Down-Regulation of a Manganese Transporter in the Face of Metal Toxicity

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The yeast Smf1p Nramp manganese transporter is posttranslationally regulated by environmental manganese. Smf1p is stabilized at the cell surface with manganese starvation, but is largely degraded in the vacuole with physiological manganese through a mechanism involving the Rsp5p adaptor complex Bsd2p/Tre1p/Tre2p. We now describe an additional level of Smf1p regulation that occurs with toxicity from manganese, but not other essential metals. This regulation is largely Smf1p-specific. As with physiological manganese, toxic manganese triggers vacuolar degradation of Smf1p by trafficking through the multivesicular body. However, regulation by toxic manganese does not involve Bsd2p/Tre1p/Tre2p. Toxic manganese triggers both endocytosis of cell surface Smf1p and vacuolar targeting of intracellular Smf1p through the exocytic pathway. Notably, the kinetics of vacuolar targeting for Smf1p are relatively slow with toxic manganese and require prolonged exposures to the metal. Down-regulation of Smf1p by toxic manganese does not require transport activity of Smf1p, whereas such transport activity is needed for Smf1p regulation by manganese starvation. Furthermore, the responses to manganese starvation and manganese toxicity involve separate cellular compartments. We provide evidence that manganese starvation is sensed within the lumen of the secretory pathway, whereas manganese toxicity is sensed within an extra-Golgi/lysosomal compartment of the cell.

INTRODUCTION

Metals that are both essential and potentially toxic pose a challenge to cells in that sufficient quantities must be acquired in times of metal starvation, whereas hyperaccumulation must be minimized in times of metal surplus. To help meet this challenge, the levels and/or cellular localization of metal transporters are often regulated in response to changes in metal exposures. In studies that have been conducted in yeast, transporters for zinc, copper, and iron are all induced at the level of transcription by metal responsive transcription factors (Jungmann et al., 1993; Yamaguchi-Iwai et al., 1996; Iwai et al., 1997; Zhao and Eide, 1997; Dancis, 1998). Yet thus far, no transcriptional regulation has been described for transporters of manganese. Instead, the major manganese transporters of yeast, Smf1p and Smf2p, are regulated at the posttranslational level. Smf1p and Smf2p are members of a well-conserved family of Nramp metal transporters that from bacteria to humans are involved in the uptake and distribution of iron, manganese, and other divalent metals (Cellier et al., 1995; Gunshin et al., 1997; Thomine et al., 2000). Although Smf1p and Smf2p are both manganese transporters in yeast, they are not redundant in function. Smf2p is largely responsible for the activation of manganese enzymes in the cell (Luk et al., 2005), whereas Smf1p is more critical for oxidative stress protection by supplying the cell with critical manganese antioxidants (Reddi et al., 2009). With normal physiological levels of manganese, a large fraction of Smf1p and Smf2p are subject to rapid turnover by degradation in the vacuole (Liu and Culotta, 1999b; Portnoy et al., 2000). This continual degradation of Smf1p and Smf2p maintains these transporters at a low steady-state level that is sufficient for essential manganese acquisition but prohibits excess transport of other toxic metals (Liu et al., 1997).

The vacuolar degradation of Smf1p and Smf2p during physiological manganese has been well characterized. In the secretory pathway, the bulk of newly synthesized Smf1p and Smf2p are recognized by Tre1p/Tre2p and Bsd2p, which triggers ubiquitination of the transporters via Rsp5p. Ubiquitinated Smf1p and Smf2p then enter the multivesicular body (MVB) pathway for delivery to the vacuolar lumen for degradation (Liu et al., 1997; Liu and Culotta, 1999b; Portnoy et al., 2000; Eguez et al., 2004; Hettema et al., 2004; Stimpson et al., 2006; Sullivan et al., 2007). Yet during manganese starvation, this down-regulation of Smf1p and Smf2p is abrogated. The transporter polypeptides fail to be recognized by Tre1p/Tre2p and Bsd2p and rather than vacuolar targeting, the bulk of Smf1p and Smf2p relocate to the cell surface and intracellular vesicles to optimize manganese uptake and distribution (Liu and Culotta, 1999b; Luk and Culotta, 2001; Sullivan et al., 2007). The mechanism for the switch in Smf1p and Smf2p localization is currently unknown, but has been proposed to involve manganese binding directly to the transporters (Liu and Culotta, 1999a; Sullivan et al., 2007).
In addition to up-regulation by metal starvation, many metal transporters are down-regulated in response to metal toxicity. For example, yeast transporters for zinc, copper, and iron are turned over when cells are challenged with high doses of these metal ions (Gitan and Eide, 2000a; Gitan et al., 2003; Petris et al., 2003; Felice et al., 2005; Liu et al., 2007; Strohlic et al., 2008). In all three cases, high concentrations of metal stimulate ubiquitination and endocytosis of the cell surface transporter and targeting to the vacuole for degradation. The Smf1p and Smf2p transporters can contribute to metal ion toxicity (Liu et al., 1997), and recently cadmium has been shown to promote endocytosis of Smf1p through Rsp5p-dependent ubiquitination of specific lysine residues in the N-terminus (Nikko et al., 2008). The regulation of Smf1p by cadmium was mediated by several arrestin-like proteins that appear to function as Rsp5p adaptors. However the regulation of Smf1p by cadmium appeared distinct from previously reported regulation of this transporter, because endocytosis was not observed when cells were stressed with manganese (Nikko et al., 2008), and additional down-regulation of Smf1p and Smf2p in response to manganese toxicity has not been previously reported.

In this study, we have examined the fate of the Smf1p manganese transport protein in response to manganese starvation, physiologial manganese, and manganese toxicity. We report here that in addition to the "basal" down-regulation of Smf1p during physiological manganese, an additional tier of Smf1p down-regulation occurs when cells are subject to very high doses of the metal. Loss of Smf1p at toxic manganese is distinct from Bsd2p-dependent regulation of the protein, but nevertheless does involve vacuolar degradation through the MVB pathway. We observe that sensing of manganese for regulating Smf1p at very low and very high metal levels requires distinct intracellular pools of the metal.

MATERIALS AND METHODS

Plasmids, Strains, and Growth Conditions

The majority of yeast strains used in this study were derived from BY4741 (MATa, leu2Δ0, metα50Δ, ura3Δ0, his3Δ1). Single gene deletions in the BY4741 background including bsd2Δ::kanMX4, tre1Δ::kanMX4, and nam13Δ::kanMX4 were obtained from Research Genetics (Huntsville, AL). The BSD2 gene was deleted in the indicated kanMX4 disruption strains using the bsd2Δ::HIS3 deletion plasmid (Liu et al., 1997) resulting in LJS05 (bsd2Δ::HIS3 pyc217::URA3, LJS06 (bsd2Δ::HIS3 pyc6::kanMX4), LJS07 (bsd2Δ::HIS3 pyc6::kanMX4), LJS08 (bsd2Δ::HIS3 pyc6::kanMX4), LJS09 (bsd2Δ::HIS3 pyc6::kanMX4), LJS10 (bsd2Δ::HIS3 pyc6::kanMX4). Additional strains used include YJ472 (MATa, leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1), the tre1Δ::HIS3 strain (MATa, leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1, tre1Δ::HIS3), Stimpson et al., 2006), and YJ423 (BY4742 bsd2Δ::HIS3), LJS12 (his3Δ1, tre1Δ, lys3Δ1, ura3Δ0, bar1Δ, bar2Δ, lub1Δ, LJS13 (his3Δ1, tre1Δ, lys3Δ1, ura3Δ0, bar1Δ, lub1Δ), LJS14 (bsd2Δ::HIS3), LJS15 (bsd2Δ::HIS3, pyc6::ura3Δ::kanMX4), LJS16 (bsd2Δ::HIS3, pyc6::ura3Δ::kanMX4), LJS17 (bsd2Δ::HIS3, pyc6::URA3, LJS18 (bsd2Δ::HIS3, pyc6::URA3). Additional mutations were generated using XbaI and EcoRI (upstream) or SacI and XbaI (downstream) digestion of these mutants using SacI and EcoRI. All mutations were generated using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), and the sequence integrity of plasmids was ensured by double-stranded DNA sequencing (DNA Analysis Facility, Johns Hopkins University).

Immunobots

Cultures were grown aerobically in either YPD or in minimal defined medium depleted of manganese that was supplemented as needed with MnCl₂. Cultures were inoculated at an OD₆₀₀nm of 0.2 and grown for 16 h or grown to an OD₆₀₀nm of ~2 before addition of MnCl₂. Yeast extracts were generated with the glass bead lysis protocol (Lapinskas et al., 1996) using 10 mM NaH₂PO₄, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, pH 7.8, containing protease inhibitors. Proteins were separated by SDS-PAGE using 12% gels, and immunobots were probed with either anti-HA (Roche, Indianapolis, IN), anti-GFP (Molecular Probes, Eugene, OR), or anti-Pgk1 (Molecular Biosciences, Cincinnati, OH) antibodies at 1:500 dilution. Visualization of immunobots utilized either HRP-conjugated secondary antibodies with ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ) or the Odyssey infrared imagining system (Li-Cor Biosciences, Lincoln, NE) employing an Alexa Fluor 680 secondary antibody (Invitrogen, Carlsbad, CA). Quantitation of immunobots utilized Odyssey quantitation software (version 1.2).

β-Galactosidase Assays

β-Galactosidase activity was measured as described previously (Portnoy et al., 2002). Results from two independent transformants assayed at least in duplicate were reported in Miller units (Giacomini et al., 1992).

RESULTS

The Down-Regulation of Smf1p by Toxic Manganese

Smf1p is known to respond to the switch from manganese starvation to physiological manganese by degrading much of the newly synthesized protein in the vacuole (Liu et al., 1997; Liu and Culotta, 1999b; Eguez et al., 2004; Hettema et al., 2004; Simpson et al., 2006; Sullivan et al., 2007). In addition to this well-defined pathway of Smf1p degradation under physiological manganese conditions, we now show that Smf1p is subject to down-regulation by toxic manganese. These two stages of Smf1p regulation are shown in...
Toxic manganese regulation of Smf1p occurs independent of the gene promoter. SMF1 exhibits manganese regulation when driven by either its native promoter (sequences from −296; Figure 1B) or the nonnative TP11 promoter (Figure 1C). Moreover, when SMF1 promoter sequences were fused to LacZ, there was no down-regulation of promoter activity by high manganese (Figure 2A). SMF1 is not transcriptionally regulated by toxic manganese, and we therefore examined posttranscriptional effects. The Bsd2p-dependent regulation of Smf1p occurs through vacuolar degradation (Liu and Culotta, 1999b), and we tested whether the same was true for Bsd2-independent regulation. GFP-Smf1 protein levels were monitored in a bsd2Δ strain also containing a pep4Δ mutation blocking vacuolar proteases. As seen in Figure 2B, down-regulation of GFP-Smf1 by toxic manganese was inhibited in the bsd2Δ pep4Δ strain. By comparison, mutants that affect endoplasmic reticulum-associated degradation (ERAD) via the proteasome had no effect on toxic manganese regulation of GFP-Smf1 (Supplemental Figure S1). Visualizing GFP-Smf1 in the bsd2Δ pep4Δ strain clearly shows GFP-Smf1 localization changing from a combination of plasma membrane and vesicular localization under physiological manganese to accumulation in the vacuole in the presence of toxic manganese (Figure 2C). Hence regulation by both physiological (Bsd2p-dependent) and toxic manganese (Bsd2p-independent) involves targeting of the protein to the vacuole for degradation.

Three pathways for vacuolar targeting in yeast involve trafficking through multivesicular bodies (MVB), autophagy, and the pathway used by alkaline phosphatase (ALP; reviewed in Huang et al., 2007 and references therein). To test which if any of these were involved in toxic manganese regulation of Smf1p, we created double bsd2Δ pep4Δ (blocking the MVB pathway), bsd2Δ atg1Δ (blocking autophagy), and bsd2Δ apm1Δ (blocking ALP) mutants. An inspection of GFP-Smf1 localization in a bsd2Δ pep4Δ strain yielded a large punctate staining pattern reminiscent of class E compartments (Supplemental Figure S2) that are hallmarks of a block in trafficking through the MVB pathway (Raymond et al., 1992). Therefore as is the case with Bsd2-dependent regulation of Smf1p (Hetema et al., 2004; Sullivan et al., 2007), Bsd2-independent regulation at toxic manganese involves trafficking to the vacuole through the MVB.

To verify that the enhanced stability of Smf1p in the presence of toxic manganese was not specific for pep4Δ cells, we examined GFP-Smf1 levels in additional mutants containing blocks in the MVB pathway. Key elements of the MVB pathway are the ESCRT complexes that sort proteins destined for the vacuole into endosomes and the MVB

Figure 1A. The abundance of Smf1p tagged at the N-terminus with GFP ("GFP-Smf1") is high in manganese-deficient medium compared with growth in the same minimal medium supplemented with 20 μM manganese (Figure 1A, left, cf. lanes 1 and 2). Adding increasing levels of manganese up to 5 mM does not change GFP-Smf1 levels, nor does the metal cause substantial toxicity over these several orders of magnitude concentrations (Figure 1A, cf. lanes 2–7 and total growth bars below). We refer to this large range of nontoxic metal concentrations as "physiological" manganese. Yet when manganese in the growth medium becomes toxic to the cell, GFP-Smf1 levels are further decreased (Figure 1A, lanes 8 and 9). The down-regulation of Smf1p by toxic manganese is not medium specific and also occurs in enriched medium, the only difference being the effective dose. The toxic dose of manganese is much lower in enriched medium, as cells are far more efficient at accumulating the metal from enriched medium, perhaps because of formation of extracellular manganese-phosphate complexes (Yang et al., 2005). As seen in Figure 1B, lanes 1–3, the level of a Smf1p tagged at the C-terminus with HA "HA-Smf1-HA" from cells grown in enriched medium drops significantly as manganese levels begin to inhibit growth (see total growth bars below), and the same regulation at toxic manganese was observed with GFP-Smf1 (Figure 1C, lane 2).

The effect of high manganese on Smf1p does not reflect nonspecific growth inhibitory effects of metals. In Figure 1B, lanes 8 and 9). The down-regulation of Smf1p by toxic manganese involves the Bsd2p and Tre1/Tre2 proteins implicated in Smf1-regulation by physiological manganese (Liu et al., 1997; Stimpson et al., 2006). As seen in Figures 1A, A (right) and C, regulation of GFP-Smf1 by toxic manganese is preserved in a bsd2Δ strain. The same is true of GFP-Smf1 expressed in a triple bsd2Δ tre1Δ tre2Δ strain (Figure 1C). Hence, regulation of Smf1p at very high manganese is distinct from regulation by low manganese in that it does not involve Bsd2p and the Tre1 and Tre2 proteins.

Toxic manganese regulation of Smf1p does not reflect...
Regression of Smf1p at toxic manganese is posttranslational and occurs through vacuolar degradation. (A) The promoters of SMF1 and ACT1 were fused to LacZ, and expressed in WT cells treated for 16 h with the indicated concentration of manganese in YPD medium. β-Galactosidase activity was measured using o-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. Results are the averages of two trials; error, SD. (B–D) GFP-Smf1 was expressed in the indicated strains grown in YPD medium (+) or the same medium supplemented with 200 μM MnCl2 (+) for 16 h. (B and D) The abundance of GFP-Smf1 was expressed in the indicated strains grown in YPD medium (–) or the same medium supplemented with 200 μM MnCl2 (+) for 16 h. (B and D) The abundance of GFP-Smf1 was monitored using immunoblots. (C) Cellular localization of GFP-Smf1 was observed by fluorescence microscopy at 100× magnification. Strains utilized include WT, BY4741; bsd2Δ, 5738; bsd2Δ pep4Δ, LJ388; bsd2Δ, LJ389; bsd2Δ aptg1Δ, LJ405; and bsd2Δ apm5Δ, LJ404.

(Williams and Urbe, 2007). We observed that blocking each of the ESCRT complexes individually using vps23Δ (ESCRT-I), vps36Δ (ESCRT-II), and snf7Δ (ESCRT-III) mutations in combination with bsd2Δ resulted in enhanced stability of GFP-Smf1 grown with exposure to toxic manganese (Figure 3A). In addition to the ESCRT complexes we monitored GFP-Smf1 levels in bsd2Δ yeast lacking genes involved in entry of proteins into (PEP12 and VPS27; Gerrard et al., 2000a,b) or exit from (VPS27) the prevacuolar compartment (Piper et al., 1995), and again we observed a block in the manganese-induced degradation of Smf1p (Figure 3B). Localization of GFP-Smf1 in the bsd2Δ vps27Δ strain with toxic manganese is also consistent with a large endosome structure, most likely the class E compartment (Supplemental Figure S2). These results demonstrate that the MVB pathway is involved in targeting of Smf1p to the vacuole in cells grown in the presence of toxic manganese.

Smf1p has been localized to both the plasma membrane and in internal vesicles (Liu and Culotta, 1999b; Sullivan et al., 2007). To determine if vacuolar targeting during manganese exposure involves one or both of these Smf1p locations, we utilized mutants that either blocked the endocytic pathway (end3Δ; Benedetti et al., 1994) or Golgi-to-vacuolar trafficking (vps45Δ; Bryant et al., 1998). We observed that blocking either endocytosis or Golgi to vacuolar transport was capable of inhibiting some degradation of Smf1p during exposure to toxic manganese (Figure 3C), yet effects of blocking Golgi to vacuolar transport were greater than that of blocking endocytosis (see legend Figure 3C). These results suggest that the Bsd2-independent vacuolar targeting of Smf1p largely involves protein derived from the exocytic pathway, but also from the endocytic pathway.

Two Steps for Down-Regulating Smf1 by Toxic Manganese

The aforementioned studies with secretory pathway mutants suggest that both cell surface and intracellular Smf1p are subject to down-regulation by toxic manganese. In wild-type (WT) cells, steady-state Smf1p is predominately intracellular, whereas in bsd2Δ mutants and also tre1 tre2 mutants, the protein can accumulate at the cell surface (Liu and Culotta, 1999b; Stimpson et al., 2006; Sullivan et al., 2007; also see Figure 4B). We therefore analyzed Smf1p levels and localization in WT versus bsd2Δ cells as a function of time of manganese treatment. The experiments of Figures 1–3 were all conducted with long-term “chronic” manganese exposures (16 h). Yet we observed that the addition of toxic manganese to WT cells has no effect during short periods and requires 4 h to see any change in GFP-Smf1; even so Smf1p loss is no greater than 50% (Figure 4A, lane 7). By comparison, the response of GFP-Smf1 in bsd2Δ cells to toxic manganese is more rapid and GFP-Smf1 levels are reduced...
transporter of iron (Portnoy et al., 2000; Luk and Culotta, 2001). As seen in Figure 5A (lanes 3 and 4), Smf2-HA expressed from its native promoter is not normally down-regulated during chronic exposures to toxic manganese. However, Smf2p is expressed at very low levels compared with Smf1p (cf. lanes 1 and 3, Figure 5A) and when placed under control of the strong TP11 promoter, Smf2p did exhibit some toxic manganese regulation, but effects were only seen during chronic (Figure 5B, lanes 3 and 4, bottom panel), and not short-term (top panel) exposures to manganese. Smf2p may have some capacity to respond to toxic manganese, but only when expressed at nonphysiological high levels. Compared with Smf1p and Smf2p, Smf3p is not regulated by manganese under any conditions tested, either driven by its own promoter (Figure 5A, lane 6) or the TP11 promoter (Figure 5B, lane 6). We also tested the effects of manganese on the uracil permease Fur4p and the tryptophan/tyrosine permease Tat2p. The abundance of both Fur4-GFP and Tat2-GFP are not significantly changed by growth in the presence of toxic manganese (Figure 5B). Toxic manganese preferentially down-regulates Smf1p.

Previous studies have shown that regulation of Smf1p by physiological manganese (Stimpson et al., 2006) or by cadmium (Nikko et al., 2008) involves ubiquitination by the E3 ligase Rsp5p; we tested whether the same was true for toxic manganese regulation. To monitor the effects of rps5 we first used a temperature-sensitive rps5-1 allele. As seen in Figure 6A, the manganese-induced endocytosis of GFP-Smf1 in the bsd2Δ strain was attenuated by the rps5-1 mutation at the restrictive temperature. To test the effects of RSP5 loss at longer chronic periods of manganese toxicity, we used a viable promoter mutant of rps5. In this allele, the HIS3 gene was inserted in the RSP5 promoter to cause a dramatic reduction in Rsp5p levels; this mutant is similar to other RSP5 promoter mutants containing insertion of a Ty element

Figure 4. Two modes for down-regulating Smf1p by toxic manganese. (A) GFP-Smf1 levels were monitored from cells grown in YPD with no added metal (~, lanes 1 and 8) or the same medium supplemented with 200 μM MnCl₂ (16 h, lanes 2 and 9). Cultures were also grown in YPD to an OD 600 nm of ~1 and then supplemented with 200 μM MnCl₂ (lanes 3–7 and 10–14), and samples were removed at the indicated times after addition of manganese for immunoblot analysis. (B) Cellular localization of GFP-Smf1 was monitored using fluorescence microscopy at ×100 magnification. (C) GFP-Smf1 protein levels and cellular localization in bsd2Δ, tre1Δ trec2Δ, and bsd2Δ tre1Δ trec2Δ strains were monitored in cells grown in YPD as described in A and B. Strains utilized include WT, BY4741; bsd2Δ, 5738, bsd2Δ, IJ423; tre1Δ tre2Δ, tre1/2; and bsd2Δ tre1Δ tre2Δ, IJ420.
exposure of the rsp5 mutant to toxic manganese. Hence, Rsp5 appears necessary for manganese-induced endocytosis of Smf1p, but may not be as critical for vacuolar degradation of intracellular Smf1p during chronic exposures to manganese (lane 16), implying an alternative mechanism.

The Smf1p N-Termminus and Toxic Manganese Regulation

We sought to determine sequences of Smf1p that modulate regulation during manganese toxicity. Smf1p and Smf2p contain N-terminal extensions that are absent in Smf3p, and in the case of Smf1p, residues K33 and K34 within this N-terminus were found to be involved in Smf1p regulation by cadmium (Nikko et al., 2008). Specifically these residues were implicated in Rsp5p-mediated ubiquitination of Smf1p as a prelude to endocytosis (Nikko et al., 2008). We tested whether these same residues were involved in toxic manganese regulation of Smf1p. As seen in Figure 7A, lane 7, the K33,34R variant of GFP-Smf1 was degraded to a degree similar to that of WT GFP-Smf1 under chronic (16 h) exposures to manganese. Moreover, under shorter periods of manganese toxicity, the protein was endocytosed (Figure 7B) and degraded (Figure 7A, lanes 2–5) with roughly the same kinetics as WT GFP-Smf1. Although these N-terminal lysines are not absolutely essential for manganese regulation, we did observe a requirement for the Smf1p N-terminus. As seen in Figure 7A, a Δ63 variant of GFP-Smf1 lacking the cytosolic N-terminus was not down-regulated during 4 h of manganese treatment (lanes 2–5), nor was the protein subject to any endocytosis during this period (Figure 7B). Nevertheless, Δ63 GFP-Smf1 did eventually exhibit some down-regulation by toxic manganese during chronic exposures (Figure 7A, lane 7, middle panel). The N-terminus appears to facilitate the rapid response to toxic manganese, and residues other than K33 and K34 are involved.

Sensing of Manganese for Regulating Smf1p by Manganese Starvation versus Manganese Toxicity

An attractive model for the manganese regulation of Smf1p involves Smf1p as a sensor itself whereby metal binding to the transporter signals targeting to the vacuole for degradation. To begin to address this, we tested whether metal transport activity was necessary for the regulation of Smf1p during chronic manganese toxicity. Previous mutagenesis studies with bacterial and human Nramp transporters have identified conserved residues important for metal ion transport. On the basis of these findings, we introduced mutations D92G and E344A into TM1 of Smf1p and the response to toxic manganese. The protein levels (A) and cellular localization (B) of wild type, Δ63, and K33,34R GFP-Smf1 were monitored in bsd2Δ cells grown in YPD as described in Figure 4. Strain bsd2Δ, 5738 was used in these experiments.

Figure 6. The role of the ubiquitin ligases RSP5 and TUL1 in the down-regulation of Smf1p during toxic manganese stress. (A) Cultures of bsd2Δ and bsd2Δ rsp5-1 strains containing GFP-Smf1 were grown in YPD at 25°C to an OD600 nm of 1 then shifted to 37°C for 30 min before addition of 200 μM MnCl2, and samples were removed at the indicated times. Immunoblot analysis and cellular localization of GFP-Smf1 was done as in Figure 4. (B) Cellular localization of GFP-Smf1 was monitored using fluorescence microscopy as in A but in bsd2Δ pep4Δ or in a triple mutant containing an insertion of the HIS3 gene within the promoter of RSP5 (bsd2Δ pep4Δ rsp5). (C) Immunoblot analysis of GFP-Smf1 expressed in WT, bsd2Δ, rsp5, and tul1Δ grown in YPD alone (−) or supplemented with 200 μM MnCl2 (+) for either 3 or 16 h as in Figure 5. Strains utilized include WT, BY4741; bsd2Δ, 5738; rsp5, LJ424; tul1Δ, 4883; bsd2Δ pep4Δ, LJ388; and bsd2Δ pep4Δ rsp5, LJ426; bsd2Δ, LJ431; bsd2Δ rsp5-1, LJ432.

Figure 7. The N-terminus of Smf1p and the response to toxic manganese. The protein levels (A) and cellular localization (B) of wild type, Δ63, and K33,34R GFP-Smf1 were monitored in bsd2Δ cells grown in YPD as described in Figure 4. Strain bsd2Δ, 5738 was used in these experiments.
and TM8 of Smf1-HA, whose equivalent substitutions in human Nramp2 disrupt metal transport without affecting protein localization (Lam-Yuk-Tseung et al., 2003). We also introduced N95T into Smf1p TM1, which was shown to disrupt manganese transport in Escherichia coli Nramp (Chaloupka et al., 2005). All three Smf1-HA mutants were stably expressed in yeast (Figure 8B), yet were unable to complement the oxidative stress defect of a yeast mutant (Figure 8A), a phenotype specifically associated with loss of Smf1p-transport of manganese-based antioxidants, which substitute for Cu/Zn SOD1 (Reddi et al., 2009). Transformation with WT Smf1 complements this defect and allows for aerobic growth on medium lacking lysine (left), whereas the corresponding Smf1p mutants do not. (B) Smf1-HA and the D92G, N95T, and E344A derivatives were expressed in the WT BY4741 strain and protein abundance was monitored using immunoblot. Cultures were grown in YPD medium alone (−) or supplemented with 200 μM MnCl₂ (+; top panel) or in minimal defined medium depleted of manganese (−) or supplemented with 20 μM MnCl₂ (+) Bottom, strain utilized include WT, BY4741; and sod1Δ bsd2Δ smf1-1, XBS851.

Figure 8. Effects of metal transport mutations on manganese regulation of Smf1p. (A) The ability of the indicated Smf1-HA mutants or WT Smf1-HA to complement a smf1-1 mutation was tested. A sod1Δ bsd2Δ smf1-1 is unable to grow aerobically without lysine because of loss of Smf1p-transport of manganese-based antioxidants, which substitute for Cu/Zn SOD1 (Reddi et al., 2009). Transformation with WT Smf1 complements this defect and allows for aerobic growth on medium lacking lysine (left), whereas the corresponding Smf1p mutants do not. (B) Smf1-HA and the D92G, N95T, and E344A derivatives were expressed in the WT BY4741 strain and protein abundance was monitored using immunoblot. Cultures were grown in YPD medium alone (−) or supplemented with 200 μM MnCl₂ (+; top panel) or in minimal defined medium depleted of manganese (−) or supplemented with 20 μM MnCl₂ (+) Bottom, strain utilized include WT, BY4741; and sod1Δ bsd2Δ smf1-1, XBS851.

What are the pools of manganese that are being sensed for Smf1p regulation? Because Smf1p localizes at both the cell surface and secretory pathway, Smf1p regulation could involve manganese pools that are extracellular, cytosolic, or intraluminal secretory pathway. To begin to resolve this, we tested Smf1p regulation in yeast mutants affecting manganese homeostasis. We have previously shown that yeast mam3Δ mutants accumulate lower cytosolic manganese under manganese surplus conditions and are resistant to manganese toxicity (Yang et al., 2005). As seen in Figure 9A, it takes greater concentrations of manganese to down-regulate Smf1-HA in a mam3Δ mutant compared with the WT strain. Because it is intracellular and not extracellular manganese that is affected in mam3Δ mutants (Yang et al., 2005), Smf1-HA is responding to an intracellular pool of toxic manganese during chronic manganese toxicity. Another mutant affecting cellular pools of manganese is pmr1Δ. PMR1 encodes a Golgi transporting ATPase for Mn. Mutants lacking Pmr1p accumulate high manganese in the cytosol and low Golgi manganese, and are exquisitely sensitive to manganese toxicity (Rudolph et al., 1989; Lapinskas et al., 1995; Durr et al., 1998; Mandal et al., 2000). As seen in Figure 9B, pmr1Δ mutants down-regulate Smf1-HA at much lower manganese concentrations than the WT strain. As such,

Figure 9. Smf1p is sensing intracellular manganese. (A–C) Smf1-HA was expressed in WT, mam3Δ, or pmr1Δ strains and protein abundance, and cell growth was monitored as in Figure 1. Cultures for A and B were grown in YPD medium, and the cells for C were grown in manganese-depleted minimal defined medium for 16 h. In each case the medium was supplemented with the indicated concentration of manganese (μM). ev, empty vector–transformed cells to illustrate position of nonspecific band cross-reacting with anti-HA. Strains utilized include WT, BY4741; mam3Δ, 1752; and pmr1Δ, 4534.
Smf1p appears to sense cytosolic or extra Golgi manganese during chronic manganese toxicity. We also used the pmr1Δ mutant to test whether cytosolic or Golgi luminal manganese is being sensed with manganese starvation. In WT cells, Smf1-HA is normally expressed at high levels with manganese starvation, and down-regulated in response to 5–10 μM (nontoxic/physiological) levels of manganese (Figure 9C). Yet in a pmr1Δ mutant, there is no such response to manganese; the polypeptide remains at high levels (Figure 9C). Because a pmr1Δ mutant blocks manganese uptake into the secretory pathway, these studies indicate that under manganese starvation conditions, Smf1p is sensing intraGolgi manganese. Hence, the manganese for regulating Smf1p at manganese starvation and chronic manganese toxicity appears to arise from different cellular compartments.

DISCUSSION

Yeast cells respond to differential manganese exposures by altering the polypeptide levels and localization of the major Nramp manganese transporter Smf1p. Under manganese starvation, the transporter stably accumulates at the cell surface, whereas with physiological manganese, a bulk but not all of the protein is degraded in the vacuole via the MVB pathway. We now show that under manganese toxicity conditions, the remainder of Smf1p is also targeted to the MVB for vacuolar degradation, but through a distinct mechanism. As we show for Smf1p, the Ftr1p iron transporter of yeast also undergoes two tiers of vacuolar degradation (Strochlic et al., 2008). With physiological iron, Ftr1p is subject to “basal MVB sorting” and with higher iron levels, a “stimulated MVB sorting” occurs where a larger proportion of the transporter is degraded (Strochlic et al., 2008). Both basal and stimulated MVB sorting of Ftr1p employ the same pathway involving ubiquitination by the E3 ligase Rsp5p (Strochlic et al., 2008). Although Smf1p is similarly subject to basal and stimulated MVB sorting, distinct pathways are utilized. Basal MVB sorting with physiological manganese clearly involves recognition by Bsd2p/Tre1p/Tre2p, whereas stimulated MVB sorting at high manganese does not. Furthermore, basal MVB sorting of Smf1p clearly requires ubiquitination by Rsp5p, whereas a role for Rsp5p in stimulated MVB sorting of Smf1p is less obvious. Our studies thus far suggest that Rsp5p is required for manganese-induced endocytosis of Smf1p, but may not be required for ultimate delivery of Smf1p to the vacuole. However, since the rsp5 mutants used in these studies are not nulls, we cannot totally exclude the possibility that a low level of Rsp5p is sufficient to trigger vacuolar targeting of Smf1p during manganese toxicity. In any case, the lack of a requirement for Bsd2p and Tre1/Tre2 demonstrate that basal and stimulated MVB targeting of Smf1p are distinct. The need for dual pathways may reflect the second Nramp transporter, Smf2p. Smf2p is subject to the same basal MVB, Bsd2p-mediated degradation as Smf1p (Portnoy et al., 2000), but is not typically turned over with toxic manganese. Smf2p is critical for activating manganese enzymes in the cell (Luk and Culotta, 2001) and the use of a Bsd2-independent pathway for regulating Smf1p during toxic manganese may help keep Smf2p levels constant.

Proteins can enter the MVB through either endocytic (from cell surface) or exocytic (from Golgi) pathways. Our studies show that both pathways can contribute to Smf1p degradation during manganese toxicity, but the kinetics for the two are different. In the case of cell surface Smf1p that accumulates in bsdΔ strains, treatment with toxic manganese can trigger complete endocytosis of the transporter within 1–3 h. But when steady-state Smf1p is largely intracellular, as with WT cells, the kinetics for vacuolar targeting are remarkably slow and require ≥4 h. Furthermore, the Smf1p that is endocytosed upon manganese treatment is not immediately targeted to the vacuole, but requires sustained metal exposures to reach the vacuolar lumen. It is possible that this endocytic Smf1p merges with exocytic Smf1p into the same “holding” pool of the transporter that is ultimately moved into the vacuole after prolonged periods of toxic manganese exposure. In any case, the kinetics are quite slow in comparison to other reports of vacuolar targeting of metal transporters. With yeast transporters for copper, zinc and iron, the response time for transporter endocytosis and/or vacuolar targeting typically ranges from minutes to 2 h (Gitan and Eide, 2000b; Pena et al., 2000; Felice et al., 2005). Moreover, cadmium was recently shown to induce endocytosis of Smf1p within minutes and vacuolar degradation within 1–2 h (Nikko et al., 2008). The degradation of Smf1p by toxic manganese, particularly in WT cells is indeed very slow. The rationale for this slow response may be based on the role of Smf1p in oxidative stress protection. Exceedingly high levels of manganese that approach toxicity can provide a unique benefit to cells by promoting formation of nonproteinaceous manganese antioxidants (Archibald and Fridovich, 1981, 1982; Chang and Kosman, 1989; Al-Maghrebi et al., 2002; Barnese et al., 2008). In S. cerevisiae, Smf1p serves as the primary source of these manganese antioxidants (Reddi et al., 2009), and therefore Smf1p can be both protective and detrimental in the face of high manganese. The immediate degradation of Smf1p during high manganese exposures may not always be advantageous.

Our studies with manganese accumulation mutants (i.e., pmr1 and mam3) show that during chronic manganese toxicity, intracellular, presumably cytosolic pools of the metal are being sensed for down-regulation of Smf1p. As one possibility, the metal directly reacts with Smf1p in the secretory pathway to stimulate its movement to the vacuole. The manganese translocating residues of the transporter are not likely to be involved, as Smf1p mutants defective in manganese transport were still subject to toxic manganese regulation. Other, perhaps low-affinity manganese binding sites on Smf1p may trigger trafficking to the vacuole. Alternatively, manganese binding to an as-of-yet unidentified partner protein for Smf1p may direct vacuolar targeting in the face of manganese toxicity. Lastly, these studies have provided evidence for distinct modes of manganese sensing under very high and very low manganese. Our experiments with pmr1Δ mutants disrupted for Golgi uptake of manganese indicate that under manganese starvation conditions, the cell senses intra-Golgi manganese for up-regulation of Smf1p. By comparison, manganese pools outside the secretory pathway, presumably cytosolic are being sensed for the strong down-regulation of Smf1p during long-term manganese toxicity. This separate compartmentalization for manganese sensing is logical when one considers the consequences of too much or too little manganese. During manganese starvation, the critical manganese sugar transferases of the Golgi are at risk for inactivation (Nakajima and Ballou, 1975; Parodi, 1979; Durr et al., 1998). The cytosol on the other hand appears to be a prime target of manganese toxicity (Lapinskas, 1995; Durr et al., 1998). The distinct compartmentalization for sensing extremes of metal starvation and metal toxicity are likely applicable to other transition metals that are both essential and potentially deleterious.
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