The Saccharomyces cerevisiae SMF1 gene encodes a member of the well conserved family of Nramp metal transport proteins. Previously, we determined that heavy metal uptake by Smf1p was down-regulated by the product of the S. cerevisiae BSD2 gene. We now demonstrate that this regulation occurs at the level of protein stability. In wild type strains, the bulk of Smf1p is normally directed to the vacuole and is rapidly degraded by vacuolar proteases in a PEP4-dependent manner. In bsd2Δ mutants, Smf1p fails to enter the vacuole, and the Nramp protein is stabilized. Metal ions themselves play an important role in the post-translational regulation of Smf1p. The depletion of heavy metals from the growth medium effects stabilization of Smf1p and additionally results in accumulation of this transporter at the cell surface. Supplementation of manganese alone is sufficient to trigger rapid degradation of Smf1p in a Bsd2p-dependent manner. Together, the action of Bsd2p and metal ions provide a rapid and effective means for controlling Nramp metal transport in response to environmental changes.

The Nramp family of polypeptides (for natural resistance associated macrophage protein) consists of a group of highly conserved integral membrane proteins thought to play an important role in heavy metal transport. Homologues to Nramp have been identified in animals, plants, and fungi, as well as in certain bacteria (1, 2). Among the most studied are the Nramp1 and Nramp2 genes of rodents. Nramp1 is believed to control the phagosomal accumulation of redox active iron or manganese ions, thereby contributing to an oxygen radical defense against parasitic infection (3–7). Nramp2 is expressed in all tissues and is needed for proper iron absorption and utilization (7–9). In rats, the Nramp2 isoform (DCT1) is induced following iron starvation and exhibits a broad substrate range including essential metals such as zinc, iron, manganese, and copper, as well as the nonessential metals cadmium and lead (10). Transporters such as DCT1/Nramp that act on both essential and toxic metals are expected to fall under tight cellular control.

The bakers' yeast Saccharomyces cerevisiae provides an excellent model system in which to study the function and regulation of eukaryotic metal transporters. The high affinity uptake of copper, iron, and zinc in yeast is accomplished by the action of the CTR1, FTR1, and ZTR1 gene products, respectively (11–13). Each of these transport systems is induced under metal-starvation conditions and correspondingly repressed at physiological metal concentrations, and this regulation occurs at the level of CTR1, FTR1, or ZTR1 gene transcription (13–19). S. cerevisiae also contains two Nramp homologues, SMF1 and SMF2 (1, 2, 20). Smf1p and Smf2p share approximately 40% identity with mammalian Nramp proteins (1, 2, 20), and accordingly, murine Nramp2 complements the metal transport defect of smf mutant yeast (21). Smf1p was identified by Supek et al. (6) as a manganese transporter, and our subsequent studies demonstrated that Smf1p and Smf2p additionally participate in the transport of other heavy metals including copper, cobalt, and cadmium (22). Hence like mammalian Nramp transporters, yeast Smf proteins exhibit a broad specificity for both essential and nonsential toxic metals.

We have previously shown that metal transport by Smf1p is suppressed in yeast by a process involving the product of the BSD2 gene (22). When BSD2 is inactivated by mutation, the transport of copper and cadmium by Smf1p greatly increases, and cells accumulate toxic levels of the metals (22). Bsd2p exhibits an endoplasmic reticulum (ER)1 localization (22), yet the mechanism by which Bsd2p controls Smf1p was unclear. In the present study, we demonstrate that Bsd2p and metal ions act together to facilitate the rapid turnover of Smf1p. This post-translation control of Smf1p effectively minimizes the hyper-accumulation of toxic metals and also provides a rapid switch for inducing metal uptake under conditions of metal starvation.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—Most of these studies employed the isogenic wild type (AA255) and bsd2Δ::HIS3 mutant (XL115) strains as described previously (22). Strains XL112 (22) and XL120 are smf1Δ::URA3 and bsd2Δ::URA3 derivatives of AA255. XL118 and XL119 are ubc7Δ::HIS3 mutants derived from AA255 and XL120, respectively. The isogenic wild type (L3852) and pep4Δ::URA3 (ACY17) strains were kind gifts of A. Chang. XL121 and XL125 were obtained by replacing the chromosomal BSD2 gene with HIS3 as described (22) in strains L3852 and ACY17, respectively. XL126 and XL124 are pep4Δ::HIS3 mutants derived from AA255 and XL101, respectively.

Stocks of strains were maintained on standard yeast extract/petriplate/trexidose media, and cultures for experimental analysis were obtained by growth in a synthetic minimal medium containing dextrose (SD) (23). All yeast transformations were carried out by electroporation (24). A metal-depleted minimal defined medium (MDM) was prepared through use of an ion exchange resin as described (25). MDM was supplemented with 2.4 mM MgSO4, 30 mM KCl, 2.0 mM CaCl2 and 0.86 mM MnCl2. The abbreviations used are: ER, endoplasmic reticulum; MDM, metal-depleted minimal defined medium; PCR, polymerase chain reaction; SD, synthetic minimal medium containing dextrose; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; HA, hemagglutinin.
Fig. 1. Mutations in BSD2 cause an increase in Smf1 protein levels. A, Western blot of Smf1-HA. Extracts were prepared from the indicated yeast strains transformed with either the Smf1-HA CEN plasmid pSF4 or with pRS315 vector (−) (26), as indicated. 5 μg of cell protein were subjected to 12% PAGE, followed by Western blot analysis using an anti-HA antibody. Migration of the 66-kDa Smf1-HA band was followed by co-electrophoresis of molecular weight markers (left). B, Northern blot analysis of SMF1 expression. Total RNA from the indicated strains was subjected to Northern blot analysis using SMF1 containing sequences as probe (top), followed by rehybridization with an ACT1 probe encoding actin (38) as control (bottom). Strains utilized were: wt = AA225; bsd2Δ = XL115; and smf7Δ = XL112.

Molecular Biology—Construction of the Smf1-HA expressing plasmid pSF4 involved polymerase chain reaction (PCR) amplification of SMF1 sequences −230 to the stop codon using a primer that changed the termination sequence to an SphI site (CAT ATG) in frame with HIS3. The product was ligated to the pCRII vector (Invitrogen), and following digestion with NdeI and ApaI, the SMF1-containing fragment was used to replace the BSD2 sequences of a CEN LEU2 plasmid expressing Bsd2-HA (22). The Smf1-HA URA3 CEN plasmid pSF10 was obtained by inserting the Smf1-HA containing insert of pSF4 into the XhoI and SacI sites of pRS416 (26).

The Smf1-HA plasmid was transformed into wild-type strains by the PCR-mediated gene disruption method as described (27). A ubc7Δ::HIS3 cassette was amplified by PCR using the HIS3 plasmid pRS403 (26) as template, and primers that spanned UBC7 sequences −140 to −100 and +533 to +494, with respect to the start codon, both fused at their 3′ ends to the sequence designated by Brachmann et al. (27) for amplification of yeast auxotrophic markers. Proper deletion of UBC7 was confirmed by colony PCR. The pep4Δ mutation was similarly introduced using primers that spanned PEP4 sequences +24 to +64 and +1230 to +1270.

RNA blot analysis of SMF1 expression followed standard procedures (28) and employed a probe spanning SMF1 sequences −68 to +1833 amplified by PCR and radiolabeled with 32P. Blot analysis—For Western blot analysis, yeast cells expressing the Smf1-HA fusion protein were grown to a mid-logarithmic phase (A600 = 1.0) in SD medium or MDM as needed. Extracts were prepared either by glass bead homogenization (29), or by an alkaline lysis procedure (30). Samples were resolved by 12% SDS-PAGE and analyzed by Western blot using a mouse anti-HA antibody (Babco) as described previously (22).

Immunofluorescence microscopy analysis was conducted essentially as described (22). Briefly, strains transformed with a CEN Smf1-HA plasmid were grown to a mid-logarithmic stage in SD medium, fixed with formaldehyde, digested with zymolyase, and probed with a mouse anti-HA antibody and a secondary antibody consisting of a goat anti-mouse antibody coupled to FITC (Boehringer Mannheim) as described (22). Nucleic acids were stained with DAPI (Sigma). FITC and DAPI staining were monitored by fluorescence microscopy, whereas visualization of yeast vacuoles used Nomarski optics.

Sucrose gradient fraction was conducted with cells grown to an A600 of 0.5–1.0. For examination of Golgi, ER, and vacuolar markers, cell lysates were prepared as described (22) and were fractionated over linear or step gradients of 18–54% sucrose. Assays for GDPase and NADPH cytochrome c reductase were carried out by standard proce-

Fig. 2. bsd2Δ mutations increase the stability of Smf1p. The indicated strains of yeast transformed with the Smf1-HA plasmid pSF4 were treated with 100 μg/ml cycloheximide for the designated time points prior to cell lysis and analysis of Smf1p by Western blot as described in Fig. 1A. Ten times more cell lysate was analyzed in wild type (wt) samples compared with bsd2Δ samples. Strains utilized were: wt = AA225; bsd2Δ = XL115.

Fig. 3. The role of the ubiquitin-mediated and vacuolar pathways of protein degradation in the control of Smf1p stability. The indicated yeast strains transformed with pSF4 were subjected to Western blot analysis of Smf1-HA as described in the legends to Figs. 1 and 2. A, cells were lysed without additional treatment. B, cells were treated with cycloheximide for the given time points prior to preparation of cell lysates. Strains utilized were: panel A, left, wt = AA225; bsd2 = XL120; bsd2 Δ = XL119; and bsd2 pep4 = XL125; panel A, right, and panel B, wt = L3852; bsd2 = XL121; pep4 = ACY17; and bsd2 pep4 = XL125.

RESULTS

The Effects of bsd2 Mutations on Smf1p Protein Stability—We previously reported that the S. cerevisiae BSD2 gene negatively regulates the accumulation of metal ions by the Smf1p metal transporter (22). In the present study, we monitored the accumulation and localization of this transporter using an epitope-tagged version of the protein. Two copies of the hemagglutinin (HA) epitope were fused in frame to Smf1p at the stop codon, creating a protein fusion that was fully functional in complementing smf1Δ mutations when expressed from its native promoter and present on a single copy CEN vector (not shown). To monitor the effects of bsd2 mutations on Smf1p levels, total extracts from cells expressing the fusion protein were analyzed by immunoblot. As seen in Fig. 1A,
Protein Stability Control of Nrfp

Smf1p was increased in the wild type strain AA255 expressing Smf1-HA and were subjected to sucrose gradient fractionation. Fractions were collected from the top to bottom and were assayed for GDPase activity (G) in nanomoles of P_i liberated per 20 min (a Golgi marker (G)); for NADPH cytochrome C reductase (C) in nanomoles of cytochrome c reduced per 10 min per µg of protein (an endoplasmic reticulum marker (ER)); and for Smf1-HA (H) detected by Western blot, quantitated by densitometric tracings, and shown as a percentage of the maximal level of Smf1-HA.

Smf1-HA expressed from the CEN vector was increased by approximately 10–20-fold in a bsd2 null mutant compared with a Bsd2 wild type strain.

To investigate whether bsd2Δ mutations result in a transcriptional induction of the Smf1 gene, total RNA from isogenic wild type and bsd2Δ mutants was subjected to Northern blot analysis. We observed that mutations in Bsd2Δ did not increase the expression of Smf1 at the mRNA level (Fig. 1B).

We next addressed whether bsd2 mutations affect Smf1 protein stability. Through the use of cycloheximide to inhibit new protein synthesis, we observed that Smf1-HA is normally a very unstable protein and exhibits an apparent half-life between 10 and 20 min (Fig. 2). In comparison, Smf1-HA was greatly stabilized in the bsd2Δ mutant (Fig. 2). These results demonstrated that the Bsd2 gene product is involved in controlling stability of the Smf1p polypeptide.

Bsd2 Regulation of Smf1p Depends on Vacular but Not Ubiquitin-mediated Proteolysis—Two major protein degradation pathways predominate in yeast, a ubiquitin-mediated pathway and a pathway involving proteolytic breakdown in the vacuole. In the ubiquitin-mediated pathway, proteins destined for degradation by the 26 S proteasome are tagged with ubiquitin via the action of a ubiquitin-conjugating enzyme such as Ubc7p (33). The possible involvement of this pathway in the degradation of Smf1p was examined through use of an ubc7Δ mutant. As seen in Fig. 3A, the steady state levels of Smf1-HA were increased in the ubc7Δ null strain, suggesting that the proteasome participates to some degree in Smf1p degradation. However, inactivation of Bsd2 still caused a rise in Smf1-HA protein levels in the ubc7 mutant (Fig. 3A). The strong additive effects of bsd2 and ubc7 mutations indicated that Bsd2 does not work through the ubiquitin pathway to control Smf1p stability.

To examine if Smf1p degradation involves the vacuole, we measured the steady state level of Smf1-HA in strains containing mutations in PEP4 necessary for vacuolar proteolysis (34). As shown in Fig. 3A, Smf1-HA accumulated to a high level in the pep4Δ mutant. Furthermore, an additional mutation in Bsd2Δ did not increase this high level of Smf1-HA. We next compared the turnover rates of Smf1-HA in these strains. A pep4Δ mutation was seen to increase Smf1-HA stability, and there was no additive effect of double mutations in Bsd2Δ and PEP4 (Fig. 3B). Thus, Bsd2Δ works through vacuolar protein degradation to control Smf1p stability.

The stabilization of Smf1p in a bsd2Δ mutant results in hyper-accumulation of copper and cadmium (22). Because pep4 mutations also cause stabilization of Smf1p, we tested whether the accumulated Smf1p in the pep4Δ mutant results in elevated metal uptake. Unlike bsd2Δ mutations, mutations in PEP4 did not effect a rise in metal accumulation (not shown). Thus the hyper-accumulated Smf1p in a pep4Δ mutant appears nonfunctional for metal transport.

Localization of Smf1p—The subcellular localization of Smf1-HA was examined by indirect immunofluorescence microscopy using a secondary antibody coupled to FITC. As seen in Fig. 4A, Smf1-HA expressed in a wild type strain exhibited rimming surrounding the nucleus (defined by DAPI) and a rimming around the nucleus (defined by DAPI) and a light punctate staining pattern that was absent in control cells not expressing Smf1-HA (not shown). This staining pattern suggested localization to the ER and Golgi, and this was confirmed by biochemical fractionation studies: Smf1-HA colocalized with markers for the Golgi and ER in sucrose gradient fractionation (Fig. 4B). It is noteworthy that our immunofluorescence studies failed to reveal the anticipated plasma membrane localization for Smf1p. To ascertain if the intracellular localization of Smf1-HA represented rapid internalization of a cell surface protein, we tested Smf1-HA localization in an end4Δ mutant defective for endocytosis (35). We observed that Smf1-HA staining was not affected by the end4 mutation (not shown), demonstrating that the bulk of Smf1p is not normally...
present on the cell surface. In a *bsd2* mutant, Smf1-HA exhibited a much brighter staining pattern because of elevated levels of this polypeptide, but the perinuclear and punctate staining pattern was still evident (Fig. 4A). A fraction of Smf1p appeared to be surface-localized in some *bsd2* cells, although this represented a minor component (Fig. 4A).

Smf1-HA localization was additionally examined in a *pep4D* mutant defective for vacuolar degradation. In this mutant, very intense staining of Smf1-HA was found within the vacuole identified by Nomarski optics (Fig. 5A), and in sucrose gradient fractionation, the bulk of Smf1-HA co-migrated with the vacuolar a-mannosidase marker (Fig. 6A). A quite distinct pattern of Smf1-HA localization was achieved in an isogenic *pep4D bsd2D* double mutant. By immunofluorescence microscopy, Smf1-HA was absent from the vacuole, and the protein exhibited a punctate staining pattern (Fig. 5B). Smf1-HA expressed in these cells failed to co-migrate with the vacuolar marker during sucrose gradient centrifugation and, instead, superimposed the markers for Golgi and ER (Fig. 6B). Together, these studies demonstrate that Smf1p is normally targeted to the vacuole for degradation by *PEP4*-dependent proteases. Furthermore, this delivery of Smf1p to the vacuole is dependent upon the product of the *BSD2* gene.

**Metal Ions and the Rapid Turnover of Smf1p**—We addressed whether metal ions can influence the stability of Smf1p. Treating the growth medium with elevated concentrations of heavy metals such as copper and manganese did not change the steady state levels of Smf1-HA (not shown). However, depletion of the heavy metals zinc, cobalt, copper, manganese and iron from the growth medium mimicked the effects of a *bsd2* mutation and resulted in high accumulation of Smf1-HA (Fig. 7A). The addition of manganese back to the growth medium was sufficient to down-regulate Smf1-HA protein levels in the wild type strain (Fig. 7, A and B), but not in the isogenic strain containing a *bsd2* mutation (Fig. 7B). Down-regulation of Smf1-HA was also observed upon supplementation of iron to the metal-depleted medium, although to a lesser extent than was observed with manganese (Fig. 7A). The individual addition of zinc, copper or cobalt to the metal-depleted medium had no effect on Smf1-HA levels (Fig. 7A).

We next tested whether the down-regulation of Smf1p by manganese ions occurs at the level of gene transcription or...
protein stability. As seen in Fig. 7C, manganese depletion had no effect on SMF1 mRNA levels, and the same was seen with iron depletion (not shown). To test for protein stability effects, the time course of Smf1-HA degradation was monitored in strains grown under manganese deplete or replete conditions. As seen in Fig. 7D, Smf1-HA expressed in the wild type strain was stabilized in the metal-depleted medium, and supplementation of manganese to the growth medium resulted in instability of Smf1-HA in the wild type strain but not in the bsd2 mutant (Fig. 7D). These findings demonstrate that metal ions can induce degradation of Smf1p through a mechanism dependent upon the BSD2 gene product.

To examine further the effect of metal ions on Smf1p, the localization of Smf1-HA was examined in cells grown in metal-starved versus metal-replete conditions. These studies were conducted with a pep4 mutant such that vacuolar Smf1-HA could be readily discerned. Under metal-starvation conditions, intense staining of Smf1-HA was observed at the cell surface (Fig. 8). Supplementation of metals back to this metal-depleted medium resulted in a dramatic shift in Smf1-HA localization where the bulk of this transporter was now found in the vacuole and was absent from the cell surface (Fig. 8). Therefore, the cellular localization of Smf1-HA is strongly influenced by the metal ion status of the cell. When metal ions are ample, Smf1p is targeted to the vacuole in a manner dependent on Bsd2p (Fig. 6, A and B). Yet Bsd2p is not required for the recruitment of Smf1p to the plasma membrane upon metal deprivation as metal starvation still triggered the cell surface localization of Smf1-HA in cells lacking Bsd2p (Fig. 6C). Therefore, factors other than Bsd2p are involved in the trafficking of Smf1-HA to the cell surface under metal-starvation conditions.

**Discussion**

These studies have addressed the regulation of metal transport by the yeast Nramp protein Smf1p. This transporter was previously shown to fall under negative control by the S. cerevisiae BSD2 gene (22), and we now demonstrate that this repression occurs at the level of Smf1p stability. The bulk of Smf1p is normally targeted to the vacuole for degradation by vacuolar proteases, yet in bsd2 mutants, the transporter fails to arrive at the vacuole and the bulk of the protein remains within the secretory pathway. Metal ions also play an important role in Smf1p regulation. Exposure of cells to physiological concentrations of metals such as manganese induces the degradation of Smf1p in a Bsd2p-dependent manner. Under conditions of metal starvation, the yeast Nramp protein fails to arrive at the vacuole and accumulates at the cell surface. This switch in localization to the cell surface occurs independent of Bsd2p.

Regulation of other metal transporters in yeast (e.g. the Ctr1p, Ftr1p, and Zrt1p/Zrt2p (30–36). We have found no evidence of transcriptional control of SMF1 by metals. The strong regulation of Smf1p at the protein stability level may be particularly important for a transporter that acts on both essential (e.g. manganese) and nonessential toxic (e.g. cadmium) metals. Targeting Smf1p to the vacuole ensures cessation of harmful metal uptake, yet when cells are starved for essential metals, Nramp activity can be rapidly induced by protein stabilization and redistribution to the plasma membrane, bypassing the need for new Smf1p synthesis.

How is Smf1p directed to the vacuole? When metals are abundant, the transporter appears to move directly through the secretory pathway to the vacuole without plasma membrane routing since a block in endocytosis fails to effect a plasma membrane localization for Smf1p under these conditions. Furthermore, Smf1p targeting to the vacuole is completely dependent on Bsd2p in the ER. Bsd2p does not appear to be a general receptor for the transport of polypeptides to the vacuole because bsd2 mutations do not impact on the delivery of carboxypeptidase Y (CPY) to the vacuole (37). However, Chang and co-workers have noted that a mutant allele of the plasma membrane proton ATPase, Pma1p (Pma1–7p), is targeted to the vacuole in a Bsd2-dependent manner (37). Presum-
ably mutant Pma1–7p adopts a specific conformation that is recognized by the Bsd2p-dependent machinery for vacuole targeting. We therefore propose that Bsd2p (or an auxiliary factor dependent on Bsd2p) recognizes a specific conformation of a subset of ion transporters as they pass through the secretory pathway and triggers the trafficking of these transporters to the vacuole for degradation.

Our studies are consistent with a model in which the metal bound or active form of Smf1p adopts a conformation that is recognized by Bsd2p for targeting to the vacuole. First, the trafficking of Smf1p to the vacuole is absolutely dependent on the presence of metals. Second, our recent studies indicate that vacuolar targeting requires a Smf1 polypeptide that is functional for metal transport. It is conceivable that the apo form of Smf1p adopts an alternative conformation that fails to be recognized by Bsd2p and additionally favors trafficking to the plasma membrane independent of Bsd2p. Although the mechanism underlying the switch for Smf1p trafficking is unknown, it may involve the unmasking of putative signal sequences on the Smf1 polypeptide for plasma membrane localization.

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