Manganese activation of superoxide dismutase 2 in *Saccharomyces cerevisiae* requires *MTM1*, a member of the mitochondrial carrier family

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Manganese-containing superoxide dismutase (SOD2) plays a critical role in guarding against mitochondrial oxidative stress and is essential for survival of many organisms. Despite the recognized importance of SOD2, nothing is known regarding the mechanisms by which this nuclear-encoded protein is converted to an active enzyme in the mitochondrial matrix. To search for factors that participate in the posttranslational activation of SOD2, we screened for yeast genes that when mutated lead to SOD2 inactivation and identified a single ORF, *YGR257c*. The encoded protein localizes to the mitochondria and represents a member of the yeast mitochondrial carrier family. *YGR257c* was previously recognized as the homologue to human CGI-69, a widely expressed mitochondrial carrier family of unknown function. Our studies suggest a connection with SOD2, and we have named the yeast gene *MTM1* for manganese trafficking factor for mitochondrial SOD2. Inactivation of yeast *MTM1* leads to loss of SOD2 activity that is restored only when cells are treated with high supplements of manganese, but not other heavy metals, indicative of manganese deficiency in the SOD2 polypeptide. Surprisingly, the mitochondrial organelle of *mtm1*Δ mutants shows no deficiency in manganese levels. Moreover, *mtm1*Δ mutations do not impair activity of a cytosolic version of manganese SOD. We propose that Mtm1p functions in the mitochondrial activation of SOD2 by specifically facilitating insertion of the essential manganese cofactor.

Eukaryotic cells typically possess two evolutionarily distinct forms of superoxide dismutases (SODs) that function to remove superoxide: a copper-zinc containing SOD (SOD1) that localizes primarily to the cytosol and a manganese containing SOD (SOD2), which resides in the mitochondrial matrix. SOD2 lies in close proximity to the superoxide-generating mitochondrial respiratory chain, and deficiency of this SOD has dramatic consequences. Mice homozygous for SOD2 disruption die at the neonatal stage with damage to the heart and other organs, suggesting mitochondrial dysfunction (1–3). Absence of SOD2 in *Drosophila* also leads to early adult lethality (4). Therefore, it seems critical to maintain SOD2 activity at physiological levels.

Although much is known regarding the importance of SOD2 in eukaryotic growth and survival, the *in vivo* pathways leading to active SOD2 assembly remain largely unknown. SOD2 is a nuclear-encoded polypeptide that must be transported to the matrix of the mitochondria. The fully functional enzyme exists as a homotetramer, with each subunit harboring a single manganese cofactor coordinated to amino acids His-52, His-107, His-198, and Asp-194 (5). Given the wide spacing of the ligands, the enzyme is not likely to coordinate the manganese during the protein unfolding process associated with mitochondrial import. In fact, metal binding to metalloproteins has been shown to prohibit mitochondrial uptake of proteins (6). Hence, formation and loading of the manganese active site of SOD2 is expected to occur within the mitochondrial matrix.

Clues to the maturation process of SOD2 may be obtained from what is known about Cu/Zn-containing SOD1. Assembly of the active copper site in SOD1 requires the trafficking of copper to the SOD1 polypeptide and direct insertion of the metal ion by its copper chaperone, CCS (7, 8). Genetic studies in bakers' yeast (*Saccharomyces cerevisiae*) have shown that the copper for SOD1 can be derived from a variety of upstream copper transporters (9); however, there is just one copper chaperone for SOD1 (7, 8). In fact, the only instance in which yeast SOD1 can acquire copper independent of the chaperone is when intracellular copper accumulates to toxic levels and the ion is freely available to associate with the enzyme (8).

A similar scenario is expected for SOD2. Manganese is potentially toxic (10–12), and metal trafficking to SOD2 should require a specific relay system involving manganese transporters and a single downstream manganese chaperone. The only factor identified thus far to participate in manganese trafficking to SOD2 is yeast Smf2p, a member of the NRAMP family of metal transporters. This protein localizes to intracellular vesicles and helps traffic manganese to various cellular locations, including the mitochondria (13). In addition to Smf2p, it is expected that mitochondrial transporter(s) for manganese and a putative metallochaperone ultimately deliver manganese to SOD2. Nothing is known about these downstream factors for manganese trafficking.

In this study, we used a yeast genetics approach to identify potential manganese trafficking proteins for SOD2. We identified a single gene that we have named *MTM1* (manganese trafficking factor for mitochondrial SOD2), which is crucial for SOD2 activity. Mtm1p localizes to the mitochondria and represents a member of the mitochondrial carrier family (MCF) of proteins that is needed to activate SOD2 with manganese. Surprisingly, this MCF does not function simply as a general manganese transporter for the mitochondria, but appears to be specific for SOD2.

Materials and Methods

Yeast Strains and Growth Conditions. The majority of yeast strains used in this study are isogenic to the parental strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). The *smf2*Δ::*kanMX4* (1878), *ygr257c*Δ::*kanMX4* (7288), *sod2*Δ::*kanMX4* (6605), and *isa1*Δ::*kanMX4* (1515) mutant variants were purchased from Research Genetics (Huntsville, AL). The *MATα* and *MATa rho*$^-$ strains LJ104 and LJ109 were constructed as described with ethidium bromide (14). The *mtm1*Δ::*LEU2* MC101 strain was derived from BY4741 by using the pVC257Δ deletion plasmid linearized by *Pst*I. Yeast transformations were performed by the lithium acetate procedure (15), and transformants were selected

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Abbreviations: SOD, superoxide dismutase; *MTM1*, manganese trafficking factor for mitochondrial SOD2; MCF, mitochondrial carrier family; YPD, yeast-peptone-based medium supplemented with 2% glucose.

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on a minimal synthetic-defined media under anaerobic conditions. Successful gene deletion was verified by PCR.

Stocks of yeast were maintained in enriched yeast-peptone-based medium supplemented with 2% glucose (YPD) (16) or in synthetic-defined media at 30°C. Liquid YPD cultures were grown without shaking, and cultures on solid YPD plates were maintained in an anaerobic culture jar (BBL Gas Pak) (17).

Plasmids. Construction of pVC257Δ (*ygr257c*Δ::*LEU2*) involved PCR amplification of *MTM1* sequences −827 to −24 and +633 to +1595 using primers that created *Pst*I, *Hin*dIII, and *Bam*HI sites at −819, −39, and +648, respectively. The PCR products were digested at these sites and an internal *Pst*I site at +1482, and then ligated at the *Bam*HI and *Hin*dIII sites of the *LEU2* integrating vector pRS305 (18). The resulting pVC257Δ plasmid was then linearized with *Pst*I and used to delete the endogenous *YGR257C* ORF sequences −24 and +633. The Mtm1-GFP fusion was derived from the Mtm1 expression plasmid pLJ063 harboring *MTM1 YGR257c* sequences −824 to +1660 amplified by PCR and inserted into the *Bam*HI and *Not*I sites of the *URA3* vector pRS426. To create Mtm1-GFP, sequences surrounding the *YGR257c* stop codon TGA ATA AAG were first converted to a *Not*I site GGC GGC CGC in pLJ063. This plasmid was subsequently digested with *Not*I and *Sac*I and fused to a GFP gene with a downstream *TIM23* terminator liberated from pAA1 (19) by using the same restriction sites. The resulting in-frame Mtm1-GFP fusion construct was in turn subcloned into pRS415 and pRS416 through the *Hin*dIII and *Sac*I sites, generating pEL47G and pEL48G, respectively.

Biochemical and Immunofluorescence Techniques. For SOD activity assays and SOD2 immunoblot analysis, WT, *smf2*Δ, *rho*$^-$, and *mtm1*Δ strains were inoculated into 50 ml of YPD media at a starting A_{600} of 0.05, 0.05, 0.1, and 0.15, respectively. Cultures were incubated at 30°C without shaking for 16 h to a final A_{600} of 3.5–4.0 for WT, *smf2*Δ, and *rho*$^-$ strains and 2.5–3.0 for the *mtm1*Δ strain. The higher inoculation of *rho*$^-$ and *mtm1*Δ strains ensured that the four strains grew to similar growth states. This was particularly important for comparing specific activities of SOD2 because SOD2 polypeptide levels are lower when cell density was <2.0 OD units (data not shown). Cells were lysed by glass-bead agitation as described with the addition of 1% (vol/vol) protease inhibitor mixture (P8340, Sigma) to ensure the stability of the apo-SOD2 polypeptide. Cell lysates were subject to native gel electrophoresis for SOD activity analysis (20) or to denaturing gels for immunoblot analysis of SOD2, Mas2p, and Pgk1p as described (13). *Candida albicans* SOD3 was detected by an anti-SOD2 antibody diluted 1:5,000. For SOD2 antibody production, recombinant yeast SOD2 was purified from *Escherichia coli* transformed with pEL001 (13) according to procedures in ref. 21 and was used to prepare rabbit-generated anti-SOD2 antibodies (Cocalico Biologicals, Reamstown, PA).

For steady-state manganese and iron analyses, total cell lysate and mitochondrial and postmitochondrial supernatant fractions prepared as described (13) were subjected to analysis on a Perkin–Elmer AAnalyst 600 graphite furnace atomic absorption spectrometer according to the manufacturer's specifications.

For immunofluorescence studies, WT cells expressing Mtm1-GFP (pEL47G) were grown aerobically in synthetic-defined medium lacking leucine to a final A_{600} of 3.0. Immunofluorescence microscopy was conducted as described (22) except fixed cells were digested with 5 μg/ml zymolyase (ICN) for 10 min at 30°C. Mitochondria were probed with an antiporin antibody at a final concentration of 1:10,000 followed by staining with an anti-rabbit antibody conjugating to Alexa Fluor 594 (Molecular Probes) at 1:1,000 dilution. Nuclear and mitochondrial DNA was stained with 4′,6-diamidino-2-phenylindole as described (23). Fluorescence microscopy was conducted with a Zeiss Axiovert 135TV microscope (Microscopy Facility, Johns Hopkins Medical Institutions) at a magnification of ×1,000.

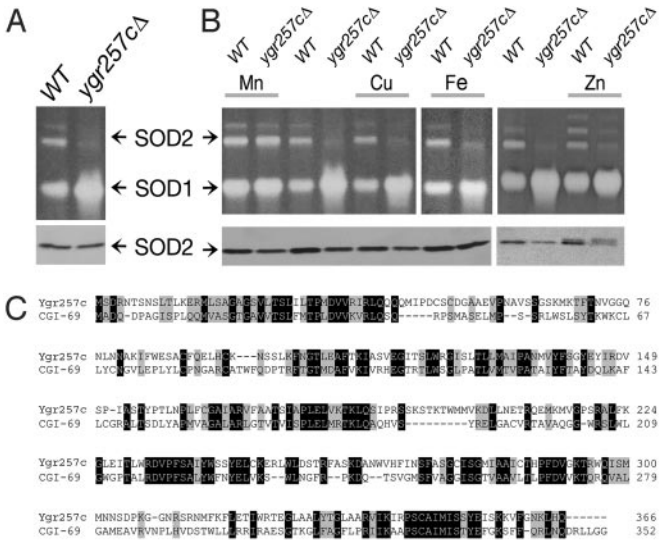

Fig. 1. The yeast *YGR257c* homologue to human CGI-69 is essential for manganese activation of SOD2. (*A* and *B*) Total cell lysates were prepared from BY4741 (WT) and the isogenic MC101 (*ygr257c*Δ) strains grown as described in *Materials and Methods*. Where indicated, the medium was supplemented with 250 μM $MnCl_2$, 10 mM $CuSO_4$, 1 mM $FeCl_3$, or 1 mM $ZnCl_2$. (*Upper*) Forty micrograms of lysate protein was analyzed by native gel electrophoresis, and SOD activity was visualized by nitroblue tetrazolium staining (13, 20). SOD1 and SOD2 activities were indicated by arrows. (*Lower*) Fifty micrograms of lysate protein was analyzed by denaturing gel electrophoresis followed by immunostaining using an anti-SOD2 antibody. (*C*) An alignment of the predicted *YGR257c* amino acid sequence with its reported human homologue CGI-69. Gray indicates similarity and black indicates amino acid identity.

Results

An MCF Protein Is Required for Manganese Activation of SOD2 in the Mitochondria. To search for the manganese transporter for mitochondrial SOD2, we used a yeast genetics approach. From the commercially available collection of yeast deletion strains, we selected ≈50 mutants representing putative membrane transporters and assessed their effects on SOD2 activity (Table 1, which is published as supporting information on the PNAS web site, www.pnas.org). The vast majority represented the MCF of proteins. These transporters, located in the inner mitochondrial membrane, function to regulate the exchange of small molecules between the matrix and cytosol. Eukaryotes typically express ≈30–40 distinct MCF proteins, and although substrates for many have been identified [e.g., components of the tricarboxylic acid cycle, ADP/ATP, flavins, etc. (24–26)], the substrates for the bulk of MCF transporters are unknown. In addition to the MCF proteins, 10 ATP-binding cassette transporters, 6 P-type ATPases, and 3 other transporters were tested for their effects on SOD2 activity (Table 1). Total extracts were prepared from the corresponding mutants, and each was individually analyzed for SOD activity by a native gel assay. Of all of the mutants tested, only one mutation, corresponding to a previously uncharacterized ORF *YGR257c*, reproducibly showed a consistent strong loss of SOD2 activity (Table 1). Some loss of SOD2 activity was also observed with mutations in *YDL198c* (*YHM1*) (27) and *YPL270w* (*MDL2*) (28); however, the effects were quite small (Table 1). The strong SOD2 defect associated with the commercially available *YGR257c* mutant was reproduced in a *ygr257c*Δ::*LEU2* mutant strain that we had engineered. SOD2 activity was virtually absent (Fig. 1*A Upper*), whereas the SOD2 polypeptide was still expressed (Fig. 1*A Lower*). Interestingly,

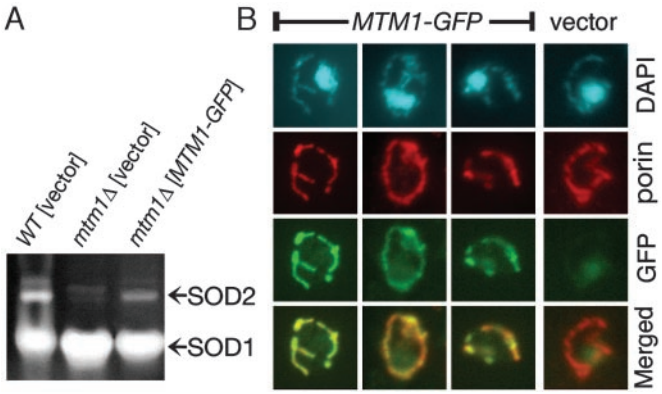

Fig. 2. A functional Mtm1-GFP fusion protein localizes to the mitochondria. The BY4741 WT or MC101 *mtm1*Δ strain were transformed with either pRS416 or pRS415 vectors or the pEL48G (*A*) or pEL47G (*B*) plasmids expressing *MTM1-GFP*. (*A*) Lysates were prepared from the indicated strains and subjected to analysis of SOD activity as in Fig. 1. (*B*) The indicated transformants of BY4741 were grown and prepared for microscopy as described in *Materials and Methods*. 4′,6-Diamidino-2-phenylindole (DAPI) staining (cyan) indicates mitochondrial and nuclear DNA. Porin immunofluorescence staining (red) identifies the mitochondria. GFP fluorescence (green) represents the location of the Mtm1-GFP fusion protein. The localization of Mtm1-GFP and porin are superimposed in the bottom row (merged).

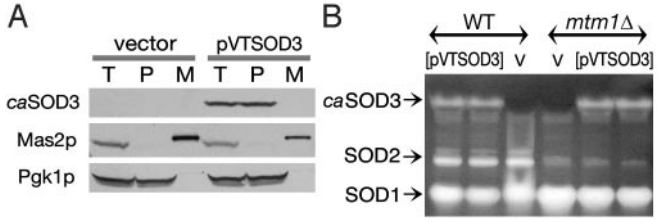

Fig. 3. MTM1 is needed to activate mitochondrial manganese-containing SOD2, but not a manganese SOD enzyme in the cytosol. (*A*) Total cell lysates (T) of the *S. cerevisiae sod2*Δ mutant strain 6605 transformed with an empty vector (pRS416) or with pVTSOD3 expressing *C. albicans* SOD3 (*ca*SOD3) were separated into mitochondrial fraction (M) and postmitochondrial supernatant fraction (P) by differential centrifugation as described (14). Sixty micrograms of total protein and the same cell equivalents of M and P protein samples were subjected to immunostaining with antibodies directed against *S. cerevisiae* SOD2 (cross-reacts with *C. albicans* SOD3) (*Top*), the mitochondrial processing protease Mas2p (*Middle*), or the cytosolic phosphoglycerate kinase Pgk1p (*Bottom*). (*B*) Total extracts prepared from WT yeast and *mtm1*Δ mutants transformed with the pVTSOD3 expressing *C. albicans* SOD3 or the empty vector pRS416 (V) were subjected to SOD activity analysis as conducted in Fig. 1. Two independent transformants with pVTSOD3 are shown. The activities corresponding to *S. cerevisiae* SOD1 and SOD2 and *C. albicans* SOD3 (*ca*SOD3) are indicated by arrows. On nondenaturing gels, *C. albicans* tetrameric SOD3 runs at a higher mobility than *S. cerevisiae* SOD2 even though the two molecules are highly homologous.

Cu/Zn SOD1 activity (Fig. 1*A*) and SOD1 protein (not shown) was increased in *ygr257c*Δ mutants, perhaps compensating for the loss of SOD2 activity.

YGR257c encodes a member of the *S. cerevisiae* MCF of proteins. The function of *YGR257c* was unknown, but it has previously been published as the yeast homologue to a widely expressed mammalian MCF represented by numerous EST fragments (29). This human clone known as CGI-69 was recently proposed to function as an uncoupler protein, but this could not be confirmed experimentally and the function of CGI-69 remained enigmatic (30). The complete sequence of CGI-69 aligned with yeast YGR257c is shown in Fig. 1*C*.

We addressed whether the SOD2 defect of *ygr257c*Δ mutants was caused by loss of manganese in the enzyme. If so, the activity defect might be overcome by high manganese supplements as has been shown for the loss of SOD2 activity in strains lacking the *SMF2* manganese transporter (13). Indeed, the SOD2 defect of the *ygr257c*Δ mutant was fully rescued by supplementation of 250 μM manganese to the growth medium. In contrast, similar supplements of other metals, such as copper, iron, and zinc (Fig. 1*B*) and magnesium (data not shown) were unable to restore the activity. It is noteworthy that the reactivation of SOD2 by manganese *in vivo* also reversed the up-regulation of Cu/Zn SOD1 in the *ygr257c*Δ mutant (Fig. 1*B*). Because of the effect of *YGR257c* on manganese activation of SOD2, we named the corresponding gene *MTM1* for manganese trafficking factor for mitochondrial SOD2.

MTM1 Is a Mitochondrial Protein and Specifically Affects the Mitochondrial Form of Manganese SOD. We wanted to confirm that yeast Mtm1p is localized in the mitochondria. These studies used a Mtm1-GFP fusion in which a GFP tag was fused to the C terminus of Mtm1p. The tagged construct was functional and capable of complementing the SOD2 deficiency of *mtm1*Δ mutants (Fig. 2*A*). As seen in the fluorescence microscopy images of Fig. 2*B*, Mtm1-GFP localization coincided with tubular structures recognized by an antibody raised against the mitochondrial porin protein. These studies confirm the mitochondrial localization of Mtm1p and are consistent with the mitochondrial location of the Mtm1p human homologue, CGI-69 (30).

Because Mtm1p localizes to the mitochondria, it is expected to specifically facilitate manganese activation of a mitochondrial form of SOD. To test this idea, we used a cytosolic version of manganese SOD. In our preliminary studies, *S. cerevisiae* SOD2 was found to be largely inactive when expressed in the cytosol of WT cells (unpublished work), making it difficult to assess the effects of Mtm1p on nonmitochondrial SOD2. However, *C. albicans* expresses a cytosolic form of Mn-SOD that is reported to be active when expressed in the cytosol of *S. cerevisiae*. This Mn-SOD, named SOD3, shares 60% sequence identity with *S. cerevisiae* mitochondrial SOD2 and clearly requires manganese for activity (31). In fact, *C. albicans* SOD3 cross-reacts well with an antibody directed against *S. cerevisiae* SOD2 (Fig. 3*A Top*). As seen in Fig. 3*A*, SOD3 is indeed cytosolic when it is expressed in *S. cerevisiae*, as it colocalizes with cytosolic Pgk1p and not with mitochondrial Mas2p. As published (31), SOD3 exhibits activity when expressed in WT *S. cerevisiae* (Fig. 3*B*). However, unlike the mitochondrial *S. cerevisiae* SOD2, the activity of the cytosolic Mn-SOD was not affected by a *mtm1*Δ deletion (Fig. 3*B*). This result strongly suggests that Mtm1p specifically works to control activation of manganese SOD that is present in the mitochondria.

Mitochondrial Metal Levels and MTM1. We originally hypothesized that Mtm1p functions as a mitochondrial transporter for manganese by delivering the metal to the mitochondrial matrix. If so, *mtm1*Δ deletions should effect a decrease in mitochondrial manganese. To our surprise, there was no obvious deficiency in mitochondrial manganese of *mtm1*Δ mutants. If anything, manganese levels were slightly elevated in the crude mitochondria of these cells (Fig. 4*A*) as measured by atomic absorption spectrometry. The same trends were observed with mitochondria purified by a Nycodenz gradient (32) (data not shown). Cytosolic (postmitochondrial supernatant) manganese was not affected by *mtm1*Δ mutations (Fig. 4*B*). Therefore, it appears that a mitochondrial deficiency in total manganese cannot account for the SOD2 defect in *mtm1*Δ mutants.

In addition to the slight elevated manganese accumulation,

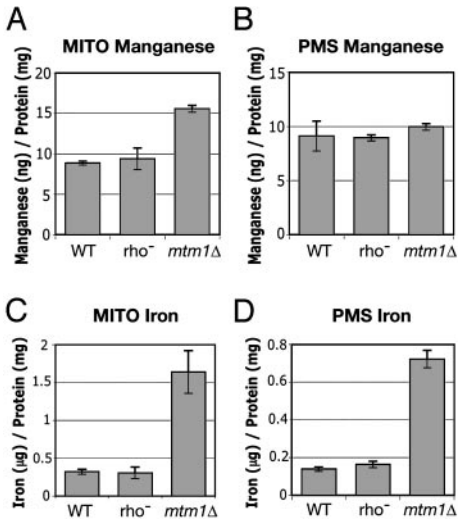

Fig. 4. Manganese and iron accumulation in *mtm1*Δ mutants. Mitochondrial fractions (MITO) and the postmitochondrial supernatant (PMS) were isolated as described in *Materials and Methods* from the indicated yeast strains cultured as described in Fig. 1. Samples were analyzed for iron and manganese content by atomic absorption spectrometry. Data represent averages of triplicate samples from three experimental trials. Error bars indicate SD. Yeast strains used were: WT, BY4741; *rho*$^-$, LJ109; *mtm1*Δ, MC101.

*mtm1*Δ mutants accumulate very high levels of iron in both the mitochondria and cytosol (Fig. 4 *C* and *D*). Currently, it is not clear why the mitochondria of *mtm1*Δ mutants hyperaccumulate manganese and iron, but the yeast may attempt to compensate for loss of Mtm1p by up-regulation of a mitochondrial metal transporter(s) (see *Discussion*). In any case, the elevated mitochondrial iron of *mtm1*Δ mutants is not responsible for the SOD2 deficiency because other yeast mutants that accumulate high mitochondrial iron [e.g., the *isa1*Δ mutant (14)] show no defect in SOD2 (Fig. 5*B*).

Accumulation of high mitochondrial iron in yeast generally leads to mtDNA mutations (33–39). A hallmark of such mutations is the inability to use glycerol as a carbon source. Mutants

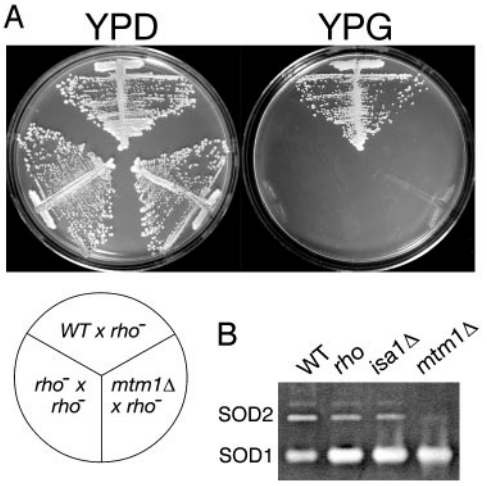

Fig. 5. Mutants of MTM1 accumulate mtDNA mutations. (*A*) Diploid yeast strains resulting from the indicated crosses (see circle template) were tested for growth on solid medium containing glucose (YPD) or glycerol (YPG) as the sole carbon source. (*B*) Total lysates from the indicated yeast strains were prepared and assayed for SOD activity as described in Fig. 1. Yeast strains used were: WT, BY4741; rho$^-$a, LJ109; rho$^-\alpha$, LJ104; *mtm1*Δ, MC101; *isa1*Δ, 1515.

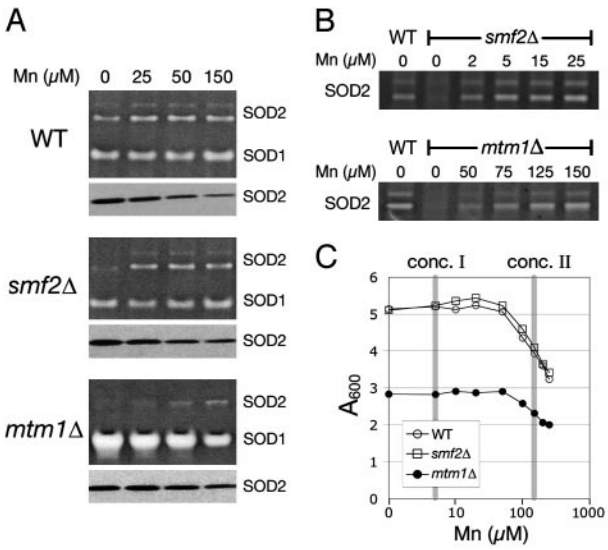

Fig. 6. Restoration of SOD2 activity in mtm1Δ mutants requires toxic supplements of manganese. (*A* and *B*) The indicated yeast strains were grown in YPD supplemented with the specified concentrations of $MnCl_2$ as described in *Materials and Methods* and were assayed for SOD activity and SOD2 protein as in Fig. 1. (*C*) Total cell growth after a 28.5-h culture was measured at A_{600}. Minimum manganese required to reactivate SOD2 in *smf2*Δ strains is 5 μM (conc. I) and in *mtm1*Δ strains is 150 μM (conc. II). Yeast strains used were: WT, BY4741; *mtm1*Δ, MC101; *smf2*Δ, 1878.

of *mtm1*Δ display this defect (40), and this phenotype is not complemented by crossing *mtm1*Δ mutants to *rho*$^-$ mutants that lack mtDNA (Fig. 5*A*). Therefore, *mtm1*Δ mutants also appear to accumulate mtDNA mutations. Yet mutations in mtDNA cannot explain the SOD2 deficiency associated with loss of *MTM1* because a *rho*$^-$ mutant lacking mtDNA exhibits normal SOD2 activity (Fig. 5*B*).

Reactivation of SOD2 in mtm1Δ Mutants Requires Toxic Levels of Manganese. Thus far we have identified two proteins needed to activate *S. cerevisiae* SOD2: the Smf2p manganese transporter and Mtm1p. The SOD2 defect associating with loss of either gene can be restored by manganese supplements. However, the SOD2 defect of *smf2*Δ mutants is clearly caused by low mitochondrial manganese (13), whereas there is no obvious manganese deficiency in the mitochondria of *mtm1*Δ mutants (Fig. 4*A*). We therefore directly compared manganese activation of SOD2 in *smf2*Δ and *mtm1*Δ mutants. As seen in Fig. 6*A*, it takes far more manganese to reactivate SOD2 in *mtm1*Δ mutants compared with *smf2*Δ mutants. With a more detailed titration, reactivation of SOD2 in *smf2*Δ strains requires only 2–5 μM manganese, whereas *mtm1*Δ mutants require as much as 150 μM (Fig. 6*B*). In fact, this latter concentration is approaching a toxic dose of manganese (Fig. 6*C*). Therefore, the SOD2 activity defect of *mtm1*Δ mutants is corrected only by manganese concentrations that approach toxic levels. This finding is reminiscent of what is observed with yeast mutants lacking the CCS copper chaperone for Cu/Zn SOD1; only toxic copper levels can compensate for loss of CCS (8). By analogy, Mtm1p would appear to behave more like a metal ion "chaperone" for SOD2 than a mitochondrial transporter for manganese (see *Discussion*).

Discussion

In the present study, we describe a mitochondrial factor that facilitates activation of manganese SOD2. This factor, named Mtm1p, is a member of the yeast MCF. Mtm1p localizes to the

mitochondria, and when the corresponding gene is mutated in yeast, SOD2 is largely inactive. This loss in SOD2 activity is fully rescued by high manganese supplements, indicative of manganese deficiency in the SOD2 polypeptide. It is noteworthy that the manganese defect of *mtm1*Δ mutants appears specific for mitochondrial SOD2. The mitochondrial organelle itself shows no deficiency in manganese, and moreover, *mtm1*Δ mutations have no effect on a cytosolic version of manganese SOD. We therefore propose that Mtm1p specifically functions to facilitate activation of holo-SOD2 in the mitochondria.

In addition to loss of SOD2 activity, *mtm1*Δ mutations are associated with an elevation in mitochondrial iron and manganese along with mutations in mtDNA, presumably resulting from the high mitochondrial iron. It is possible that other mitochondrial metal transporters are up-regulated in *mtm1*Δ mutants, in an attempt to compensate for the block of manganese delivery to SOD2.

How does Mtm1p modulate the activity of SOD2? A comparison of the known metal transporters and metallochaperones for SOD molecules may provide some clues. If Mtm1p were a metal transporter for the mitochondria, then we would expect the corresponding mutant to exhibit lower mitochondrial manganese and be rescued by low nontoxic manganese supplements that activate low-affinity metal transporters. This certainly is the case for the Smf2p manganese transporter for SOD2 (13). However, *mtm1*Δ mutants exhibit no obvious deficiency in mitochondrial manganese, and the SOD2 defect is rescued only when cells are treated with toxic levels of manganese, a condition when the metal ion may become freely available. Based on these findings and also the close proximity of Mtm1p to the SOD2 target, Mtm1p would appear to behave more like a specific manganese chaperone for SOD2 than a global manganese transporter for the mitochondria. It is possible that Mtm1p either directly inserts the metal into apo-SOD2 or delivers the metal to an accessory factor, which in turn inserts the cofactor into SOD2. We also do not exclude the possibility that Mtm1p may transport another mitochondrial substrate that is required for assembly of the manganese site in SOD2. In any case, SOD2 is not expected to acquire its metal from a free ionic pool, because despite the ample mitochondrial manangese found in *mtm1*Δ mutants, SOD2 is still unable to secure its cofactor.

Mtm1p is predicted to lie in the inner mitochondrial membrane, placing Mtm1p in close proximity to the site of SOD2 translocation into the matrix. Our preliminary studies indicate that manganese insertion into apo-SOD2 requires new protein synthesis and that protein unfolding during mitochondrial import may assist in this metal insertion process (unpublished work). As such, it is possible that manganese insertion takes place as the polypeptide enters the mitochondrial matrix, and that the inner membrane localization of Mtm1p could possibly facilitate this process.

Factors that control metalloenzyme assembly in the mitochondria can certainly have an important impact on mitochondrial function and cell fitness. For example, the SCO2 protein is required for copper insertion into mitochondrial cytochrome oxidase and mutations in the human gene have been associated with fatal infantile hypertropic cardiomyopathy and encephalopathy (41). Like cytochrome oxidase, SOD2 is an essential component of the mitochondria, and proteins like Mtm1p that modulate metal insertion into SOD2 could be an important risk factor for disease.

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