to define the Mn$^{2+}$ selectivity of the PMR1 ion pump (21). Also this residue is conserved in all species.

The N-terminal region upstream of the first transmembrane domain of *C. elegans* PMR1 has the same length as in the human PMR1 sequence reported by Sudbrak et al. (23), whereas the human sequence predicted by Hu et al. (22) is 16 residues longer. The length of the C-terminal part is more similar to the product of the human splice variant ATP2C1a than to the shorter ATP2C1b described by Hu et al. (22). As mentioned above, there is no evidence for the generation of C-terminal PMR1 protein variants in *C. elegans*. The C-terminal sequence does not contain an eleventh hydrophobic region as in the SERCA2b splice variant. The EF hand-like domain near the N terminus of the *S. cerevisiae* sequence has been shown to play a role in modulating ion transport (37). Because its primary structure is poorly conserved, it remains to be demonstrated whether a similar function occurs in other species.

Characterization and Functional Analysis of *C. elegans PMR1* Protein—The Celpmrloop antibody, raised against the large cytosolic loop between transmembrane segments 4 and 5 of PMR1 of *C. elegans*, clearly demonstrated the expression of the protein in COS-1 cells transfected with the corresponding cDNA, both by Western blot analysis and immunocytchemistry (Fig. 3). On Western blots, the immunoreactive band migrated slightly below the predicted theoretical Mr value of 98,505. A strong immunoreaction was also seen on blots of fragmented membranes prepared from whole worms. Hence, it is clear that our antibody is able to recognize the PMR1 protein both in *C. elegans* and after its ectopic expression in COS-1 cells. The vertebrate PMR1 homologue found in untransfected COS-1 cells appears not to react with the antisera as shown by the controls of untransfected COS-1 cells. Furthermore, immunocytchemistry of PMR1-overexpressing and control cells reveals the correct targeting of PMR1 to the Golgi compartment of the COS-1 cells (Fig. 3B). In conclusion, by using our polyclonal anti-PMR1 antisera it became clear that PMR1 is expressed in the worms, that it can be overexpressed in COS-1 cells and that it contains all the information needed to target the PMR1 protein to the Golgi membranes.

Because the fraction of Golgi-derived membranes in microsomes of COS-1 cells is relatively small compared with that of ER, we could not rely on conventional techniques used to measure Ca$^{2+}$ transport, like those for the ER-based SERCA transport ATPases (25). Instead we took advantage of the 45Ca$^{2+}$-flux system utilizing detergent-permeabalized cells (30).

In a first series of experiments we tested PMR1 of *C. elegans* for its ability to transport Ca$^{2+}$. Control COS-1 cells, cells overexpressing rabbit SERCA1a (as a positive control for Ca$^{2+}$ pumping), and cells overexpressing PMR1 were permeabilized with saponin in a medium mimicking a cytosolic composition and loaded with $^{45}$Ca$^{2+}$ for 45 min in the presence of NaN$_{3}$ to prevent mitochondrial Ca$^{2+}$ uptake. Ca$^{2+}$ transport via SERCA-type Ca$^{2+}$-ATPases was determined by comparing the
FIG. 2. Alignment of the predicted amino acid sequences of PMR1 homologues of four distant species. Amino acids conserved in all four species are in bold. The function of some critical residues demonstrated in SERCA and conserved in PMR1 are indicated above the aligned sequences: the phosphate accepting Asp (Asp 336 in C. elegans), residues involved in binding of ATP and fluorescein isothiocyanate, and parts of structurally important loops. The conserved DPPR motif is part of the loop (NP connection) between two major cytosolic domains, the nucleotide-binding domain and the phosphorylation domain (36). The 10 hydrophobic stretches that presumably form the transmembrane domain are underlined and labeled M1-M10. Near the N terminus, an EF hand-like stretch in S. cerevisiae that has been shown to bind metals (37) is indicated below the alignment. The asterisks (*) indicate conserved amino acids in transmembrane domains M4 and M6 that form in SERCA the site II Ca\textsuperscript{2+}-binding site. Site I residues are not conserved in PMR1. Mn indicates Glu\textsuperscript{335} in S. cerevisiae that has been shown to contribute to the selectivity for Ca\textsuperscript{2+} and Mn\textsuperscript{2+} (40). Dmelanog, D. melanogaster (see text); Hapios, H. sapiens, the longer ATP2C1a alternative transcript (22); Celegans, C. elegans (see text); Scerevis, S. cerevisiae, SwissProt P13586 (14).

\textbf{M10}
uptake in the presence and absence of 2 μM thapsigargin. The loading of the cells was followed by an efflux for 20 min in a Ca2+-free medium. IP3 (10 μM) was administered after 10 min of efflux. Fig. 4A shows that control and SERCA1a-transfected cells exhibit a Ca2+ uptake, which is blocked by 2 μM thapsigargin. In control COS-1 cells and in PMR1-expressing cells, the difference in Ca2+ content with thapsigargin and without thapsigargin represents the Ca2+ pump activity of the endogenous SERCA2b. In SERCA1a-transfected cells, it represents that of endogenous SERCA2b plus overexpressed SERCA1a. In contrast, COS-1 cells overexpressing PMR1 show an additional Ca2+ pump activity even in the presence of thapsigargin. This suggests that PMR1 is a Ca2+-transporting protein residing in the Golgi and that it is insensitive to thapsigargin. Fig. 4A also shows that Golgi membranes contain IP3 receptors, since 10 μM IP3 induced a more rapid decrease in the store Ca2+ content.

Fig. 4B shows the time course of Ca2+ uptake by PMR1-expressing cells. Transfected cells were loaded with 45Ca2+ for different time intervals in the presence of thapsigargin to block Ca2+ uptake by endogenous SERCA2b. Ca2+ pump activity reaches almost a plateau after 20 min of Ca2+ loading. Subsequent experiments were performed after 10 min of loading with Ca2+. Fig. 4C shows the Ca2+ dependence of Ca2+ uptake by PMR1. The Ca2+ concentration needed for half-maximal activation was 0.25 μM Ca2+.

In a second series of experiments we explored the possibility of PMR1 of C. elegans to function as a Mn2+ pump, since previous reports based on the activation of ATP hydrolysis by Mn2+ and on the inhibition of Ca2+ transport by Mn2+ suggested that yeast PMR1 could act as a Mn2+ pump. Control cells and PMR1-expressing cells were loaded with radioactive Mn2+ for 10 min in the presence of thapsigargin. The efflux was followed for 10 min (Fig. 5A). Fig. 5, A and C, provide direct evidence that PMR1 can indeed act as a Mn2+-transporting protein. PMR1-overexpressing cells show an enhanced uptake of Mn2+. The accumulated Mn2+ was released by the ionophore A23187 (10 μM), demonstrating that the Mn2+ has been transported into a membrane-delineated compartment. However, the addition of IP3 did not have a significant effect on the rate of efflux. The Mn2+ uptake was inhibited by Ca2+ (Fig. 5C), and conversely the Ca2+ uptake by PMR1 was inhibited by Mn2+ (Fig. 5B). It is clear from Fig. 5B that at higher Ca2+ concentrations more Mn2+ was needed to inhibit the transport of Ca2+. Half-maximal inhibition was observed at 1, 0.5, and 0.25 μM Mn2+ for loading at, respectively, 1.0, 0.32, and 0.1 μM Ca2+.

Formation of the Phosphoenzyme Intermediate—A determining characteristic of all P-type ion-transport ATPases is the transient transfer of the γ-phosphate of ATP to the protein.
forming a covalent bond with the carboxyl group of a conserved aspartic acid residue in the large cytosolic domain. The radioactively labeled phosphoprotein can be preserved during SDS-gel electrophoresis by quenching the reaction in acid and maintaining acid conditions throughout electrophoresis. Fig. 6 shows the radioactively labeled phosphointermediate in an SDS gel of microsomes from COS-1 cells overexpressing PMR1. The labeling was completely removed by treatment with hydroxylamine, demonstrating that the phosphate was bound to a carboxyl and not to a hydroxyl group (data not shown). As for the protein detected on Western blots, the phosphoprotein migrated slightly faster relative to the markers than expected from the predicted Mr of 98,505. The anomalous migration is probably due to the gel system because a similar shift is also observed for the SERCA2a Ca2+-transport ATPase, which has a predicted Mr of 109,720 (Fig. 6A). The phosphointermediate formation was stimulated by Ca2+ and Mn2+. The maximum levels were observed below 1 mM for both Ca2+ and Mn2+ and these maximum levels were not significantly different (data not shown). There remains a small residual amount of phosphoprotein also in the presence of EGTA without added Ca2+ or Mn2+. At present we do not have a straightforward explanation for this background labeling. Possibly, a small fraction of the transport-protein molecules in the COS-1 cell membranes is able to reach a conformational state that allows phosphorylation without occupation of the transport sites. The Ca2+- or Mn2+-dependent phosphoprotein formation was strongly inhibited by 50 mM La3+ (Fig. 6B). The phosphorylation experiments thus confirm the high affinity of the C. elegans PMR1 transporter for both Ca2+ and Mn2+.

**FIG. 6. Autoradiogram of the phosphoprotein intermediate of C. elegans PMR1 expressed in COS-1 cells.** A, the phosphorylation reaction was carried out on microsomes from PMR1- or SERCA2a-transfected COS-1 cells. The phosphorylation medium contained 0.5 mM total EGTA, a calculated free Mg2+ concentration of 0.5 mM, in the absence or presence of 1 μM free Ca2+ or Mn2+. In the case of PMR1-overexpressing microsomes, 1 μM thapsigargin was included. The PMR1 phosphoenzyme could not be seen in SERCA2a-transfected cells (left lanes) nor in nontransfected cells (data not shown). B, the effect of 50 μM La3+ on the phosphoprotein formation of C. elegans PMR1 in the presence of 1 μM free Ca2+ or Mn2+.

**FIG. 5. Mn2+ transport by C. elegans PMR1 and competition between Ca2+ and Mn2+ for transport.** A, Mn2+ loading of COS-1 cells transfected with native vector (control, ▲) or C. elegans PMR1 (●) in the presence of 2 μM thapsigargin. Cells were loaded at 10 μM free Mn2+ for 10 min, washed three times with efflux medium, and subjected to a passive efflux for 10 min. After 4 min of efflux 10 μM A23187 was added. B, inhibition of Ca2+ uptake of C. elegans PMR1 by Mn2+. Control cells and C. elegans PMR1-transfected COS-1 cells were incubated with loading medium containing 1 μM free Ca2+ (▲), 0.316 μM (●) or 0.1 μM (▲) free Ca2+ and different free Mn2+ concentrations. The Ca2+ content of the stores is plotted as a function of the Mn2+ concentration and is represented as the mean ± S.E. of four independent experiments. C, inhibition of Mn2+ uptake of C. elegans PMR1 by Ca2+. Control COS-1 cells and C. elegans PMR1-expressing cells were loaded with 54Mn2+ at 1 μM in the presence of 2 μM thapsigargin and at different free Ca2+ concentrations (▲, nominal Ca2+ free; ▲, 2 μM Ca2+; ●, 10 μM Ca2+). Cells were flushed for 10 min following three quick washes with efflux medium to reduce passive binding of Mn2+. 10 μM IP3 was added after 4 min of efflux. The values represent the difference between C. elegans PMR1-transfected and control COS-1 cells.

**DISCUSSION**

The analysis of the C. elegans genome has resulted in the previous identification of several members of the superfamily of P-type Ca2+-transport ATPases. A single gene encoding a member of the SERCA-type subfamily is found on chromosome III (cosmid K9D11) and three genes (mca-1–3) encoding members of the PMCA subfamily reside on chromosome IV (38). In this work, another gene (designated CePMR1) is identified encoding a P-type Ca2+-transport ATPase that is located on chromosome I. The conservation of some intron positions, the overall sequence similarity of the encoded protein, and the conservation of major domains and critical motifs of the pri-
mary sequence unequivocally place it in the PMR1 subfamily of P-type Ca\(^{2+}\)-transport ATPases. Besides the CePMR1 genomic sequence, we have in this work also characterized its transcripts and protein product.

With respect to the number of exons, the CePMR1 gene takes an intermediate position between the human and D. melanogaster orthologues. Only some of the exon/intron borders are conserved between these different species. All splice sites follow the GT...AG rule. Surprisingly, with the exception of the intron between exon 2 and 3, all introns in this gene are relatively long considering the fact that most of the introns in C. elegans genes have a length of only about 50 nucleotides (39).

A particularly long intron (\(-7\) kb) is that between exons \(-3\) and \(-2\), both located in the 5′ UTR. Interestingly, we observed the possibility of alternative splicing in the 5′ UTR, which was coupled to trans-splicing to SL1 or SL2. The meaning of such an alternative trans-splicing which only affects the 5′ UTR remains unknown. It should be noted that for the human PMR1 orthologue (gene ATP2C1), 5′-end alternative splicing has also been suggested but here it affects the open reading frame (23), whereas in C. elegans it does not result in the formation of distinct protein isoforms. At the 3′ end of the CePMR1 gene three polyadenylation sites were predicted and also experimentally detected by PCR analysis. However, there was neither any predicted nor any experimental indication for alternative splicing. Both for the corresponding human gene and for the rat gene alternative splicing and different protein tails have been suggested. However, the alternative splice sites in human and rat appear not to be conserved.

At the protein level, the major domains described in other P-type transport ATPases can be recognized in C. elegans PMR1. Also sequence motifs demonstrated to be critical for function in other P-type transport ATPases are conserved in the C. elegans sequence (Fig. 2). On the basis of these comparisons, it can be firmly concluded that the coding sequence identified in the C. elegans genome and whose protein product has been investigated in this study is a member of the family of PMR1-ion-transport ATPases. This conclusion is further substantiated by the transport and phosphorylation studies on the protein expressed in COS-1 cells.

Functional data on the PMR1 transporter are up till now available only for the yeast S. cerevisiae (21, 37, 40). In the present work the first characterization of the ion transporting activity of an animal PMR1 enzyme is presented. The C. elegans PMR1 protein overexpressed in COS-1 cells showed a predominantly Golgi-like distribution as shown by immunocytochemistry. ATP-dependent uptake of \(^{45}\text{Ca}^2+\) and \(^{54}\text{Mn}^2+\) was demonstrated in cells permeabilized with saponin. Cells overexpressing PMR1 accumulated more \(^{45}\text{Ca}^2+\) and \(^{54}\text{Mn}^2+\) than control cells, and this additional uptake was not diminished by the SERCA-specific inhibitor thapsigargin, suggesting that PMR1 is not only able to transport \(^{45}\text{Ca}^2+\) but also \(^{54}\text{Mn}^2+\). Part of this thapsigargin-insensitive \(^{45}\text{Ca}^2+\) pool was released by \(1\mu\text{M} \text{Ca}^2+\), which is slightly higher than the value determined from the ATPase activity of the purified PMR1 protein of S. cerevisiae (21). The \(^{45}\text{Ca}^2+\) uptake was progressively inhibited by increasing concentrations of Mn\(^{2+}\). Half-maximal inhibition occurred at a 

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\frac{1}{2} \text{max} = 0.25 \ \mu\text{M} \text{Ca}^2+\]

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The Golgi PMR1 P-type ATPase of *Caenorhabditis elegans*: IDENTIFICATION OF THE GENE AND DEMONSTRATION OF CALCIUM AND MANGANESE TRANSPORT

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