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The *medial*-Golgi Ion Pump Pmr1 Supplies the Yeast Secretory Pathway with Ca^{2+} and Mn^{2+} Required for Glycosylation, Sorting, and Endoplasmic Reticulum-Associated Protein Degradation

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The yeast Ca^{2+} adenosine triphosphatase Pmr1, located in *medial*-Golgi, has been implicated in intracellular transport of Ca^{2+} and Mn^{2+} ions. We show here that addition of Mn^{2+} greatly alleviates defects of *pmr1* mutants in N-linked and O-linked protein glycosylation. In contrast, accurate sorting of carboxypeptidase Y (CpY) to the vacuole requires a sufficient supply of intralumenal Ca^{2+}. Most remarkably, *pmr1* mutants are also unable to degrade CpY*, a misfolded soluble endoplasmic reticulum protein, and display phenotypes similar to mutants defective in the stress response to malfolded endoplasmic reticulum proteins. Growth inhibition of *pmr1* mutants on Ca^{2+}-deficient media is overcome by expression of other Ca^{2+} pumps, including a SERCA-type Ca^{2+} adenosine triphosphatase from rabbit, or by Vps10, a sorting receptor guiding non-native luminal proteins to the vacuole. Our analysis corroborates the dual function of Pmr1 in Ca^{2+} and Mn^{2+} transport and establishes a novel role of this secretory pathway pump in endoplasmic reticulum-associated processes.

INTRODUCTION

Compared with the knowledge on mechanisms that distribute distinctive sets of proteins into specific organelles along the eukaryotic secretory pathway, relatively little is known about how the ionic milieu within these organelles contribute to their function. This scarcity of information stems, in part, from the lack of convenient tools with which to individually alter the intracellular distribution of specific ions. A23187, an ionophore widely used to dissipate Ca^{2+} gradients in vivo, binds Mn^{2+}, Zn^{2+}, and other heavy metals with 100- to 1000-fold higher affinity than Ca^{2+} and fails to sufficiently discriminate between Mg^{2+} and Ca^{2+} (Pfeiffer and Lardy, 1976). This note of caution is underscored by a recent study in *Saccharomyces cerevisiae* demonstrating that Mn^{2+} can effectively replace Ca^{2+} ions to promote growth of yeast cells. In fact, Mn^{2+} ions may be the physiological mediator of processes previously thought to uniquely require Ca^{2+} (Loukin and Kung, 1995).

Despite this ambiguity, the Ca^{2+} concentration in the endoplasmic reticulum (ER) has been implicated in retention of resident luminal proteins (Booth and Koch, 1989), in export of secretory proteins (for review, see Sambrook, 1990), in protein folding and degradation (reviewed in Lodish and Kong, 1990; Wileman *et al*., 1991; Gething and Sambrook, 1992), and in the association of the ER chaperone BiP with misfolded proteins (Suzuki *et al*., 1991). Recent studies have demonstrated that some misfolded luminal proteins are exported from the ER into the cytosol for ubiquitination and subsequent degradation by the proteasome (for review, see Brodsky and McCracken, 1997; Sommer and Wolf, 1997). Yeast CpY*, a mutant

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form of carboxypeptidase Y (CpY), is subjected to this degradative route, and the ubiquitin-conjugating enzymes (Ubc6, Ubc7) have been identified (Hiller *et al.*, 1996). In contrast, other non-native luminal proteins appear to exit the yeast ER via the secretory pathway and interact with Vps10, a transmembrane receptor normally engaged in sorting of soluble hydrolases, including CpY, from the Golgi to the vacuole (Marcusson *et al.*, 1994; Cooper and Stevens, 1996). Apparently, Vps10 exerts a dual role in intracellular targeting, i.e., it participates in the sorting of specific vacuolar enzymes and in a more general salvage pathway guiding non-native luminal proteins to the vacuole (Hong *et al.*, 1996). It is not known whether Ca^{2+} ions in the ER affect export of substrates for degradation by the proteasome. Similarly, it is unclear how intralumenal Ca^{2+} influences the Vps10-mediated salvage pathway.

Accumulation of unfolded proteins in the ER, experimentally induced by the addition of tunicamycin to block glycosylation or by treatment with reducing agents such as DTT, activates a universal signal transduction cascade that allows eukaryotic cells to alter the conditions in the ER (Lee, 1987; Kozutsumi *et al.*, 1988). This unfolded protein response (UPR), best understood in the yeast *Saccharomyes cerevisiae* (for review, see Shamu *et al.*, 1994; Nunnari and Walter, 1996; Shamu, 1997), coordinates the transcription of genes encoding ER-resident chaperones with the synthesis of glycerophospholipids destined for the ER membrane (Cox *et al.*, 1997). Inactivation of UPR components, like Ire1 (a transmembrane kinase of the ER and/or inner nuclear membrane) or Hac1 (a transcription factor binding to UPR-regulated promoters), leads to a complete block of the UPR. Such yeast mutants are sensitive to DTT and tunicamycin and fail to grow on media lacking inositol, a precursor in the synthesis of phosphatidylinositol.

Within the Golgi complex, high Ca^{2+} concentrations are found in all cisternae along the *cis–trans* axis (Pezzati *et al.*, 1997). The available evidence for a role of Ca^{2+} in important Golgi functions, including luminal and membrane protein traffic (Carnell and Moore, 1994), cargo condensation, and precursor processing (Chanat and Huttner, 1991; Fuller *et al.*, 1989; Oda, 1992), suggests that Golgi Ca^{2+} transport and storage are specifically regulated, independently from the control of ER Ca^{2+}. As inferred from the in vitro requirement for Mn^{2+} by numerous enzymes involved in oligosaccharide addition in animal cells and yeast, the Golgi complex hosts reactions that depend on Mn^{2+} (Sharma *et al.*, 1974; Nakajima and Ballou, 1975; Parodi, 1979; Sugiura *et al.*, 1982; Elhammer and Kornfeld, 1986; Haselbeck and Schekman, 1986). Depletion of manganese in vivo inhibits O-linked and N-linked protein glycosylation in mammalian cells (Kaufman *et al.*, 1994). Similar results were obtained with thapsigargin, a potent inhibitor of the SERCA-type Ca^{2+}-adenosine triphosphatases present in sarco/endoplasmic reticulum membranes, suggesting a role of these ion pumps in maintaining intralumenal Ca^{2+} and Mn^{2+} concentrations (Kaufman *et al.*, 1994).

In yeast, the only Ca^{2+} pump known to reside in the early secretory pathway (ER, Golgi) is Pmr1 (Rudolph *et al.*, 1989; Antebi and Fink, 1992; Cunningham and Fink, 1994, 1996; Sorin *et al.*, 1997). In subcellular fractionation and immunofluorescence microscopy, Pmr1 almost entirely coincides (> 98%) with the steady-state pool of Emp47, a lectine-like transmembrane protein of the *medial*-Golgi, which recycles, in contrast to Pmr1, between the ER and the Golgi (Schröder *et al.*, 1995). These findings, consistent with previous work demonstrating a Golgi-like distribution for Pmr1 (Antebi and Fink, 1992), imply that only minute amounts of Pmr1 reside in the ER membrane. Since no other candidate gene that could encode an ATP-driven Ca^{2+} pump of the ER, is present in the genome of *Saccharomyces cerevisiae*, the yeast ER appears to lack a spezialized Ca^{2+} pump (Sorin *et al.*, 1997). This raises interesting questions on how yeast cells achieve the appropriate distribution of Ca^{2+} and Mn^{2+} ions within organelles of the early secretory pathway, which is thought to critically depend on the adequate presence of both ions.

Inactivation of Pmr1 leads to conspicuous defects, compatible with the loss of an ion pump shared by Ca^{2+} and Mn^{2+}. *pmr1* mutants display a subtle CpY sorting defect and are blocked in pheromone maturation; both defects are fully reversed by the addition of extracellular Ca^{2+} (Antebi and Fink, 1992). However, *pmr1* mutants also accumulate Mn^{2+} and are hypersensitive to this metal cation (Lapinskas *et al.*, 1995). Finally, *pmr1* mutants are sensitive to EGTA, an effective chelator of Ca^{2+}, Mn^{2+}, and other divalent metal ions (Rudolph *et al.*, 1989). In this study, we provide evidence for a dual role of Pmr1 in the transport of Ca^{2+} and Mn^{2+} into the secretory pathway and show that both ions, albeit interchangeable to support growth, exert distinct functions. Furthermore, we demonstrate that Pmr1-mediated ion transport, despite the prominent location of this ion pump in the *medial*-Golgi, is an important determinant of processes hosted in the lumen of the ER.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Yeast strains used in this study are shown in Table 1. Standard yeast culture medium was prepared as described (Sherman *et al.*, 1986). Media containing the ion chelator bis-(*O*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (BAPTA) used in the analysis of CpY sorting were derived from standard defined synthetic medium SD (Wickersham, 1951.) without the salts of calcium, manganese, zinc, copper, and iron; Ca^{2+} pantothenate was replaced by the sodium salt. Ammonium sulfate was replaced by ammonium chloride (37

mM), potassium sulfate was replaced by the chloride salt, phosphate was reduced to 200 μM, and the pH was adjusted to pH 6.5 with 50 mM potassium 2-[*N*-morpholino]ethane sulfonic acid (MES). All components were used at the highest purity available (Ultrapure, Merck, Darmstadt, Germany). Growth medium contained ammonium sulfate (200 μM), which was omitted in the medium (1 M sorbitol; 0.1% glucose) used for spheroblasting and labeling. Before the addition of BAPTA, both media were analyzed for their metal content (calcium, manganese, iron, copper, and zinc) by inductively coupled plasma spectroscopy. From the total metal concentrations, the necessary additions of calcium, manganese, and zinc (all as chlorides) and K-BAPTA were calculated (MAXChelator by Chris Patton, Stanford University, Stanford, CA) to keep the concentrations of free zinc (2.5×10^{-10} M), free iron (6.3×10^{-21} M), and free BAPTA (1.8×10^{-3} M) constant, but achieve desired concentrations for calcium and manganese. Binding constants used in the calculations are described by Loukin and Kung (1995). Manganese and calcium concentrations in the media used are given in the legend to Figure 1.

Plasmids

Plasmids used in this study are shown in Table 2. Plasmid br434 was constructed by inserting the *PMA1* promoter fragment (*Hin*dIII/blunt–*Bam*HI) from pSPPMA1C (Supply *et al.*, 1993) into the pRS316 polylinker (*Eag*I/blunt, *Bam*HI). *ADC1* transcription terminator sequences (*Bam*HI–*Hin*dIII) from pAAH5 (Ammerer, 1983) were added into the *Sal*I site (blunt-ended), and the *SERCA1a* cDNA (Moutin *et al.*, 1994) was added into the *Eco*RI site (blunt-ended). Plasmid br435 was constructed by moving the expression casette (*Apa*I/blunt–*Sac*I) from br434 into the *Kpn*I(blunt)/*Sac*I sites of YEplac195 (Gietz and Sugino, 1988).

Analysis of CpY Sorting

Cultures (15 ml) in the defined growth medium were inoculated at 0.1 optical density (OD) units/ml and grown to 1–1.5 U/ml of OD at 600 nm. Cells were washed twiced with defined labeling medium and resuspended at 3.5 OD units/400 μl. Cells were treated with DTT (10 min), washed, resuspended, and converted to spheroblasts (zymolyase 100T) as described (Vida *et al.*, 1990; Horazdovsky and Emr, 1993). After washing, spheroblasts were incubated in defined labeling medium for 30 min in the presence of protease inhibitors, BSA and α2-macroglobulin. After the addition of ^{35}S-methionine (15 μCi/OD cells), aliquots (400 μl) were withdrawn at the times indicated and separated into supernatant and pellets. Processing of samples for immunoprecipitation of CpY (commercially available anti-CpY antibody; Molecular Probes, Eugene, OR), SDS-PAGE, and autoradiography was as described previously (Vida *et al.*, 1990; Horazdovsky and Emr, 1993).

Analyis of CpY Degradation and Deg1-β-Galactosidase Activity*

To assay CpY* degradation by pulse-chase analysis, cells were grown at 30°C in complete synthetic medium containing 2% glucose. For each time point, cells corresponding to 2.5 U of OD at 600 nm (0.75×10^8 to 1×10^8 cells) were taken and labeled with 62.5 μCi ^{35}S-methionine. Labeling and chase conditions, as well as all other experimental procedures, e.g., cell lysis, immunoprecipitation, and SDS-PAGE, were performed as described previously (Finger *et al.*, 1993). To monitor β-galactosidase activity in strains expressing Deg1-β-galactosidase, cells were grown as described for the analysis of CpY* degradation. After cycloheximide was added to a final concentration of 0.5 mg/ml at zero time (t = 0) to the logarithmically growing culture, cells corresponding to 0.3 U of OD at 600 nm (0.9×10^7 to 1.2×10^7 cells) were taken for each time point, mixed with lysis buffer (0.6% Triton-X 100, 0.75% M *O*-nitrophenyl β-D-galactopyranoside, 2.25% β-mercaptoethanol, 0.15 M Tris/HCl, pH 7.5), and kept at −80°C for 30 min. After a 60- to 90-min incubation at 37°C, 75 μl 1 M $NaHCO_3$ were added to the samples, debris was removed by centrifugation (20,000 × *g*, 3 min), and the OD_{405} was determined.

RESULTS

Mn^{2+} Can Replace Ca^{2+} to Promote Growth, but Is Unable to Sustain Vacuolar Protein Sorting

As a basis for subsequent studies, we first examined the growth of our wild-type strain in media depleted for either Ca^{2+} or Mn^{2+}. Following the published procedure (Loukin and Kung, 1995), we used BAPTA, a chelator of divalent cations (Tsien, 1980), to manipulate the free concentrations of Ca^{2+} and Mn^{2+} in liquid culture media, maintaining fixed free concentrations for other essential cations (see MATERIALS AND METHODS). As shown in Figure 1A, a Mn^{2+}-depleted medium (free $Mn^{2+} \approx 0.16$ pM) with a concentration of ≈1.4 μM free Ca^{2+} allows growth comparable to the BAPTA-free control medium. Similarly, efficient growth is observed in a Ca^{2+}-depleted medium (free $Ca^{2+} \approx 0.84$ nM) with a concentration of free Mn^{2+} of ≈ 20 nM. The growth observed in both media is dependent on the presence of free Ca^{2+} or Mn^{2+}, since simultaneous depletion of both ions completely inhibits growth (Figure 1A). Thus, our strain grows well at very low Ca^{2+} (≈ 0.84 nM) or Mn^{2+} (≈ 0.16 pM), in very good agreement with the data reported for another strain of *S. cerevisiae* (Loukin and Kung, 1995).

Since mutants lacking the Golgi Ca^{2+} adenosine triphosphatase Pmr1 partially secrete the vacuolar hydrolase CpY (Antebi and Fink, 1992), we wished to evaluate the individual effects of Ca^{2+} and Mn^{2+} ions on this sorting reaction. Wild-type cells were grown in BAPTA-free control medium and in BAPTA media depleted for either Ca^{2+} or Mn^{2+}. To monitor CpY sorting, cells were converted to spheroplasts and labeled with ^{35}S-methionine, maintaining the defined ionic conditions during all incubations. After 20 and 40 min, CpY secreted due to missorting was recovered from the culture supernatants by immunoprecipitation and analyzed by SDS-PAGE. As seen in Figure 1B, cells growing in control medium (no BAPTA, left panel in Figure 1B) with regular concentrations of total Ca^{2+} (≈5.7 μM) and Mn^{2+} (≈73 nM) fail to secrete CpY into the medium. Depletion of free Mn^{2+} from this medium has no apparent effect on sorting: CpY is not secreted into the medium (Figure 1B, right panel). However, the reduction of free Ca^{2+} to ≈ 0.84 nM, a concentration demonstrated to allow effficient growth (see Figure 1A, $-Ca^{2+}$), significantly impairs vacuolar sorting. Approximately 15–20% of total CpY is secreted into the medium; the molecular mass of the secreted material (69 kDa) is characteristic for the Golgi-associated p2 form of CpY (Figure 1B, middle

Table 1. Yeast strains

Strain	Genotype	Source
YGD57	YR98 *pmr1-Δ1::LEU2*/pKC46 [2μ, *PMC1*]	This study
YR98	*MATα ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52* (AA255)	Rudolph *et al.*, 1989
YR122	YR98 *pmr1-Δ1::LEU2* (AA274)	Rudolph *et al.*, 1989
YR123	YR98 *pmr1-Δ2::HIS3* (AA298)	Fink lab
YR437	W303-1B *pmr1::HIS3 pmc1::TRP1 cnb1::LEU2* (K616)	K. Cunningham
YR439	YR98 *pmr1-Δ1::LEU2*/YEp24	This study
YR441	YR98 *pmr1-Δ1::LEU2*/br327 [2μ *PMR1*]	This study
YR469	W303-1B *pmr1::HIS3 pmc1::TRP1 cnb1::LEU2*/YEp24	This study
YR472	W303-1B *pmr1::HIS3 pmc1::TRP1 cnb1::LEU2*/br327 [2μ *PMR1*]	This study
YR477	W303-1B *pmr1::HIS3 pmc1::TRP1 cnb1::LEU2*/br349 [2μ *VPS10*]	This study
YR480	YR98 *pmr1-Δ1::LEU2*/br356 [2μ *VPS10*]	This study
YR537	YR98 *pmr1-Δ1::LEU2 vps10::HIS3*	This study
YR547	YR98 *pmr1-Δ1::LEU2 vps10::HIS3*/YEp24	This study
YR549	YR98 *pmr1-Δ1::LEU2 vps10::HIS3*/br358 [*SUC2::VPS10-ΔN*]	This study
YR550	YR98 *pmr1-Δ1::LEU2 vps10::HIS3*/br375 [2μ *VPS10-1385*]	This study
YR551	YR98 *pmr1-Δ1::LEU2 vps10::HIS3*/br356 [2μ *VPS10*]	This study
YR657	YR98 *pmr1-Δ1::LEU2*/pKC11 [2μ, *PMR1*]	This study
YR663	YR98 *pmr1-Δ1::LEU2*/br434 [*CEN PMA1::SERCA1A::ADC1*]	This study
YR664	YR98 *pmr1-Δ1::LEU2*/br435 [2μ *PMA1::SERCA1A::ADC1*]	This study
YRP023	W303 *prc1-1 pmr1::HIS3*	This study
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
W303-1C	W303 *prc1-1*	Knop *et al.*, 1996

panel). Analysis of intracellular CpY under both conditions indicates that the bulk of CpY reaches the vacuole and is properly processed (Dürr and Rudolph, unpublished observations). Although we have not explored the kinetics of intracellular transport under both conditions, our data allow a firm conclusion: Ca^{2+} ions are required to sustain accurate sorting of CpY in wild-type cells.

Mn^{2+} Ions Stimulate Protein Glycosylation

pmr1 strains produce and secrete a form of invertase essentially lacking high-mannose outer chains (Rudolph *et al.*, 1989; Antebi and Fink, 1992). Due to this reduction in outer-chain glycosylation, invertase isolated from *pmr1* mutants (Figure 2A; two panels, *pmr1::LEU2* and *pmr1::HIS3*) migrates on native gels significantly faster than invertase isolated from a wild-type strain (Figure 2A; left panel, *PMR1*). However, we have found that the presence of Mn^{2+} ions (450 μM) during induction of invertase synthesis in low-glucose medium strongly stimulates invertase glycosylation in the *pmr1* mutants. As demonstrated in Figure 2A, invertase secreted from *pmr1* cells in Mn^{2+}-rich medium migrates similarly to the heterogeneous, high-molecular mass material seen with wild-type cells. In contrast, even high concentrations of Ca^{2+} (20 mM) exert only a modest effect on invertase glycosylation, in complete agreement with previous work (Antebi and Fink, 1992). This finding strongly suggests that the known defect in N-linked glycosylation in *pmr1* cells primarily results from an insufficient supply of Mn^{2+} ions.

Similarly, we analyzed the gel mobility of secreted chitinase, a protein exclusively O-glycosylated (Kuranda and Robbins, 1991) and a convenient indicator of O-glycosylating activity in vivo (Immervoll *et al.*, 1995; Gentzsch and Tanner, 1996). As shown in Figure 2B, chitinase secreted from *pmr1* mutants growing in regular YPD medium migrates faster than chitinase produced by a *PMR1* wild-type strain (Figure 2B; see lanes marked "−"). Apparently, *pmr1* cells are also defective in O-linked glycosylation. Interestingly, this increased mobility can, in part, be reversed by supplementing the medium with Mn^{2+} (250 μM), which causes *pmr1* cells to produce two forms of chitinase: a high-molecular mass form with essentially wild-type mobility, and a form migrating even faster than chitinase produced by *pmr1* in the absence of Mn^{2+}. In contrast, addition of Ca^{2+} (20 mM) to the *pmr1* cultures yields only one form of chitinase with intermediate mobility, indicating that Ca^{2+} is unable to restore production of fully glycosylated chitinase in *pmr1* cells. Taken together with the results on invertase, our findings suggests that the Pmr1 ion pump functions in vivo to supply the Mn^{2+} ions required for N-linked and O-linked glycosylation.

Two Ca^{2+} Pumps, Pmc1 and SERCA1a, Restore Growth of pmr1 on Ca^{2+}-Deficient and EGTA-Containing Media

pmr1 null mutants fail to grow on Ca^{2+}-deficient media (Rudolph *et al.*, 1989). In a search for yeast genes that, upon overexpression, could compensate this defect, we transformed a *pmr1* mutant with a YEp24-

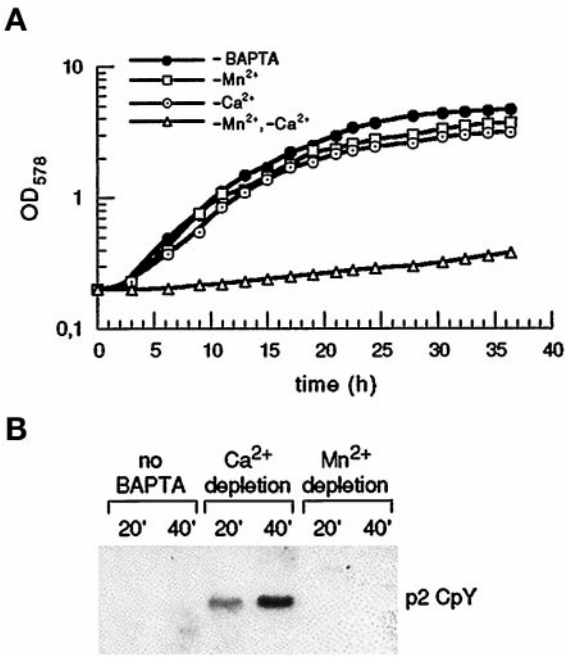

Figure 1. Growth and vacuolar sorting of *S. cerevisiae* in ion-depleted media. (A) Growth in Ca^{2+}- or Mn^{2+}-depleted media. Wild-type strain YR98 was inoculated into media containing BAPTA to buffer the free ion concentrations of Ca^{2+} and Mn^{2+} as described. Media: $-Mn^{2+}$ medium (1.4×10^{-6} M Ca^{2+}, 1.6×10^{-13} M Mn^{2+}), $-Ca^{2+}$ medium (8.4×10^{-10} M Ca^{2+}, 2.0×10^{-8} M Mn^{2+}), $-Mn^{2+}$, $-Ca^{2+}$ medium (8.4×10^{-10} M Ca^{2+}, 1.8×10^{-11} M Mn^{2+}). For the BAPTA-free control medium (−BAPTA), total ion concentrations (5.7×10^{-6} M Ca^{2+}; 7.3×10^{-8} M Mn^{2+}) were determined. (B) Depletion of Ca^{2+}, but not Mn^{2+}, induces partial secretion of CpY in wild type. *PMR1* cells (YR98), pregrown in the defined media given in panel A, were converted to spheroblasts and labeled with ^{35}S-methionine maintaining the defined free ion concentrations. After 20 min and 40 min, supernatants were analyzed for the presence of total CpY by immunoprecipitation, SDS-PAGE, and autoradiography. All samples showed a single band migrating with the molecular mass (69 kDa) of the Golgi form, p2 CpY. Each lane corresponds to 3.5 OD of cells.

based genomic library (Carlson and Botstein, 1982). In addition to *PMR1*, we found two distinct groups of library plasmids able to promote growth under these conditions. All plasmids also alleviated the hypersensitivity of the *pmr1* strain to EGTA. The first group of plasmids was found to harbor the *PMC1* gene, which encodes a Ca^{2+} pump in the yeast vacuolar membrane (Cunningham and Fink, 1994). To confirm that *PMC1* was solely responsible for the suppression, we transformed the *pmr1* mutant with the 2μ-based plasmid pKC46, carrying *PMC1* on a 4.9 kilobase (kb) *Hin*dIII fragment (gift from K. Cunningham). As evident from Figure 3A, this plasmid allowed vigorous growth of *pmr1* cells in the presence of EGTA, indicating that the vacuolar Ca^{2+} pump Pmc1 can indeed compensate the loss of Pmr1 during growth on Ca^{2+}-deficient or EGTA-containing media.

To confirm that suppression was indeed related to the activity of Pmc1 as a Ca^{2+} pump, we also tested SERCA1a from rabbit, a sarcoplasmic Ca^{2+} pump, for its ability to suppress the EGTA hypersensitivity of the *pmr1* mutant. To express SERCA1a in yeast, we inserted the *SERCA1a* cDNA (Moutin *et al.*, 1994) into a single-copy plasmid behind the strong, constitutive *PMA1* promoter and upstream of *ADC1* sequences to ensure transcription termination. As shown in Figure 3B, a single-copy plasmid with this casette (*CEN-SERCA1a*) strongly supports growth of *pmr1* cells on EGTA plates. A 2μ-based derivative (2μ-*SERCA1a*) harboring the same *PMA1::SERCA1a::ADC1* casette is much less effective (see Figure 3B), indicating that SERCA1a overexpression may exert toxic effects on *pmr1* cells. The data show that SERCA1a is functionally expressed and replaces Pmr1 under these conditions. To find suppression by two different Ca^{2+} pumps, Pmc1 and SERCA1a, a highly Ca^{2+}-specific ion pump, strongly suggests that changes in intracellular Ca^{2+} flux are sufficient to relieve the growth inhibition of *pmr1* on EGTA-containing media.

Vps10, a Receptor for Vacuolar Sorting, Also Suppresses EGTA hypersensitivity of pmr1

The second group of plasmids, which restored growth of *pmr1* on Ca^{2+}-deficient media, contained a copy of the *VPS10* gene. Transformation of the *pmr1* mutant

Table 2. Plasmids

Plasmid		Source
br327	2μ *PMR1*, isolate from library in Yep24 (Carlson and Botstein, 1982)	This study
br349	2μ *VPS10*, isolate from library in YEp24	This study
br356	*VPS10* (6-kb *Bgl*II fragment in pRS426; pEMY10-2)	E. Marcusson/S. Emr
br358	*SUC2::VPS10-ΔN* (pEMY101-pSEYc350)	E. Marcusson/S. Emr
br375	*VPS10-1385* (pEMY10-7)	E. Marcusson/S. Emr
br434	*CEN PMA1::SERCA1A::ADC1*	This study
br435	2μ *PMA1::SERCA1A::ADC1*	This study
pKC11	2μ PMR1 (4.38-kb *Pvu*II-*Spe*I fragment, in B2205)	K. Cunningham
pKC46	2μ PMC1 (4.9-kb *Hin*dIII-*Hin*dIII fragment, in B2205)	K. Cunningham

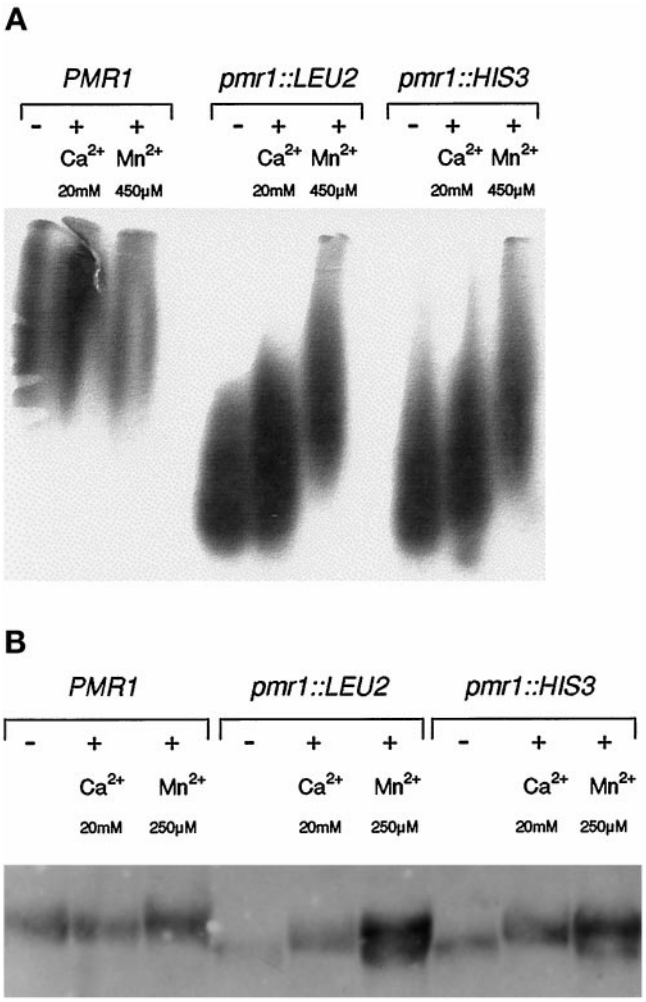

Figure 2. Mn^{2+} ions effectively stimulate glycosylation in *pmr1* mutants. (A) N-glycosylated invertase secreted by wild-type (*PMR1*) and *pmr1* mutant cells in unsupplemented medium (−), and after stimulation with Ca^{2+} or Mn^{2+} ions. Strains were pregrown in YPD (5% glucose), transferred to YPD (5% glucose, 1 h), supplemented with Ca^{2+} or Mn^{2+}, and induced for invertase production in YPD (0.1% glucose, 1 h) supplemented with Ca^{2+} or Mn^{2+} as indicated. Control cells were grown and induced for invertase in unsupplemented media (−). Analysis of external invertase by native-gel electrophoresis and subsequent activity staining was as described (Rudolph *et al.*, 1989). (B) O-glycosylated chitinase secreted by wild-type and *pmr1* cells upon stimulation with Ca^{2+} or Mn^{2+}. Cultures were grown overnight in YPD medium supplemented with Ca^{2+} or Mn^{2+} as indicated; control cells were grown in unsupplemented YPD medium (−). Secreted chitinase was affinity-purified using chitin powder (Guthrie and Fink, 1991) and analyzed by SDS-PAGE and Western blotting as described (Immervoll *et al.*, 1995). Strains: YR98 (*PMR1*), YR122 (*pmr1-Δ1::LEU2*), YR123 (*pmr1-Δ2::HIS3*)

with a plasmid solely containing *VPS10* (gift from E. Marcusson) confirmed that *VPS10* was indeed responsible for the suppression (shown in Figure 4A). The suppression of *pmr1* by elevated expression of Vps10 is not mediated through the vacuolar pump Pmc1, since suppression was still observed in a *pmr1 pmc1 cnb1* mutant (Figure 4B). The known functions of Vps10 in vacuolar sorting and in salvage of malfolded luminal proteins suggest that the *pmr1* growth defect in Ca^{2+}-deficient media may reside in the lumen of the secretory pathway.

To test whether Vps10 was indeed resolving a luminal defect, we examined two Vps10 derivatives (see Figure 4D) for their ability to suppress *pmr1*. A truncated form, Vps10–1385, consists of the first 1385 residues encompassing essentially the luminal domain of Vps10, but lacks the transmembrane segment and the cytoplasmic tail. The second variant, Suc2-Vps10(ΔN), represents an Invertase-Vps10 fusion protein, wherein the luminal domain of Vps10 is replaced by invertase sequences (gift from E. Marcusson). To avoid interference from wild-type Vps10 expressed from the chromosomal *VPS10* locus, these plasmids were transformed into a *pmr1 vps10* double mutant. As shown in Figure 4C, Vps10–1385 mediates suppression similar to wild-type Vps10, whereas replacement of the luminal part by invertase in Suc2-Vps10(ΔN) essentially abolishes suppression. Evidently, the luminal domain of Vps10 is necessary and sufficient to allow vigorous growth of the *pmr1 vps10* strain on EGTA. As shown in Figure 4E, *pmr1* cells secrete the bulk of Vps10–1385 in intact form during growth in EGTA-containing medium, i.e., under conditions where growth is dependent on Vps10–1385. Taken together, our data show that Vps10-mediated suppression resolves a growth-limiting step located within the lumen of early secretory organelles, either in the ER or in Golgi compartments. One of the known functions of Vps10, the salvage of malfolded proteins to the vacuole (Hong *et al.*, 1996), suggests that *pmr1* cells may have a defect in protein folding and/or in the degradation of malfolded luminal proteins, thereby increasing the need for Vps10 function.

DER5, a Gene Involved in ER-Associated Protein Degradation, Is Identical to PMR1

In a genetic approach to dissect components of ER-associated protein degradation, we previously isolated different *der* mutants unable to degrade CpY*, a mutant form of carboxypeptidase Y (Finger *et al.*, 1993; Knop *et al.*, 1996). One mutant in this collection, *der5*, showed phenotypes similar to *pmr1*, such as hypersensitivity to EGTA and Mn^{2+}. Cloning of *DER5*, DNA sequencing, and tetrad analysis of a diploid heterozygous for the *der5* mutation and a *LEU2*-marked *pmr1* allele demonstrated that *DER5* is identical to *PMR1* (our unpublished observations). Therefore, we introduced a *pmr1::LEU2* null allele (Rudolph *et al.*, 1989) into our wild-type strain engineered to express CpY* and used this *pmr1* null mutant together with its congenic parent for further comparisons.

The wild-type strain degrades CpY* with a half-time of 20 min (Finger *et al.*, 1993). However, introduction of the *pmr1::LEU2* mutation drastically reduces the rate of CpY* degradation as demonstrated by the pulse-chase analysis shown in Figure 5A. Quantitation of these results reveals that the half-life of CpY* is increased nearly fourfold in the *pmr::LEU2* strain (Figure 5B). To gain further insight into the nature of this degradation defect, we introduced into our strains a plasmid directing synthesis of a purely cytosolic protein, Deg1-β-galactosidase. As was demonstrated for CpY* (Hiller *et al.*, 1996), Deg1-β-galactosidase is degraded by the proteasome exclusively via an *UBC6*-, *UBC7*-dependent mechanism (Chen *et al.*, 1993). As shown in Figure 5C, the kinetics of degradation for this substrate, as reflected in the decrease in β-galactosidase activity, are virtually identical in both strains. Since Deg1-β-galactosidase is not stabilized in the *pmr1* mutant, the ubiquitin–proteasome pathway for degradation of CpY* appears to be functional in the *pmr1* mutant. Thus, the observed stabilization of CpY* suggests that loss of Pmr1 function diminishes export of CpY* from the ER into the cytosol.

Loss of PMR1 Causes Phenotypes Characteristic for Perturbations in the UPR

Based on the reduced capability of a *pmr1* mutant to clear malfolded CpY* from the ER, we hypothesized that *pmr1* cells might be particularly sensitive to an increase in misfolded proteins within the ER. To test this idea, we examined the growth of *pmr1* cells in the presence of DTT and tunicamycin, two drugs known to induce accumulation of malfolded proteins in the ER. As demonstrated in Figure 6, A and B, the *pmr1* mutant is hypersensitive to both drugs. Similar phenotypes have been reported for mutants defective in the UPR pathway, which coordinately regulates synthesis of ER chaperones and ER membrane biogenesis in response to several stress signals. Mutants blocked in UPR also exhibit, in addition to hypersensitivity toward DTT and tunicamycin, auxotrophy for inositol (for review, see Shamu *et al.*, 1994). As demonstrated in Figure 6B (left panel), *pmr1* cells also require inositol. According to these data, *pmr1* cells could have some general defect in the UPR response.

To examine the nature of this defect in more detail, we monitored induction of Kar2 in wild-type and *pmr1* cells in response to tunicamycin. As shown in Figure 6, C and D, Kar2 is induced in the *pmr1* mutant, throughout a 3-h challenge with tunicamycin, to a level even slightly higher than in wild type. The modest induction of Kar2 in these experiments might be due to the genetic background of our strains or reflect experimental conditions. However, we obtained very similar kinetics with reporter plasmids containing UPR elements, the DNA sequences mediating transcriptional activation during UPR (Mori *et al.*, 1992; Strayle and Rudolph, unpublished observations). Our findings indicate that the apparent sensitivity of *pmr1* cells to experimentally induced accumulation of malfolded proteins in the ER, if indeed related to UPR function, is not due to an immediate, complete block in signaling through the UPR cascade.

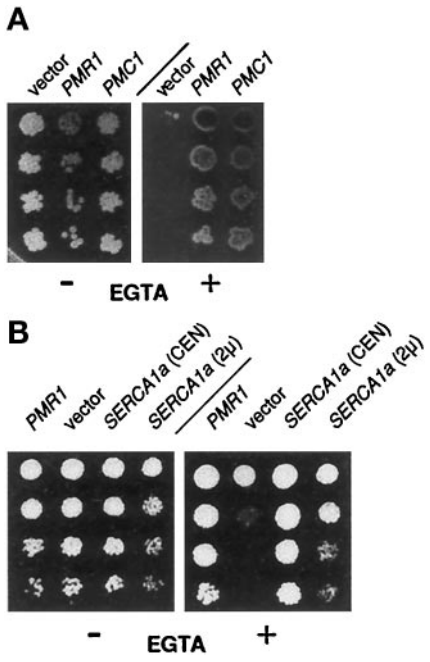

Figure 3. Expression of Ca^{2+} pumps restores growth of a *pmr1* mutant on EGTA-containing media. (A) Expression of the vacuolar ion pump Pmc1 in *pmr1* cells. Serial fivefold dilutions of saturated cultures were spotted onto complete medium lacking uracil to maintain selection for the plasmids. Addition (+) or omission (−) of 6.5 mM EGTA is indicated. Plates were photographed after 3 d incubation at 30°C. Strains (from left to right): YR439 (*pmr1*; 2μ-vector); YR657 (*pmr1*, 2μ-*PMR1*) and YGD57 (*pmr1*, 2μ-*PMC1*). (B) Expression of a sarcoplasmic Ca^{2+} pump, SERCA1a (rabbit). Conditions as in panel A; strains are (from left to right): YR441 (*pmr1*, 2μ-*PMR1*), YR439 (*pmr1*, 2μ-vector), YR663 (*pmr1*, *CEN-SERCA1a*), and YR664 (pmr1, 2μ-*SERCA1a*).

DISCUSSION

S. cerevisiae can grow at very low Ca^{2+} concentrations ($< 10^{-9}$ M) if a sufficient supply of free Mn^{2+} is available. Vice versa, growth at very low Mn^{2+} concentrations ($< 10^{-12}$ M) is sustained by free Ca^{2+} (Loukin and Kung, 1995). This apparent interchangeability of Ca^{2+} and Mn^{2+}, which we confirmed for the wild-type strain used in the present study, implies that both ions have access to secretory organelles expected to host vital processes dependent on one of the two cations. We have provided evidence that the Golgi-resident ion pump Pmr1 operates as a common transporter for Ca^{2+} and Mn^{2+} in vivo. Mn^{2+} ions are

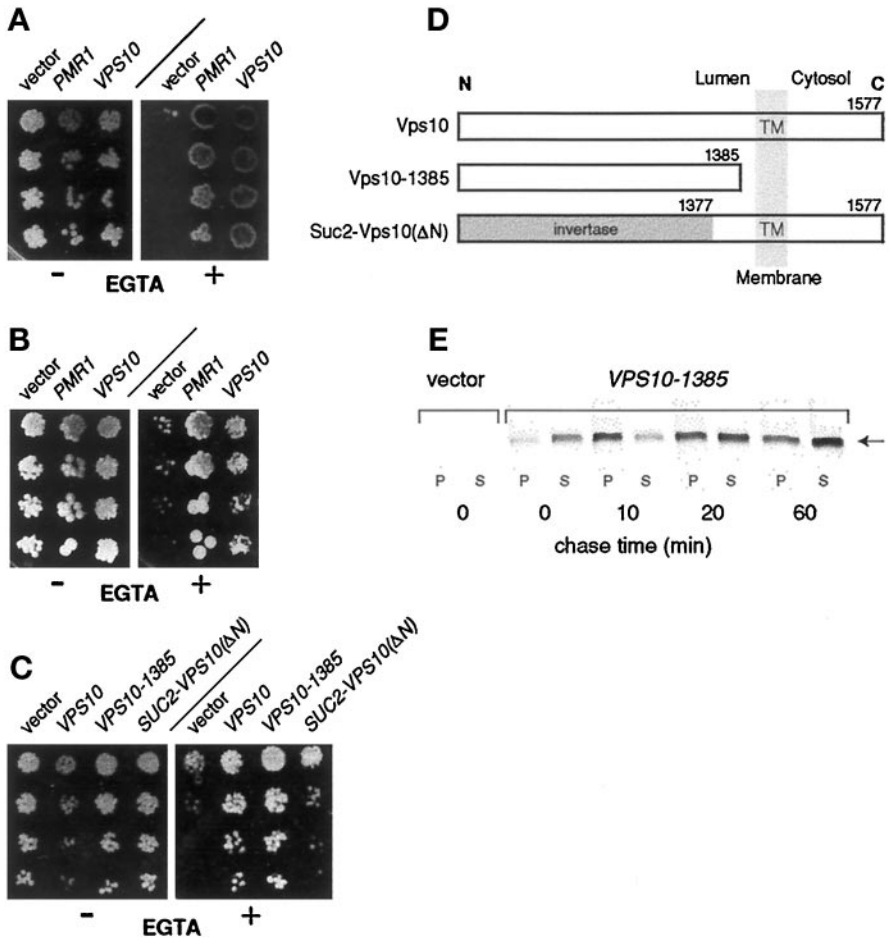

Figure 4. Suppression of EGTA hypersensitivity in *pmr1* cells by the sorting receptor Vps10 is mediated through its luminal domain. (A) Suppression by wild-type Vps10. Serial fivefold dilutions of saturated cultures were spotted onto complete medium lacking uracil. Addition (+) or omission (−) of 6.5 mM EGTA is indicated. Plates were photographed after 3 d incubation at 30°C; strains are (left to right): YR439 (*pmr1*, 2μ-vector), YR657 (*pmr1*, 2μ-*PMR1*), and YR480 (*pmr1*, 2μ-*VPS10*). (B) Vps10-mediated suppression does not require Pmc1. Conditions are as in panel A. Strains (from left to right): YR469 (*pmr1 pmc1 cnb1*, 2μ-vector), YR472 (*pmr1 pmc1 cnb1*, 2μ-*PMR1*), and YR477 (*pmr1 pmc1 cnb1*, 2μ-*VPS10*). (C) The luminal domain of Vps10 is necessary and sufficient for suppression. Same conditions as in panel A; strains are (from left to right): YR547 (*pmr1*, 2μ-vector), YR551 (*pmr1*, 2μ-*VPS10*), YR550 (*pmr1*, 2μ-*VPS10–1385*), and YR549 (*pmr1*, 2μ-*SUC::VPS10*-Δ*N*). (D) Vps10 derivatives used in the analysis. The transmembrane topology of Vps10 and its derivatives is depicted; numbers indicate the Vps10 residues present in each derivative. (E) Vps10–1385 is secreted by *pmr1* cells during growth in EGTA-containing medium. Cells were cultured in synthetic complete medium, converted to spheroblasts, and labeled (15 μCi/OD cells) with ^{35}S-methionine as described (Horazdovsky and Emr, 1993), but EGTA (3 mM) was present during these steps. At the times indicated, cells and media supernatants were analyzed for the presence of Vps10–1385 by immunoprecipitation, SDS-PAGE, and autoradiography. The band corresponding to Vps10–1385 in SDS-PAGE is marked (arrow). Strains: YR547 (*pmr1*, 2μ-vector) and YR550 (*pmr1*, 2μ-*VPS10–1385*).

required for the addition of complex carbohydrates onto N- and O-glycosylated proteins in the Golgi, whereas intralumenal Ca^{2+} is a prerequisite for accurate vacuolar sorting. Most important, we have demonstrated that Pmr1-mediated ion transport also affects processes in the ER. In particular, we have shown that CpY*, a malfolded protein normally degraded in the cytosol after export from the ER, is stabilized in *pmr1* mutants due to an intralumenal defect. Furthermore, we have demonstrated that *pmr1* mutants are sensitive to conditions inducing accumulation of malfolded proteins in the ER. We propose that the cation content of the yeast ER is critically determined by the secretory pathway pump Pmr1, which also appears to be necessary for an appropriate response of the ER to stress conditions.

The function of Pmr1 as an ATP-driven Ca^{2+} pump was recently demonstrated by assaying Ca^{2+} uptake into purified Golgi-derived vesicles (Sorin *et al*., 1997). Genetically, the role of Pmr1 in Ca^{2+} transport is manifest in *pmr1* mutants in several Ca^{2+}-related phenotypes, one of which is a partial defect in CpY sorting. In media with approximately 200 μM Ca^{2+}, *pmr1* cells mis-sort and secrete a small fraction ($< 7\%$) of the

Golgi form of CpY, normally routed to the vacuole in wild-type cells. Addition of Ca^{2+} (10 mM) fully restores CpY sorting (Antebi and Fink, 1992). In addition, the free cytosolic Ca^{2+} in *pmr1* cells is known to be elevated relative to wild type, and cytosolic Ca^{2+} increases even further upon addition of extracellular Ca^{2+} (Halachmi and Eilam, 1996). Therefore, the reversibility of the CpY-sorting defect in *pmr1* cells by external Ca^{2+} suggests that a low intralumenal Ca^{2+} content within some secretory organelle(s), rather than an elevated cytosolic Ca^{2+} concentration, is causing mis-sorting of CpY. This view is now corroborated by our finding that severe Ca^{2+} depletion in wild-type cells induces the same effect on CpY sorting as a *pmr1* mutation, i.e., partial secretion of CpY. These data also provide a clear demonstration that chelators can effectively induce cation depletion in secretory organelles of growing yeast cells.

The aberrant secretion of CpY from wild-type cells is not caused by the Mn^{2+} ions present in the Ca^{2+}-depleted medium to support growth, since chelator-free medium with a similar concentration of Mn^{2+} allows for accurate sorting. In addition, the growth rates in Ca^{2+}- or Mn^{2+}-depleted media are very similar, suggesting that either cation alone does not drastically alter bulk flow through the secretory pathway. Based on the ER-associated changes we observed in *pmr1* cells (see Figure 5), it seems likely that Ca^{2+} depletion leads to an increased production or reduced turnover of malfolded proteins, which might then utilize the Vps10-mediated salvage pathway to the vacuole and could thereby compete with CpY for binding to the Vps10 receptor. Likewise, the biogenesis or function of Vps10 might be compromised under low intralumenal Ca^{2+} conditions. Alternatively, luminal Ca^{2+} could directly promote specific sorting steps, such as partitioning of receptor–cargo complexes into a budding vesicle or binding of CpY to Vps10, but the Ca^{2+} dependence of these reactions has not been explored in vitro (for binding of CpY to Vps10, see Cooper and Stevens, 1996).

A role of Pmr1 in Mn^{2+} transport was first suggested based on changes in intracellular Mn^{2+} distribution observed in *pmr1* cells, which appear to possess an increased cytosolic Mn^{2+} level (Lapinskas *et al.*, 1995). The present study extends this observation by showing that *pmr1* cells display Mn^{2+}-related defects in glycosylation reactions that take place in the lumen of the Golgi. We have shown that chitinase, a solely O-glycosylated secretory protein, is produced by *pmr1* cells in an aberrant form migrating during SDS-PAGE significantly faster than chitinase isolated from a wild-type strain. A similar behavior was previously reported for N-glycosylated invertase, which in *pmr1* cells lacks the complex carbohydrate chains normally added in the Golgi (Antebi and Fink, 1992). As we have shown, addition of Mn^{2+} stimulates *pmr1* cells to produce high-molecular mass forms of chitinase and invertase. Since the mannosyltransferase activities responsible for carbohydrate addition onto N- and O-glycosylated precursors in the yeast Golgi require

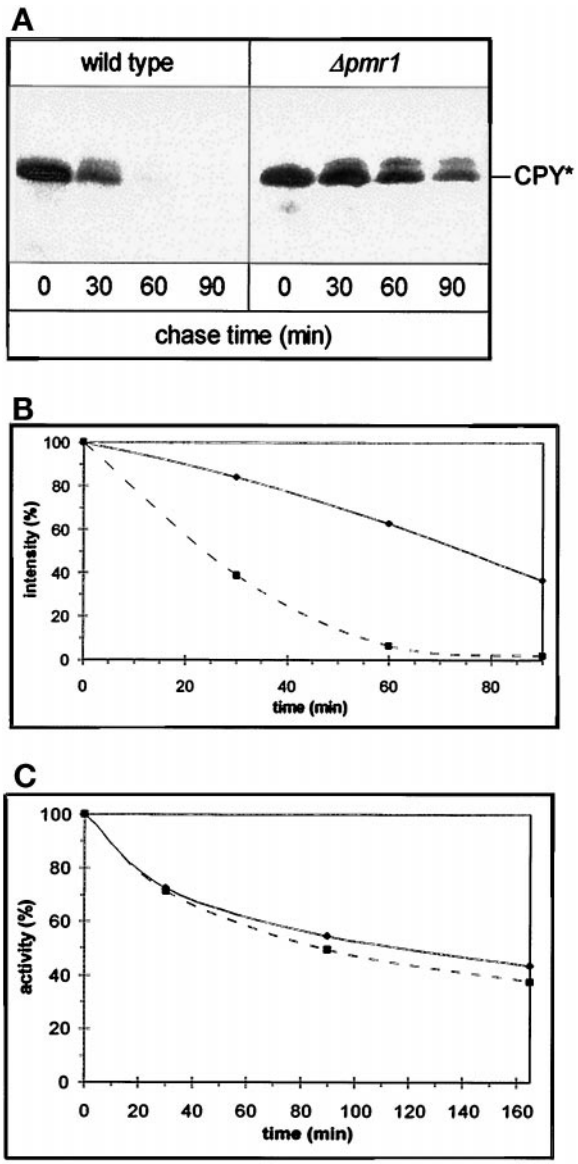

Figure 5. Pmr1 is required for ER-associated degradation. (A) CpY* is stabilized in the *pmr1* mutant. Pulse-chase analysis of CpY* was performed using the congenic strains W303–1C (wild type, ■) and YRP023 (Δ*pmr1*, ◆). Cells were grown at 30°C. At the indicated chase times, cells were lysed, and CpY* was immunoprecipitated. Antigenic material was separated by SDS-PAGE; the band corresponding to CpY* is indicated. (B) Quantification of the results shown in panel A using a Molecular Dynamics imaging system. (C) The Ubc6/Ubc7-dependent proteasome degradation system is functional in *pmr1*. β-Galactosidase activity was tested after alkaline lysis of transformants of W303–1C (*PMR1*, ■) and YRP023 (Δ*pmr1*, ◆) expressing the plasmid-encoded fusion protein Deg1-β-galactosidase (Chen *et al.*, 1993).

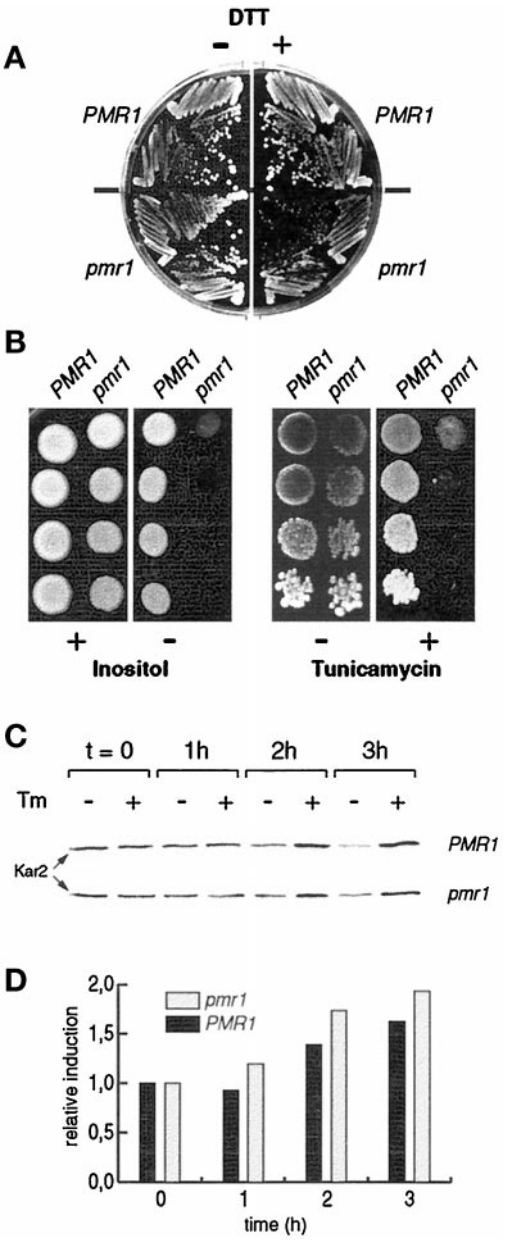

Figure 6. Mutations in *PMR1* affect the response to induced accumulation of malfolded proteins in the ER. (A) The *pmr1* mutant is sensitive to DTT. *PMR1* (YR98) and *pmr1* (YR122) cells were streaked onto YPD media, supplemented with 0.2% DTT (+) or without addition of DTT (−). Plates were photographed after 3 d incubation at 30°C. (B) The *pmr1* mutant displays inositol auxotrophy and hypersensitivity to tunicamycin. Serial fivefold dilutions of saturated cultures were spotted onto synthetic media (left panel) containing (+) or lacking (−) inositol and onto YPD media containing (+) or lacking (−) tunicamycin (0.3 μg/ml, right panel). Plates were photographed after 3 d incubation at 30°C. Strains: YR98 (*PMR1*) and YR122 (*pmr1*). (C) Induction of Kar2 after treatment with tunicamycin. Tunicamycin (1 μg/ml) was added to cells growing in YPD (OD_{600} = 0.8). After 1 h, 2 h, and 3 h, cell extracts were prepared and analyzed for the presence of Kar2 by SDS-PAGE and Western blotting. Each lane corresponds to 20 μg of total protein. Strains: YR98 (*PMR1*) and YR122 (*pmr1*). (D) PhosphoImager quantitation of the results shown in panel C. For each strain, signals are expressed as ratio of relative induction; the values at t = 0 were arbitrarily set to 1. Strains: YR98 (*PMR1*) and YR122 (*pmr1*).

Mn^{2+} in vitro (Sharma *et al*., 1974; Nakajima and Ballou, 1975; Parodi, 1979; Haselbeck and Schekman, 1986), we assume that the altered molecular mass in the different forms of chitinase and invertase reflects changes in carbohydrate content, although this has only been demonstrated for invertase produced by *pmr1* cells (Antebi and Fink, 1992).

Glycosylation reactions in the Golgi complex of mammalian cells display similar Mn^{2+} requirements (Sugiura *et al*., 1982; Elhammer and Kornfeld, 1986). In one study, defects in O- and N-linked glycosylation were generated in vivo by A23187-induced cation depletion and shown to be partially reversible by the addition of Mn^{2+}, but not Ca^{2+}. Interestingly, Mn^{2+} addition in the presence of A23187 produced two distinct populations of secreted molecules (macrophage colony-stimulating factor): one form carried fully restored complex N-linked and O-linked carbohydrates; the other one lacked O-linked oligosaccharides, but displayed high-mannose N-linked carbohydrates (Kaufman *et al*., 1994). Our results with chitinase produced upon Mn^{2+} addition are very similar: *pmr1* cells also secrete two populations of chitinase under these conditions. This "all or none" behavior in glycosylation could reflect secretion of chitinase from different secretory compartments or point to some other defect within the secretory pathway, presumably caused by a partial Ca^{2+} depletion in the absence of Pmr1. The appearance of a single, intermediate form of chitinase upon addition of Ca^{2+} to *pmr1* cells favors this explanation. Likewise, Mn^{2+} addition does not restore full glycosylation onto invertase in *pmr1* cells, and the sole addition of Ca^{2+} appears to have a slight stimulatory effect. Thus, the faithful addition of carbohydrates onto O- and N-glycosylated proteins seems to require an intricate balance of intralumenal Ca^{2+} and Mn^{2+} levels within the secretory pathway. We have also noticed that the two *pmr1* strains, derived from a common *his3 leu2* parent strain by transformation, produce the two chitinase forms in different amounts (see Figure 2B). The particulary strong response to Mn^{2+} observed in the *pmr1::LEU2* strain could be due to the *his3* mutation, which is complemented in the *pmr1::HIS3* strain. Histidine is a fairly good chelator of Mn^{2+} with a dissociation constant of 20 nM (see Hughes and Poole, 1991, and references therein), and is stored in high amounts by yeast vacuoles (Kitamoto *et al*., 1988), which also accumulate divalent cations, including Ca^{2+}, Mg^{2+}, Mn^{2+}, and other heavy metals (Okorokov *et al*., 1978; Ohsumi and Anraku, 1983; Bode *et al*., 1995). The

ability to use the full capacity of the endogenous biosynthetic pathway to supply histidine for sequestration of Mn^{2+} into the vacuole might, to some extent, attenuate the high Mn^{2+} conditions in *pmr1::HIS3* cells, and thereby indirectly affect chitinase glycosylation.

Two sets of data, one of which relates to the failure of *pmr1* cells to effectively degrade CpY*, indicate that Pmr1-mediated ion transport affects intralumenal processes hosted in a secretory compartment outside the Golgi, presumably within the ER. The observed stabilization of CpY* appears to reflect an intralumenal defect, since the same *pmr1* strain is able to degrade cytosolic Deg1-β-galactosidase. Deg1-β-galactosidase is, like CpY* (Hiller *et al.*, 1996), an Ubc6-/Ubc7-dependent substrate of the proteasome due to the presence of the Deg1 domain, which is responsible for ubiquitination of Matα2 by the ubiquitin-conjugating enzymes Ubc6 and Ubc7 (Chen *et al.*, 1993). Overexpression of the luminal domain of Vps10 (Vps10–1385), which we demonstrated to suppress EGTA hypersensitivity in *pmr1* strains, does not affect the high steady-state level of CpY* in *pmr1* cells, indicating that CpY* accumulation is not indirectly caused by a potentially Ca^{2+}-controlled redistribution of malfolded proteins between Vps10-dependent and -independent degradative pathways (Plemper, Strayle, Wolf, and Rudolph, unpublished data). In contrast to this, expression of the SERCA1a Ca^{2+} pump, which in *PMR1* wild-type cells accumulates in proliferating ER membranes (Catty, unpublished data), reduces the steady-state level of CpY* in *pmr1* cells (Plemper, Strayle, Wolf, and Rudolph, unpublished data). These findings suggest that a sufficient level of ER Ca^{2+} is necessary to accomplish export of CpY* into the cytosol for degradation by the proteasome. A requirement for Kar2, the yeast BiP homolog, in CpY* export has been demonstrated (Plemper *et al.*, 1997). Thus, the observed effects of Pmr1 on CpY* degradation could result from cation-dependent functions of the Kar2 chaperone. Remarkably, mammalian BiP was shown to undergo autophosphorylation, which in vitro is stimulated by Ca^{2+} and inhibited by Mn^{2+}, and the resulting BiP isoforms display altered properties in protein binding and oligomerization in vivo (Hendershot *et al.*, 1988; Leustek *et al.*, 1991; Carlino *et al.*, 1992). Similar, cation-dependent changes in Kar2 phosphorylation could reduce the level of Kar2 available for CpY* export and presumably affect other Kar2-mediated reactions, including protein folding.

The second set of data pointing to an ER-related function of Pmr1 concerns the EGTA hypersensitivity of *pmr1* cells. EGTA and BAPTA both lower the divalent cation content of secretory organelles in animal cells, and the partial mis-sorting of CpY we observed here with wild-type cells provides direct evidence for a similar effect of chelators on yeast cells. The strong suppression of EGTA hypersensitivity by Vps10–1385, an entirely luminal polypeptide secreted under these conditions, further confirms the inferred intralumenal nature of the EGTA-induced growth inhibition in *pmr1* cells. Based on the function of Vps10 in salvage of non-native luminal proteins to the vacuole (Hong *et al.*, 1996), we hypothesize that EGTA-induced cation depletion in some secretory organelle(s) of *pmr1* cells might generate malfolded proteins, which above a certain threshold could cause growth inhibition. As shown here, accumulation of CpY* is already occurring during growth of *pmr1* cells in a normal ionic milieu, indicating that the ER of *pmr1* cells is particularly sensitive to cation depletion. Thus, we suspect that the EGTA-induced growth inhibition originates in this compartment. It is not clear, however, whether reduced export of misfolded proteins from the ER to the cytosol, a prerequisite for degradation of CpY* and perhaps other malfolded ER proteins, is responsible for the growth inhibtion by EGTA. Alternatively, a low cation content in the ER could compromise protein folding to an extent exceeding the capacity of the Vps10 salvage pathway, which might also be affected under these conditions. Nevertheless, expression of either Ca^{2+} pump, yeast Pmc1, or heterologous SERCA1a restores growth of *pmr1* cells in the presence of EGTA, suggesting that the mechanisms underlying EGTA hypersensitivity are Ca^{2+}-dependent.

Interestingly, the loss of Pmr1 leads to phenotypes also observed in yeast mutants with a blocked UPR, which coordinates synthesis of ER-resident chaperones and ER membrane biogenesis under a variety of ER stress conditions (Cox *et al.*, 1997; see Shamu, 1997 and references therein). As we have shown, induction of Kar2 after tunicamycin challenge proceeds unperturbed for at least 3 h in *pmr1* cells, suggesting that the regulatory branch in the UPR to increase chaperone synthesis is functional. It needs to be tested whether *pmr1* cells are unable to sustain prolonged induction of chaperone synthesis or fail to induce *INO1*, the gene encoding inositol-1-phosphate synthase required for synthesis of phosphatidylinositol. It is also possible that an altered cation distribution in *pmr1* cells indirectly affects phospholipid biosynthetic enzymes requiring Mg^{2+}, Mn^{2+}, or, as in one case, Ca^{2+} (see Paltauf *et al.*, 1992 and references therein). Based on our study, which emphasizes the importance of Pmr1-mediated ion transport for early secretory organelles, including the ER, we also suspect that the Pmr1 ion pump might be directly required during UPR to uphold a favorable ionic milieu within an expanding ER.

As this study has shown, the Pmr1 ion pump sustains a variety of processes hosted in different compartments of the secretory pathway (Figure 7). Transport of Mn^{2+} ions by Pmr1 is required for the addition of complex carbohydrates onto N- and O-glycosylated

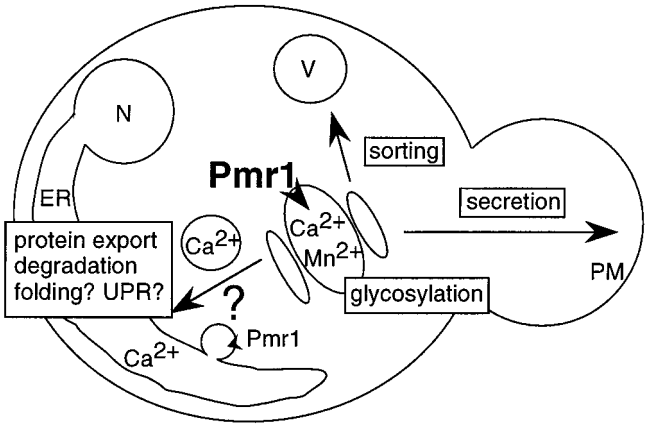

Figure 7. Model for the function of the secretory pathway pump Pmr1. Pmr1 pumps Ca^{2+} and Mn^{2+} into the secretory pathway, predominantly at the *medial*-Golgi (Pmr1, boldface). Both ions could either uniformly enter transport vesicles or be selectively recruited (or excluded). Such mechanisms could serve to enrich Ca^{2+} in the ER, or to reduce Mn^{2+}, despite the use of a single pump to transport both ions. In the ER, which contains only a small amount of Pmr1 in the membrane (Pmr1, small font), Pmr1 activity is required to export malfolded proteins into the cytosol for degradation, but other processes are likely to be affected (folding, UPR). In the Golgi, Mn^{2+} is required for protein glycosylation, whereas Ca^{2+}, directly or indirectly, sustains vacuolar sorting. Thus, Pmr1 could control the secretory pathway at several stages. Reduced Pmr1 activity could induce the secretion of nonnative proteins, otherwise retained for ER-associated degradation or salvaged by the vacuole; the first isolation of a *pmr1* mutant in a screen for "supersecretion" of heterologous proteins from yeast supports this view (Smith *et al*., 1985). Abbreviations used: N, nucleus; ER, endoplasmic reticulum; V, vacuole; PM, plasma membrane.

proteins in the Golgi. Within the ER, the export of malfolded proteins like CpY* and, presumably, other cation-dependent intralumenal processes rely on the Pmr1 ion pump to provide an adequate milieu in the lumen of this compartment. It remains unclear whether Ca^{2+} and Mn^{2+} ions both support these ER-associated processes. Even under conditions of extreme Ca^{2+} depletion, wild-type cells appear to retain about 3% of their normal total Ca^{2+} content (Loukin and Kung, 1995), a level perhaps sufficient to support strictly Ca^{2+}-dependent processes in the ER. Another interesting question is how Pmr1 exerts its function in different secretory organelles (see Figure 7). Pmr1 could simultaneously operate in the Golgi and in the ER if the very low amount of Pmr1 in the ER membrane could transport sufficient ions into this compartment. Alternatively, Pmr1 activity might be restricted to the *medial*-Golgi, where Ca^{2+} and Mn^{2+} ions would enter the secretory pathway to be appropriately distributed into other organelles, including the ER. Such remote control of the ER cation content by a distant *medial*-Golgi ion pump, which presumably lacks the high Ca^{2+} specificity of SERCA-type ER pumps, would provide yeast cells with a mechanism to evade high Mn^{2+} conditions in the ER, despite the use of a single pump for Ca^{2+} and Mn^{2+} transport. The hypersensitivity of *pmr1* cells toward Mn^{2+} is partially relieved by Vps10–1385 (Klee, Strayle, and Rudolph, unpublished observation), suggesting that an unbalanced intralumenal Mn^{2+} level perturbs processes within the early secretory pathway. Future studies on Pmr1, sometimes referred to as "the yeast secretory pathway pump," together with the use of *pmr1* cells to express SPCA, a putative rat homolog (Gunteski-Hamblin *et al*., 1992), should ultimately provide a paradigm to understand the function of the secretory pathway pump in all eukaryotes.

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