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Golgi Manganese Transport Is Required for Rapamycin Signaling in *Saccharomyces cerevisiae*

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ABSTRACT

The Pmr1 Golgi Ca^{2+}/Mn^{2+} ATPase negatively regulates target of rapamycin complex (TORC1) signaling, the rapamycin-sensitive TOR complex in *Saccharomyces cerevisiae*. Since *pmr1* causes resistance to rapamycin and *tor1* causes hypersensitivity, we looked for genetic interactions of *pmr1* with *tor1*. Deletion of *TOR1* restored two wild-type phenotypes. Loss of *TOR1* restored the ability of the *pmr1* strain to grow on media containing 2 mM $MnCl_2$ and conferred wild type as well as the wild-type sensitivity to rapamycin. Mn^{2+} additions to media partially suppressed rapamycin resistance of wild type and *pmr1 tor1*, suggesting that Tor1 and Tor2 are regulated by manganese. We parsed the roles of Ca^{2+} and Mn^{2+} transport and the compartments in rapamycin response using separation-of-function mutants available for Pmr1. A strain containing the D53A mutant (Mn^{2+} transporting) of Pmr1 is rapamycin sensitive, but the Q783A mutant (Ca^{2+} transporting) strain is rapamycin resistant. Mn^{2+} transport into the Golgi lumen appears to be required for rapamycin sensitivity. Overexpression of Ca^{2+} pump SERCA1, Ca^{2+}/H^{+} antiporter Vcx1, or a Mn^{2+} transporting mutant of Vcx1 (Vcx1-M1) failed to restore rapamycin sensitivity, and loss of Pmr1 but not other transporters of Ca^{2+} or Mn^{2+} results in rapamycin resistance. Overexpression of Ccc1, a Fe^{2+} and Mn^{2+} transporter that has been localized to Golgi and the vacuole, does restore rapamycin sensitivity to *pmr1*Δ. We conclude that Mn^{2+} in the Golgi inhibits TORC1 signaling.

PMR1 is a member of the P-type ATPase family of ion transporters (Rudolph *et al.* 1989; Antebi and Fink 1992). P-type ATPases bind ATP and are phosphorylated at a step in their transport cycles (Vanoevelen *et al.* 2005). Pmr1 transports Ca^{2+} or Mn^{2+} ions with high affinity from the cytoplasmic space into compartments of the secretory pathway (Rudolph *et al.* 1989). Pmr1 helps maintain cytoplasmic Ca^{2+} concentration at low values that inhibit signaling to Ca^{2+} effectors until a stimulus, often a cellular stress, induces a transient rise in cytoplasmic Ca^{2+} (Cyert 2003; Mulet *et al.* 2006). A *pmr1* mutant increases cytosolic Ca^{2+} and increases Ca^{2+} entry and accumulation in the vacuole. Pmr1 also provides compartments of the secretory pathway lumenally with Ca^{2+} and Mn^{2+}. There are possible roles for both ions within the Golgi, including roles in protein modification, in regulation of sorting and vesicular traffic, and in removal of toxic levels of ions. Mannosyltransferases present in the Golgi require Mn^{2+} as a metal cofactor (Lisman 2004; Lobsanov *et al.* 2004). Crystal structures of several glycosyltransferases show Mn^{2+} binds to a conserved DXD motif in the catalytic site (Gastinel *et al.* 2001; Persson *et al.* 2001; Lobsanov *et al.* 2004). Strains lacking *PMR1* have defects in N- and O-linked glycosylations (Rudolph *et al.* 1989; Olivero *et al.* 2003). The secretory pathway is also used to remove metal ions, such as Mn^{2+}, that exceed physiologic levels. *PMR1* mutants are notably sensitive to high concentrations of extracellular Mn^{2+} (Lapinskas *et al.* 1995).

Recently we reported that *Saccharomyces cerevisiae* strains with a *pmr1* deletion have increased resistance to rapamycin (Devasahayam *et al.* 2006). Rapamycin is an immunosuppressive that in complex with Fpr1 (FKBP12) inhibits a subset of functions of the *t*arget *o*f *r*apamycin (TOR) proteins (Crespo and Hall 2002). There are two *TOR* genes (*TOR1* and *TOR2*) in *S. cerevisiae* (reviewed in Crespo and Hall 2002; De Virgilio and Loewith 2006) that encode large (~280 kDa) proteins highly conserved throughout evolution that have atypical serine/threonine protein kinase activity, yet are related to phosphatidylinositol 3-kinase protein kinases. *TOR2*, but not *TOR1*, is essential in *S. cerevisiae*. TOR is found in two conserved complexes (TORC1 and TORC2) with distinct composition and function (Crespo and Hall 2002; De Virgilio and Loewith 2006); TORC1, but not TORC2, is inhibited by rapamycin. Deletion of *TOR1* consistently causes rapamycin hypersensitivity.

TOR is a central regulator of cell growth and promotes an increase in cell size but not cell number (Edgar 2006). How TOR regulates cell growth and how nutrient signals regulate TOR are intensely studied problems (Wullschleger *et al.* 2006). Given the many

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TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
TB50a	*MAT***a** *leu2-3, 112 ura3-52 rme1 trp1 his3Δ1 HML***a**	Beck and Hall (1999)
YGD3	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pmr1Δ::KanMX4*	EUROSCARF deletion set
LJ25-1A	*MAT***a** *leu2-3, 112 ura3-52 rme1 trp1 his3Δ1 HML***a** *pmr1Δ::KanMX4*	Devasahayam *et al.* (2006)
LJ25-2C	*MATα leu2-3, 112 ura3-52 rme1 trp1 his3Δ1 HML***a** *pmr1Δ::KanMX4*	This study
EG103	*MATα leu2-3,112 his3Δ1 trp-289 ura3-52* Gal^+	Lapinskas *et al.* (1995)
EG133	*MATα leu2-3,112 his3Δ1 sod1Δ::URA3 sod2Δ::TRP1* Gal^+	Lapinskas *et al.* (1995)
PJKP-1	*MATα his3Δ1 sod1Δ::URA3 sod2Δ::TRP1 pmr1Δ::LEU2* Gal^+	Lapinskas *et al.* (1995)
MH349-3d	*MAT***a** *leu2-3, 112 ura3-52 rme1 trp1 his4 HML***a** *tor1Δ::LEU2-4*	Helliwell *et al.* (1994)
YGD25	*MATα leu2-3, 112 ura3-52 rme1 trp1 HML***a** *tor1Δ::LEU2-4 pmr1Δ::KanMX4*	This study

cellular processes affected, regulation by TOR of one biological process may be paramount and effects on other cellular processes more indirect. Vesicular trafficking may be a candidate process as it is required for both isotropic and spatially restricted growth and is dependent upon actin dynamics (Neufeld 2007). Treatment with rapamycin mimics starvation for nitrogen in *S. cerevisiae* despite availability of nutrients, resulting in endocytosis and degradation of specific permeases and their substitution by the general amino acid permease, Gap1 (Crespo and Hall 2002). In *Drosophila melanogaster*, genetic studies of an eye phenotype that results from tissue-specific overexpression of TOR suggest TOR signaling suppresses endocytosis and degradation of specific proteins (Hennig *et al.* 2006). Overexpression of Rheb in *D. melanogaster*, placed in genetic models upstream of TOR, also increased cell-surface expression of Slimfast, a cationic amino acid transporter (Hennig *et al.* 2006). In human FL5.12 cells, survival and growth requires cell-surface expression of nutrient transporters whose stability at the membrane requires interleukin-3 (IL3) signaling (Edinger and Thompson 2004). Expression of an allele of mTOR having constitutive activity, but not wild-type mTOR, conferred IL3 independence to FL5.12 by an unidentified mechanism that stabilized nutrient transporters in the absence of IL3 (Edinger and Thompson 2004). These observations strongly indicate that regulation of nutrient transporters by TOR is conserved from yeast to man.

TOR localization is controversial (Martin *et al.* 2006), which may indicate the localization is dynamic and regulated. Studies in yeast and mammalian systems agree that TOR is peripherally associated with intracellular membranes. The localization of mTOR was reported as ER and Golgi (Drenan *et al.* 2004) and recently further supported by identification of putative targeting sequences (Liu and Zheng 2007). In *S. cerevisiae*, TOR1 has been localized by biochemical fractionation and immunogold electron microscopy (Martin *et al.* 2006). TOR1 was found in fractions with density of plasma membranes and in a lighter, unidentified pool. TOR2 has been reported to be associated with vacuolar membranes (Cardenas and Heitman 1995).

In this article we describe interactions of *PMR1* and *TOR*-dependent signaling pathways. We parse the roles of Ca^{2+} and Mn^{2+} transport by Pmr1 in rapamycin sensitivity. We show that multiple transporters that affect Ca^{2+} and Mn^{2+} do not affect the rapamycin response, suggesting that the effect is specific to Pmr1. We use separation-of-function mutants to show that Mn^{2+} transport by Pmr1 suffices to restore rapamycin sensitivity. We used transporters that are specific to individual compartments to demonstrate that Mn^{2+} transport by Pmr1 into the Golgi is required for rapamycin sensitivity in yeast.

MATERIALS AND METHODS

Strains: The yeast strains used in this study are listed in Table 1, except for strains in Figures 1C and 2A, which are deletion mutants from the haploid collection (replaced by *KanMX4*) in BY4741 background. The *sod1* mutants are in EG103 background (Lapinskas *et al.* 1995). YGD25 (*pmr1Δ tor1Δ*) was made by crossing MH349-3d (*tor1Δ* in JK9-3da) (Helliwell *et al.* 1994) with LJ25-2C (*pmr1Δ* in TB50α), sporulated for 2–3 days on KAc plates, and tetrads were dissected. Double mutants were selected as being G418 resistant, grown on leu− plates, and were mating-type α.

Plasmids: Plasmids to express *pmr1* point mutations D53A, Q783A, and D778A were constructed by subcloning a fragment [D53A (300 bp *Xba*I), D778A (~2.2 kb *Bam*HI–*Pst*I), and Q783A (~2.2 kb *Bam*HI–*Pst*I] from the EBc24-2L1 mutants in pRS415 (Bolton *et al.* 2002) (gift of Jeff Boeke) into the same sites of YCp pHAC111-PMR1 (Devasahayam *et al.* 2006). All three were sequenced and contained the point mutations. Ccc1 was overexpressed using plasmid pOSC10 that contains *CCC1* under control of the *MET3* promoter and tagged with FLAG epitope (gift of Jerry Kaplan) (Li *et al.* 2001). Rabbit SERCA1a cDNA (Durr *et al.* 1998) (gift of Hans Rudolph) is expressed from the constitutive yeast *PMA1* promoter in pRS316 (br434). *VCX1* and *VCX1-M1* cDNA were expressed from high-copy yeast-expression shuttle vector p2UGpd (Pittman *et al.* 2004), containing the strong constitutive *GPD* promoter. *CAX1* and *CAX2* were cloned into yeast shuttle vector piHGpd for expression in yeast.

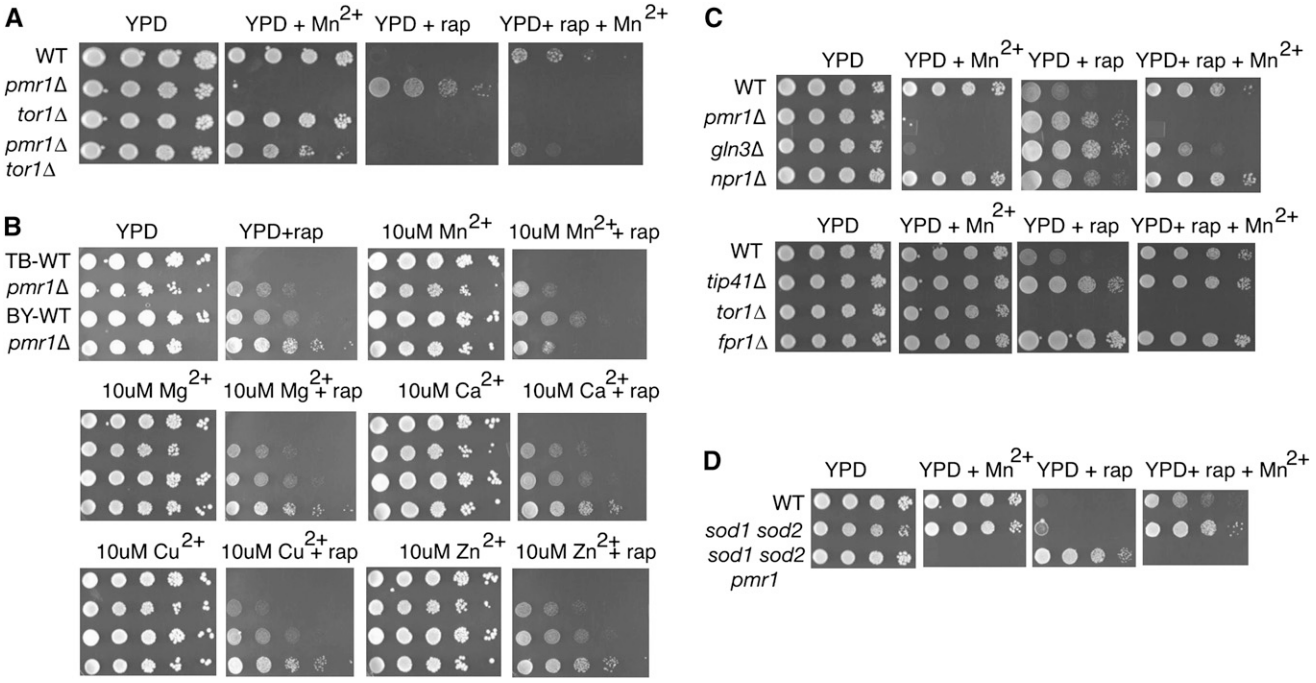

FIGURE 1.—Mn^{2+} suppresses rapamycin resistance of *pmr1*Δ. (A) *pmr1*Δ *tor1*Δ grows in the presence of extracellular Mn^{2+}; *i.e.*, *pmr1*Δ is synthetically rescued by *tor1*Δ. *tor1*Δ (MH349-3d) is not rapamycin resistant (20 ng/ml) in the presence of extracellular Mn^{2+} (2 mM) unlike WT (TB50a) and *pmr1*Δ *tor1*Δ (YGD25). *pmr1*Δ *tor1*Δ is rapamycin sensitive as is *tor1*Δ and WT. Cells were grown for 3–5 days at 30°. (B) Growth of *pmr1*Δ strains (LJ25-1A and YGD3) is compared to their WTs (TB50a and BY4741, respectively). TB-WT (TB50a) has a genetic background more sensitive to rapamycin than BY-WT (BY4741) (EUROSCARF). Extracellular Mn^{2+} suppresses rapamycin (20 ng/ml) resistance of *pmr1*Δ (LJ25-1A and YGD3) at 10 μM. Other divalent cations (chloride salts) do not suppress rapamycin resistance of *pmr1*Δ at 10 μM. Cells were grown for 3 days at 30°. (C) Mutants of the TOR pathway on rapamycin (20 ng/ml) and 2 mM Mn^{2+}. *gln3*Δ is Mn^{2+} hypersensitive and rapamycin resistant as is *pmr1*Δ (YGD3), but rapamycin resistant in the presence of extracellular Mn^{2+} (2 mM), unlike *pmr1*Δ. *tor1*Δ (BY4741) is sensitive in the presence of rapamycin and Mn^{2+} (2 mM). Cells were grown for 3 days at 30°. WT: BY4741. Deletion mutants are from the EUROSCARF deletion collection in BY4741 background. (D) *sod1*Δ *sod2*Δ (EG133) is rapamycin (20 ng/ml) resistant compared to WT (EG103) and Mn^{2+} (2 mM) enhances this phenotype. Deletion of *PMR1* in *sod1*Δ *sod2*Δ (PJKP-1) also enhances the rapamycin-resistant phenotype.

Media: Rapamycin (Sigma, St. Louis) was in 90% ethanol/10% Tween-20. 1, 2-bis (2-aminophenoxy) ethane-*N, N, N′, N′*-tetraacetic acid (BAPTA) and ethylene glycol-bis (2-aminoethylether)-*N, N, N′, N′*-tetraacetic acid (EGTA)-containing media were prepared as described and pH adjusted to 6.0 (with 1 N KOH) and 5.5 (0.5 M MES buffer), respectively (DURR *et al.* 1998; WEI *et al.* 2000). Mn^{2+} plates contained $MnCl_2$; Ca^{2+} plates, $CaCl_2$. For spottings, cells were 10-fold serially diluted from an initial OD_{600} of 1.0. Yeast transformations were done and standard yeast media prepared as described (AMBERG *et al.* 2005).

Western blotting: *CCC1* expression was induced in *pmr1*Δ (LJ25-1A/a) in the absence of methionine and detected by immunoblot with mouse anti-FLAG M2 antibody (Sigma) at 0.5 μg/ml. Cells were grown in 5 ml SC −leu or SC −leu met medium and protein was extracted by the alkali lysis method (KUSHNIROV 2000). Lysis buffers contain 350 μg/ml p-methyl sulfonyl fluoride, 1×. Complete protease inhibitor (Roche, Indianapolis) and 1 μM phosphatase inhibitor (microcystin-LR).

RESULTS

***tor1*Δ suppresses the manganese sensitivity of *pmr1*Δ:** Pmr1 is a negative regulator of TORC1 signaling (DEVASAHAYAM *et al.* 2006). Since loss of *TOR1* causes rapamycin hypersensitivity (T. F. CHAN *et al.* 2000), we looked for synthetic interactions of *tor1*Δ with *pmr1*Δ. Throughout this article, we infer TORC1 signaling from how cells respond to the drug. Reduction of TORC1 signaling results in rapamycin resistance and enhanced TORC1 signaling results in rapamycin sensitivity. Strains with a *pmr1* mutation do not grow on media containing millimolar concentrations of the divalent cation Mn^{2+} (LAPINSKAS *et al.* 1995). The Mn^{2+} sensitivity of *pmr1*Δ cells was completely suppressed by deleting *TOR1* (Figure 1A), suggesting Mn^{2+} sensitivity of Pmr1 was due to hyperactive Tor1. In addition, *tor1*Δ *pmr1*Δ was sensitive to rapamycin unlike *pmr1*Δ. Thus, deletion of *TOR1* restored the wild-type phenotype in each case. Namely, loss of *TOR1* restored the ability of the *pmr1*Δ strain to grow on Mn^{2+}-containing media such as WT, and loss of *TOR1* restored the rapamycin sensitivity of the *pmr1*Δ strain to wild-type sensitivity. Interestingly, rapamycin sensitivity of wild type (TB50a) and *tor1*Δ *pmr1*Δ was partially suppressed in media containing 2 mM Mn^{2+} (Figure 1A).

These data are consistent with a proposed genetic model in which Pmr1 is a negative regulator of TORC1

signaling (Devasahayam *et al.* 2006) and suggest that the mechanism may be through regulating manganese homeostasis. Intracellular Mn^{2+} increases dramatically in *pmr1*Δ because Mn^{2+} fails to enter the secretory pathway, whereby excess Mn^{2+} can be removed (Culotta *et al.* 2005). Since *TOR1* is required for Mn^{2+} toxicity in *pmr1*Δ, TORC1 appears toxic in this circumstance. Smaller increases in TORC1 activity by Mn^{2+}, in the absence of *pmr1*Δ or in the presence of *pmr1*Δ with *tor1*Δ, may allow weak growth in the presence of rapamycin.

Extracellular Mn^{2+} increases rapamycin resistance: TOR proteins are members of the phosphatidylinositol 3-kinase-related protein kinases that include ataxia-telangiectasia mutated (ATM) (Wullschleger *et al.* 2006). Both the TOR proteins (Alarcon *et al.* 1999) and ATM (D. W. Chan *et al.* 2000) have been reported to require Mn^{2+} as a cofactor with ATP for maximal activity. Mn^{2+} entry into cells is suppressed by feedback inhibition of expression of the Mn^{2+} transporter Smf1p, but high concentrations of Mn^{2+} still favor entry by alternative, less efficient mechanisms allowing Mn^{2+} to reach toxic levels in *pmr1*Δ (Culotta *et al.* 2005).

To distinguish whether low or high extracellular Mn^{2+} was required to increase rapamycin resistance, we studied the dose response for Mn^{2+} in two genetic backgrounds, BY4741 and TB50a (Figure 1B). TB50a is more rapamycin sensitive than BY4741, possibly due to the greater number of auxotrophies for amino acids. The highest concentration of Mn^{2+} tested, 250 μM, increased growth of wild-type strains TB50a and BY4741 on rapamycin but the lower micromolar concentrations of Mn^{2+} did not (data not shown). The toxic effect of Mn^{2+} on growth of the *pmr1*Δ strain was detectable at 10 μM. Rapamycin resistance of *pmr1*Δ is suppressed by 10 μM Mn^{2+}, in both strain backgrounds. To rule out that this effect may be due to osmolarity, we tested the effect of other divalent cations, Ca^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+}, at the same concentration on *pmr1* cells. Only Mn^{2+} suppressed the rapamycin resistance of *pmr1* cells (Figure 1B). Mn^{2+} can replace Ca^{2+} in media for growth in some circumstances (Loukin and Kung 1995). Additions of Ca^{2+} to media (1–10 mM) did not increase rapamycin resistance of BY4741 or TB50a (supplemental data at http://www.genetics.org/supplemental/).

The increase in rapamycin resistance by extracellular Mn^{2+} requires *GLN3*: Yeast treated with rapamycin act as if starved of nitrogen despite the presence of a good nitrogen source in media. Cells respond to rapamycin by inducing transcription of genes for use of alternative nitrogen sources and by expressing active permeases such as Gap1 to scavenge nitrogen. Gln3, Npr1, and Tip41 positively regulate Gap1 in response to poor nitrogen sources (Magasanik and Kaiser 2002), and their activities are inhibited by TORC1 signaling. Deletions of these genes confer rapamycin resistance. Nuclear Gln3 transactivates genes including *GAP1* that enable adaptation to poor nitrogen sources. A serine/threonine protein kinase, Npr1, positively regulates Gap1, and negatively regulates the more specific amino acid permeases, by control of their sorting and stability (De Craene *et al.* 2001). Nuclear translocation of Gln3 requires Sit4 phosphatase (Beck and Hall 1999). Tip41 is a Sit4 regulator, and its loss prevents Sit4-dependent dephosphorylation and activation of Npr1 (Jacinto *et al.* 2001). We compared the effect of Mn^{2+} on the wild-type BY4741 strain of *S. cerevisiae* and otherwise isogenic strains with deletions in these genes (Figure 1C).

All of these strains grew normally on media containing 2 mM Mn^{2+} with the exception of *pmr1*Δ and *gln3*Δ. Surprisingly, both the *pmr1*Δ strain and the *gln3*Δ strain were very sensitive to Mn^{2+}. On media containing rapamycin, loss of *PMR1* induced resistance to a similar degree as loss of *NPR1*, *GLN3*, or *TIP41*. [Deletion of *FPR1* (FKBP12) serves as benchmark for rapamycin resistance.] On media additionally containing Mn^{2+}, rapamycin resistance was increased for the wild-type BY4741 strain, confirming the observation (Figure 1A) with TB50a. The suppression of rapamycin sensitivity of BY471 by Mn^{2+} (compare YPD + Rap + Mn^{2+} to YPD + Rap) was reduced by *gln3*Δ but not by *npr1*Δ or *tip41*Δ. Moreover, the growth defect of *gln3*Δ on Mn^{2+} media is partially TORC1 dependent because rapamycin decreases the Mn^{2+} sensitivity of *gln3*Δ (compare YPD + Rap + Mn^{2+} to YPD + Mn^{2+}). Extracellular Mn^{2+} was unable to suppress rapamycin hypersensitivity of *tor1*Δ.

A *pmr1* mutant is a suppressor of the aerobic growth defect that occurs in a strain lacking Sod1 (Lapinskas *et al.* 1995), a Cu^{2+} containing superoxide dismutase localizing to the cytoplasmic compartment. Suppression is due to an increase of cellular Mn^{2+} to scavenge reactive oxygen species (Sanchez *et al.* 2005). A large screen identified *sod1* from the genome-deletion set as having rapamycin resistance (Xie *et al.* 2005). We tested a *sod1 sod2* strain (gift of Valerie Culotta), lacking both cytoplasmic (Sod1) and mitochondrial superoxide dismutase (Sod2), for rapamycin resistance (Figure 1D). Indeed, the *sod1*Δ *sod2*Δ strain was slightly more resistant to rapamycin in comparison to its wild type (EG103). Either extracellular Mn^{2+} or deletion of *PMR1* suppressed the growth defect of *sod1 sod2* on rapamycin. This result implicates a reciprocal role of reactive oxygen species (ROS) and Mn^{2+} in TORC1 signaling.

Pmr1-specific Mn^{2+} transport into Golgi is essential for TORC1 signaling: Pmr1 affects both calcium and manganese homeostasis (Rudolph *et al.* 1989; Antebi and Fink 1992). To determine which divalent cation is responsible for the rapamycin responses we compared the *pmr1*Δ strain to strains deleted for other cation transporters. Cells from mutants lacking the vacuolar Ca^{2+} transporter gene *PMC1* (Cunningham and Fink 1994), the plasma membrane Mn^{2+} transporter *SMF1* (Liu and Culotta 1999), the vesicular Mn^{2+} transporter *SMF2* (Portnoy *et al.* 2000), or the ER-localized P-type ATPase *SPF1* (Cronin *et al.* 2002) all had the same rapamycin

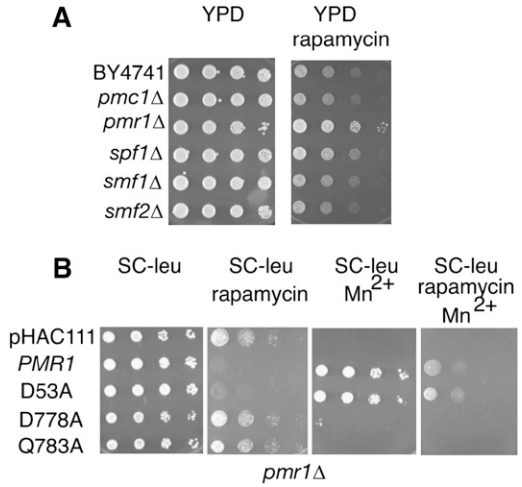

FIGURE 2.—Mn^{2+} transport into the Golgi is required for rapamycin-sensitive signaling in yeast. (A) Of the Ca^{2+} and Mn^{2+} transporters in a yeast cell, only deletion of *PMR1* confers rapamycin resistance. Deletion mutants were from the EUROSCARF yeast deletion collection in BY4741; cells were grown to midlog phase, serially diluted, and spotted on YPD and YPD containing 100 ng/ml rapamycin. (B) A Pmr1p mutant impaired for Ca^{2+} transport rescues rapamycin sensitivity. Mn^{2+} transporting mutant (D53A) of Pmr1 defective for Ca^{2+} transport suppresses rapamycin resistance. WT and D53A, Q783A, and D778A Pmr1 mutants were expressed in *pmr1*Δ (YGD3) from endogenous promoter on a YCp plasmid. Cells were spotted on SC −leu and SC −leu media containing 100 ng/ml rapamycin or 2 mM $MnCl_2$ or both.

sensitivity as a wild-type-cells strain (Figure 2A). Interestingly, only *pmr1*Δ conferred rapamycin resistance. The P-type ATPase Spf1 is involved in Ca^{2+} homeostasis (CRONIN *et al.* 2002), but did not affect rapamycin sensitivity, suggesting that manganese is the important cation that modulates the response to rapamycin.

Separation-of-function Pmr1 mutants implicate Mn^{2+} in TORC1 signaling: Point mutations of *PMR1* that confer selective transport for Ca^{2+} or Mn^{2+} have been described (WEI *et al.* 1999, 2000; MANDAL *et al.* 2000). The mutations were identified by screening for suppression of Ca^{2+} or Mn^{2+} phenotypes in *pmr1 pmc1 cnb1* cells. The D53A mutant rescued the Mn^{2+} phenotype and Q783A rescued the Ca^{2+} phenotype, and both Q783A and D53A Pmr1 were localized to Golgi (WEI *et al.* 2000). D778A is nonfunctional and serves as an additional control. We compared mutants D53A and Q783A to determine if preferential loss of Ca^{2+} or Mn^{2+} transport into Golgi was more important for the rapamycin resistance of *pmr1*Δ (Figure 2B). The *pmr1D53A* cells (defective for Ca^{2+} transport) were rapamycin sensitive, whereas the strains transformed with *pmr1Q783A* cells (defective for Mn^{2+} transport) and D778A (nonfunctional) mutants were rapamycin resistant. D53A transports Mn^{2+} normally, and it suppressed Mn^{2+} toxicity as did wild-type Pmr1 (Figure 2B). To confirm that *pmr1Q783A* was functional, we determined that it suppressed BAPTA sensitivity, the Ca^{2+} phenotype (supplemental data at http://www.genetics.org/supplemental/).

Manganese in the Golgi modulates TOR activity: We used transporters that are specific to individual compartments to demonstrate that Mn^{2+} transport by Pmr1 into the Golgi is required for rapamycin sensitivity in yeast. To rule out the lack of transport of cytosolic Ca^{2+} into the secretory pathway as the cause of rapamycin resistance, we also studied *pmr1*Δ cells expressing mammalian SERCA1. Notably, *SERCA1* did not complement the rapamycin resistance of *pmr1*Δ (Figure 3A). This P2-type Ca^{2+} ATPase restores growth of a *pmr1*Δ strain in EGTA- or BAPTA-containing media (DURR *et al.* 1998; DEGAND *et al.* 1999). In contrast, *SERCA1* was unable to restore growth of *pmr1*Δ in high Mn^{2+} (TON and RAO 2004). This suggests rapamycin resistance of *pmr1*Δ is not due to entry of Ca^{2+} into the secretory pathway.

VCX1 encodes the Ca^{2+}/H^+ exchanger in the vacuolar membrane (PITTMAN *et al.* 2004). An L208P mutant (*VCX1-M1*) has enhanced Mn^{2+}/H^+ exchange, and this mutant suppresses the Mn^{2+} toxicity phenotype of *pmr1*Δ much better than Vcx1p (PITTMAN *et al.* 2004). High-dosage expression of neither Vcx1p nor the Mn^{2+} selective mutant L208P suppressed rapamycin resistance due to loss of Pmr1 (Figure 3B). This result suggests that lowering cytosolic Ca^{2+} or Mn^{2+} by transport to the vacuolar space does not restore rapamycin sensitivity of *pmr1*Δ. The *Arabadopsis thaliana CAX1* and *CAX2* genes encode Ca^{2+}/H^+ exchangers that localize to the yeast vacuole (SHIGAKI *et al.* 2003). Cax2 transports Mn^{2+} better than Cax1, and expression of Cax1 and Cax2 decreases the Mn^{2+} toxicity phenotype of *pmr1*Δ (SHIGAKI *et al.* 2003). Neither Cax1 nor Cax2 restored rapamycin sensitivity of *pmr1* (supplemental data at http://www.genetics.org/supplemental/), confirming that lowering Ca^{2+} or Mn^{2+} in the cytoplasmic compartment by transport into the vacuole was not sufficient to restore rapamycin sensitivity. Together these data support our conclusion that cytosolic Mn^{2+} does not regulate TOR activity and suggest that the regulation is achieved in a different cellular compartment.

***CCC1* restores rapamycin sensitivity:** Pmr1 has been localized to the Golgi (RUDOLPH *et al.* 1989; ANTEBI and FINK 1992). To determine if the manganese-dependent regulation of TORC1 signaling was dependent on the Golgi, we analyzed the effect of overexpressing CCC1 in *pmr1*Δ cells. *CCC1* was isolated as a high-dosage suppressor of Mn^{2+} sensitivity of a *cnb1* strain (POZOS *et al.* 1996) and was isolated in an independent screen as a suppressor of the marked Mn^{2+} sensitivity of *pmr1* (LAPINSKAS *et al.* 1996). Furthermore, Ccc1 has been reported to localize to the Golgi (LAPINSKAS *et al.* 1996). We asked if *CCC1* could complement rapamycin resistance of *pmr1*Δ (Figure 4). Excess expression of Ccc1p from the inducible *MET3* promoter in the *pmr1*Δ strain restored wild-type sensitivity to rapamycin, fully

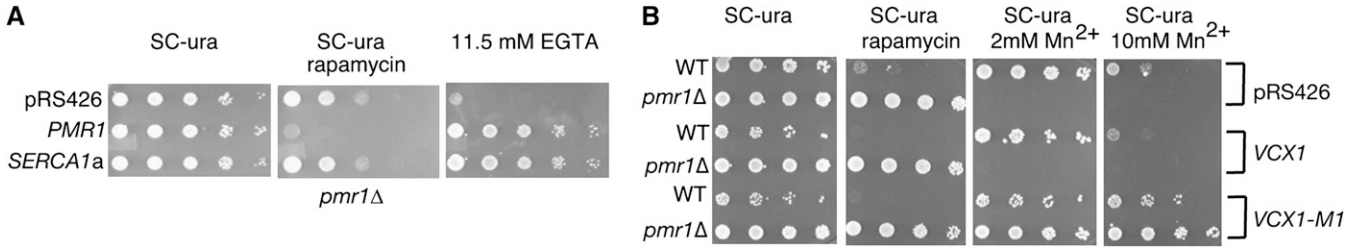

FIGURE 3.—Ca^{2+} transport into the secretory pathway is not required for rapamycin sensitivity (A) Normalizing intracellular Ca^{2+} with SERCA1a does not suppress rapamycin resistance of *pmr1*Δ. YGD3 was transformed with *PMR1* (pKC21, gift of K. Cunningham) or SERCA1a expressed on a YCp vector with yeast *PMA1* promoter (br434) or control plasmid (DURR *et al.* 1998) (gift of H. Rudolph). Cells were serially diluted and spotted; media were as labeled. (B) Vacuolar Mn^{2+} or Ca^{2+} transport does not suppress rapamycin phenotype of *pmr1*Δ. *VCX1*-M1, Mn^{2+} transporting mutant of vacuolar H^+/Ca^{2+} antiporter *VCX1*, suppresses Mn^{2+} hypersensitivity of *pmr1*Δ (YGD3) yet does not restore rapamycin sensitivity. Overexpression of *VCX1* in *pmr1*Δ also does not restore rapamycin sensitivity; rapamycin, 20 ng/ml; $MnCl_2$, 2 mM. Cells were fivefold serially diluted from a starting OD_{600} of 2.0. Plates were incubated for 5 days at 30°. WT: BY4741.

complementing *pmr1*Δ for this phenotype. We conclude that Mn^{2+} in the Golgi inhibits TORC1 signaling.

DISCUSSION

In this study we show that *TOR1* is required for Mn^{2+} toxicity of *pmr1*Δ, consistent with our model that Pmr1 is a negative regulator of TORC1 signaling (DEVASAHAYAM *et al.* 2006). Extracellular Mn^{2+} also improved growth of wild-type and *pmr1*Δ *tor1*Δ cells. Since some phosphatidylinositol 3-kinase-related protein kinases are Mn^{2+} dependent, this suggested that an increase in cytosolic Mn^{2+} increases Tor1 activity, bypassing rapamycin inhibition. Experiments to parse Ca^{2+} and Mn^{2+} in rapamycin resistance and define the compartments in which Ca^{2+} or Mn^{2+} were important led to the following conclusions:

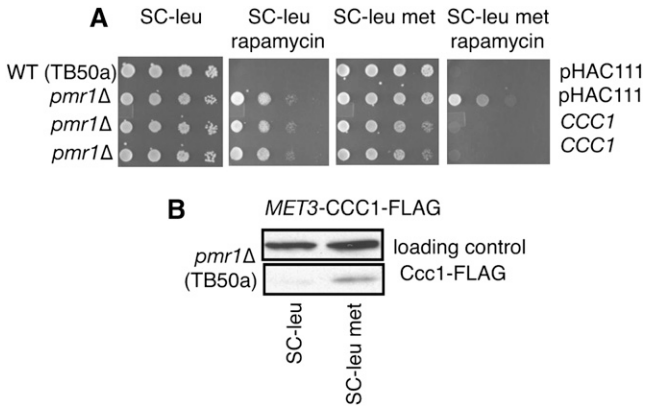

FIGURE 4.—Interactions of *PMR1* with genes involved in Mn^{2+} homeostasis. (A) Ccc1, a putative Fe^{2+}/Mn^{2+} transporter (LI *et al.* 2001), suppresses the rapamycin-resistant phenotype of *pmr1*Δ (LJ25-1A) in SC −leu, met medium containing 20 ng/ml rapamycin. Cells were 10-fold serially diluted from a starting OD_{600} of 5.0. (B) *CCC1* expression from the pMET3-*CCC1*-FLAG plasmid (pOSC10) is induced in SC −leu met medium (in the absence of methionine) but not in SC −leu medium in the LJ25-1A strain background. Mouse anti-FLAG antibody detects Ccc1 tagged with FLAG epitope.

i. Loss of Mn^{2+} transport appears more important than loss of Ca^{2+} transport for rapamycin resistance in a *pmr1* strain.
ii. Restoration of rapamycin sensitivity requires Mn^{2+} transport into the Golgi or into a vesicular compartment in communication with Golgi.
iii. Vacuolar Vcx1p-M1 sufficed to suppress Mn^{2+} sensitivity of *pmr1*Δ but failed to restore rapamycin sensitivity, disfavoring our initial hypothesis that an increase in cytosolic Mn^{2+} bypasses rapamycin to activate Tor1. The new hypothesis is that an increase in Golgi Mn^{2+} inhibits TORC1 signaling.

There were two surprising observations. First, loss of *TOR1* suppresses the Mn^{2+} sensitivity of *pmr1*Δ. Since *PMR1* is placed by epistasis upstream of TORC1 signaling (DEVASAHAYAM *et al.* 2006), the Mn^{2+} sensitivity of *pmr1*Δ can be rationalized as Mn^{2+} hyperactivity of Tor1 occurring in this context. Extracellular Mn^{2+} increased rapamycin resistance of *pmr1 tor1*. Together the data suggest Mn^{2+} regulates both Tor1 and Tor2 in yeast. Whether it is direct or indirect will have to be determined. Second, loss of *GLN3* made cells sensitive to Mn^{2+}, and sensitivity was reduced by rapamycin showing that the Mn^{2+} sensitivity of *gln3*Δ is in part TORC1 dependent. *GLN3* target genes include a number of ion transporters in addition to the set of genes for scavenging nitrogen. *ENA1*, encoding a P-type ATPase of the plasma membrane that transports monovalent cations (Na^+ and Li^+), is a Gln3 target and its transcription is induced by rapamycin (CRESPO *et al.* 2001). *PMR1* may also be a Gln3 target gene, since *gln3*Δ has a *pmr1*Δ phenotype of Mn^{2+} hypersensitivity.

Ccc1p is a putative transporter for Fe^{2+} and Mn^{2+} (LI *et al.* 2001). Ccc1 has significant similarity to archaebacteria proteins (NP_393546, YP_023568, and NP_110542) including a DMIYGISDGL motif similar to DLIIGLSDGL in Ccc1 that could coordinate a metal ion. Ccc1p is implicated in Fe^{2+} ion homeostasis from the literature but not as a Ca^{2+} transporter. Low cytosolic Fe^{2+} represses

CCC1 transcription and negatively regulates *CCC1* mRNA by inducing *CTH2* (Puig *et al.* 2005). High iron in media inhibits growth of a *ccc1*Δ strain, and high-dosage expression of *CCC1* is protective of iron toxicity (Chen and Kaplan 2000). High-dosage expression of Ccc1p-FLAG can increase iron and manganese content in a purified vacuolar fraction, and Ccc1p-FLAG is concentrated at the periphery of the vacuole (Li *et al.* 2001). Ccc1p has been localized to the Golgi as well (Lapinskas *et al.* 1996), and the latter is important for our interpretation. Both localizations are likely correct, and trafficking of Ccc1p between the Golgi and the vacuole is a distinct possibility. Specific factors may determine which localization predominates. Even if Ccc1 is in the vacuole, it does not preclude it from functioning in the Golgi. Ellis *et al.* show vacuolar zinc transporters functioning in the ER (Ellis *et al.* 2004). Transporters may have activity to transport metals into the lumen of the secretory pathway as they are being trafficked to their final destination.

There are two good ways to imagine how Mn^{2+} in the Golgi regulates rapamycin sensitivity in yeast. The first is that Mn^{2+} regulates permease routing or function. One candidate from what we know from the literature is mannosylation reactions of proteins or lipids (Lisman 2004). Glycosylations may affect sorting and plasma membrane localization of specific proteins (Proszynski *et al.* 2004). Mannosylation of proteins requires Mn^{2+}, and this suggests that mannosylation of lipids, as with proteins, could require Mn^{2+}. Indeed, sphingolipid mannosylation in yeast requires Csg2-dependent Mn^{2+} transport into the lumen of early secretory organelles, and conversion of inositol phosphoryl-ceramide (IPC) to mannosylinositol phosphoceramide in a *csg2* strain is partially suppressed by the addition of millimolar Mn^{2+} to media (Lisman 2004). IPC is synthesized in the medial Golgi from ceramide precursors made in the ER. Sphingolipids play an important role in sorting proteins, including nutrient permeases, destined for the plasma membrane via formation of detergent-resistant membrane microdomains (Proszynski *et al.* 2005). The second possibility is that some TOR may be localized to the Golgi, as suggested for mammalian Tor (Liu and Zheng 2007), where there is access to Mn^{2+} within the secretory pathway. Further work is necessary to define how Mn^{2+} is involved in sensitivity to rapamycin in yeast.

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