Mdm12p, a Component Required for Mitochondrial Inheritance That Is Conserved between Budding and Fission Yeast

Karen H. Berger, L. Farah Sogo, and Michael P. Yaffe

Department of Biology, University of California, San Diego, La Jolla, California 92093-0347

Abstract. *Saccharomyces cerevisiae* cells lacking the *MDM12* gene product display temperature-sensitive growth and possess abnormally large, round mitochondria that are defective for inheritance by daughter buds. Analysis of the wild-type *MDM12* gene revealed its product to be a 31-kD polypeptide that is homologous to a protein of the fission yeast *Schizosaccharomyces pombe*. When expressed in *S. cerevisiae*, the *S. pombe* Mdm12p homolog conferred a dominant-negative phenotype of giant mitochondria and aberrant mitochondrial distribution, suggesting partial functional conservation of Mdm12p activity between budding and fission yeast. The *S. cerevisiae* Mdm12p was localized by indirect immunofluorescence microscopy and by subcellular fractionation and immunodetection to the mitochondrial outer membrane and displayed biochemical properties of an integral membrane protein. Mdm12p is the third mitochondrial outer membrane protein required for normal mitochondrial morphology and distribution to be identified in *S. cerevisiae* and the first such mitochondrial component that is conserved between two different species.

Mitochondria are essential organelles that arise only by growth and division of preexisting mitochondria (Attardi and Schatz, 1988). Before completion of cytokinesis, a daughter cell must therefore receive a mitochondrial mass sufficient for viability. The molecular mechanisms and cellular components that mediate this mitochondrial inheritance are beginning to be elucidated through the analysis of *Saccharomyces cerevisiae* mutants exhibiting specific defects in mitochondrial distribution. These mitochondrial distribution and morphology (mdm) mutants were isolated by screening collections of temperature-sensitive strains by fluorescence microscopy to identify cells that failed to transfer mitochondria into daughter buds at the nonpermissive temperature (McConnell et al., 1990). Characterization of some of the mdm mutants has indicated that mitochondrial inheritance is a specific, active process that depends on a number of novel cellular components (Yaffe, 1996). One of these components, the Mdm1p protein, is a cytoskeletal element that exhibits structural similarities to the intermediate filament proteins of animal cells (McConnell and Yaffe, 1992, 1993). In addition, two different proteins of the mitochondrial outer membrane, Mdm10p and Mmm1p, were shown to be required for maintenance of the normal mitochondrial reticular network as well as for mitochondrial transmission to daughter cells (Sogo and Yaffe, 1994; Burgess et al., 1994). This report describes a third protein of the mitochondrial outer membrane, Mdm12p, which is essential for normal mitochondrial morphology and inheritance and which possesses a conserved homolog in the fission yeast *Schizosaccharomyces pombe*.

Materials and Methods

Strains and Genetic Techniques

*S. cerevisiae* strains used in this study were derived from wild-type strains MYY290 (*MATa, leu2, his3, ura3*), MYY291 (*MATa, leu2, his3, ura3*), or MYY298 (*MATa/α, leu2, his3, ura3*) (Smith and Yaffe, 1991; McConnell and Yaffe, 1992), *mdm12* mutant strains MYY620 (*MATa, leu2, his3, ura3, mdm12-1*), MYY621 (*MATa, leu2, his3, ura3, mdm12-1*), MYY625 (*MATa, leu2, his3, ura3, mdm12::URA3*), and MYY624 (*MATa, leu2, his3, ura3, mdm12::URA3*) are described below. *mdm10* mutant strains included MYY503 (*MATa, leu2, his3, ura3, mdm10::URA3*) (Sogo and Yaffe, 1994) and MYY505 (*MATa, leu2, his3, ura3, mdm10::LEU2*) (Berger, K., and M. Yaffe, unpublished results). *SOT1* strains MYY626 (*MATa, leu2, his3, ura3, SOT1*), MYY627 (*MATa, leu2, his3, ura3, mdm10::URA3, SOT1*), MYY628 (*MATa, leu2, his3, ura3, mdm12-1, SOT1*), and MYY629 (*MATa, leu2, his3, ura3, mdm12::URA3, SOT1*) are described below. Strain MYY461 (*MATa, leu2, his3, ura3, top1::URA3*) was isolated as a Ura* transformatant generated by transforming strain MYY290 with a top1::URA3 disruption cassette, which was a gift from C. Holm (University of California, San Diego). Growth conditions and media for *S. cerevisiae* were essentially as described (Rose et al., 1990). Yeast were transformed using lithium acetate (Ito et al., 1983). *Escherichia coli* strains DH5α and MH6 were used to amplify plasmid DNA. DNA manipulations were as described (Sambrook et al., 1989).
Identification of the mdm12-1 Mutant

The mdm12-1 mutant was isolated from a collection of temperature-sensitive strains by microscopic screening as previously described (Yaffe, 1995). The original mutant isolate was backcrossed three times to the wild-type parental strain to yield strain MYY620, and meiotic progeny from the final backcross displayed 2:2 cosegregation of temperature-sensitive growth and defects in mitochondrial distribution and morphology.

Cloning and Sequence Analysis of MDM12

The MDM12 gene was isolated by complementation of the temperature-sensitive phenotype of the mdm12-1 mutant. mdm12-1 cells were transformed with a yeast genomic DNA library in centromere vector p366 (obtained from M. Hecksträ, ICOS Inc., Bothell, WA). Leafy transformants were selected at 23°C and were replica plated to 37°C to identify temperature-resistant colonies. Six different clones were isolated, and restriction analysis revealed that these plasmids contained overlapping DNA inserts. Complementing activity was localized to a 1.3-kb Pkn–XbaI DNA fragment by subcloning and transformation of mdm12-1 cells.

The 1.3-kb fragment that complemented mdm12-1 was subcloned into plasmid pBluescript KS(−) (Stratagene Inc., La Jolla, CA) and digested with Exonuclease S1 to generate a set of nested deletions to use as sequencing templates. Nucleotide sequence of both strands of the complementing DNA region was determined by dyeoxyxynucleotide sequencing (Sanger et al., 1977). Oligonucleotide primers used for sequencing and PCR amplification (described below) were purchased from Operon Technologies Inc., (Alameda, CA). Subsequent to nucleotide sequencing of MDM12 DNA in our laboratory, the DNA sequence for the region including MDM12 was made available by the Saccharomyces Genome Database (SGD). Our DNA sequence data are largely in agreement with those provided by the SGD and are available from EMBL/GenBank/DDBJ under accession number U62252.

Mapping of MDM12 and mdm12-1

MDM12 was physically mapped by hybridization of a 32P-labeled DNA fragment containing the cloned MDM12 gene to a set of filters containing a mapped set of genomic clones provided by Dr. Linda Riles (Washington University, St. Louis, MO). The cloned sequences hybridized to two overlapping genomic clones from a region near the centromere of chromosome XV.

The mdm12-1 mutation was tested for linkage to Top1, a locus on the right arm and near the centromere of chromosome XV, by meiotic mapping. The meiotic progeny of a cross of strain MYY621 (mdm12-1 Top1) and strain MYY461 (MDM12 top1:URA3) yielded a total of 45/45 tetrads of the parental ditype (2 Ura− Ts+, 2 Ura+ Ts−), indicating linkage of the MDM12 and Top1 loci to within 1 centiMorgan (Sherman and Waksen, 1991).

MDM12 Gene Replacement

A 1.9-kb Pkn–ClaI DNA fragment containing MDM12 was subcloned into pBluescript KS(+) to yield plasmid pKB34. The URA3 gene was isolated on a HindIII fragment from plasmid pFL1 (Chevalier et al., 1980). The fragment ends were filled with Klenow, and the fragment was used to replace most of the MDM12 gene by ligation into the unique SnaB1 and MscI sites in plasmid pKB34. The resulting mdm12-1 URA3 disruption cassette was excised from the vector by digestion with KpnI and ClaI and transformed into diploid strain MYY298. Replacement of one of two chromosomal copies of MDM12 coding sequences with URA3 was confirmed by Southern blot analysis. The mdm12-1:URA3 (null) haploid strains (MYY623 and MYY624) were recovered by sporulation of the heterozygous diploid.

Analysis of Loss of Respiratory Function

Haploid meiotic progeny were obtained by sporulation of diploid cells heterozygous for either the mdm10::LEU2 mutation or the mdm12::URA3 mutation (obtained by crossing MYY291 to MYY505 or to MYY623, respectively), and the genotype of haploid segregants was determined. For each strain, individual cultures of four different haploid segregants were grown overnight in YPD liquid medium at 23°C, and dilutions were plated onto YPD agar medium. Strains of three different genotypes, MDM10 MDM12 (strain MYY290), mdm12::URA3, and mdm10::LEU2, were tested. Plates were incubated until small colonies were visible, and colonies were replica plated onto YPG medium. Colonies that grew on YPD but not on YPG were scored as having lost respiratory function (rho or rho2). For each strain a total of at least 243 colonies was counted.

Cloning and Analysis of the S. pombe MDM12 Homolog

The DNA region containing the S. pombe Mdm12p homolog, which was designated mdm12p, was amplified by PCR from genomic DNA of S. pombe strain 975 using oligonucleotide primers flanking the open reading frame. Primer sequences were 5′-GTACTAATTCGAAAGTAAAGTT-3′ (PMT-U01) and 5′-ATGTGCTAATGTGTCCTCCCA-3′ (PMT-L01). PCR amplification was performed with Taq polymerase (Fisher Scientific, Pittsburgh, PA) in Taq reaction buffer supplemented with 2.5 mM MgCl2 and 0.2 mM dNTPs (Boehringer Mannheim Corp., Indianapolis, IN) using an ERIOMP thermal cycler. The PCR product was cloned into the vector pCR2.1 (Invitrogen, San Diego, CA) to generate plasmid pPM1. Nucleotide sequencing of the cloned mdm12p was performed to verify the sequence reported in the database and to resolve ambiguities in this sequence. The DNA sequence obtained is available from GenBank under accession number U64674.

To generate a vector for expression of mdm12p in S. cerevisiae, the full open reading frame was amplified from genomic DNA of S. pombe strain 975 by PCR using a second upstream primer immediately 5′ of and including the predicted translational start site. Primers used were PMT-U02 (5′-GGGAATTCGAAACATAATGTGTCCTCCCA-3′) and PML-L01 (described above). The primer PMT-U02 introduced an EcoRI site (underlined) just upstream of the translational start site (bold). The PCR product was cloned into the pCR2.1 vector to generate plasmid pPM2. An EcoRI fragment containing mdm12p coding sequences was isolated from pPM2 and ligated into EcoRI-digested DNA of the S. cerevisiae centromere-based expression plasmid pAC1 (Hurt et al., 1985). The orientation of the mdm12p fragment in pAC1 was verified by restriction analysis. The resulting construct, plasmid pPM3, contains the S. pombe mdm12p gene under transcriptional control of the constitutive S. cerevisiae ADH1 promoter.

To construct a vector for the constitutive, high-level expression of S. cerevisiae MDM12, primers MDM12-U01 (5′-GGGTGCAAAATGTC-GTTTGTATTAAATTGGAGTA-3′) and MDM12-L01 (5′-GGGGAT-CCTTAAACCTTATTGGGTACATCAA-3′) were used for PCR amplification of S. cerevisiae genomic DNA from strain MYY290. The PCR product was cloned into plasmid pCR2.1, and a 1-kb EcoRI fragment containing MDM12 was subcloned into the EcoRI site of plasmid pAC1 to yield plasmid pKB37.

Isolation of the SOT1 Mutation

Haploid cells harboring the mdm10::URA3 mutation (strain MYY503) were grown overnight in YPD medium at 23°C to a density of ~106 cells/ml. A total of 1.5 × 109 cells was plated onto 40 YPD agar plates (4 × 106 cells/plate). Plates were incubated at 37°C, and a single temperature-resistant colony was obtained. Genetic analysis determined that temperature-resistance was caused by a single nuclear mutation unlinked to MDM10. Subsequent analysis showed that the suppressor mutation, designated SOT1, also suppressed defects caused by either the mdm12-1 or the mdm12-null mutation as well as those of the mdm10-null mdm12-null double mutant. In addition, a mutation that was determined genetically to be a second allele of SOT1 with similar suppressing activity was isolated independently in a similar selection for suppressors of the mdm12-null mutation.

Preparation of Antibodies to Mdm12p

The peptide (KGSWNLDFNDDDDE), comprising a two–amino acid linker after residues 126 and 128 of the yeast Mdm12p COOH-terminal peptide (Berger, K., unpublished results) to Affi-Gel 10. Antibodies were purified on an affinity column prepared by coupling the peptide to keyhole limpet hemocyanin, and used to immunize rabbits (Research Genetics, Huntsville, AL). For use in indirect immunofluorescence microscopy experiments, antibodies were purified on an affinity column prepared by coupling the Mdm12p COOH-terminal peptide to Affi-Gel 15 (Bio Rad Laboratories, Hercules, CA) per the manufacturer’s instructions. Affinity-purified antibodies were subsequently preabsorbed against fixed mdm12-null cells (Pringle et al., 1991) before use. For immunoblot analysis, antibodies were purified on an affinity column prepared by coupling a β-galactosidase–Mdm12 fusion protein consisting of LacZ fused to all but the first three amino acids of Mdm12p (Berger, K., unpublished results) to Affi-Gel 10.
Antibodies prepared by affinity purification against the LacZ-Mdm12p fusion protein recognized the same Mdm12p species as those purified against the COOH-terminal peptide but recognized fewer cross-reacting species (data not shown). Binding of anti-Mdm12p antibodies to both types of affinity columns and elution with 0.1 M glycine, pH 2.2, were essentially as described (Harlow and Lane, 1988), except that the column was washed with a solution of 0.14 M NaCl, 2.7 mM KCl, 5.4 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.2 (PBS), instead of Tris buffer. Preimmune sera subjected to the affinity purification protocol failed to yield antibodies with strong reaction to any of the protein species recognized by the affinity-purified immune antibodies.

Construction of Epitope-tagged Mdm12p

The 1.3-kb KpnI–XbaI DNA fragment including MDM12 was cloned into plasmid pRS316 (Sikorski and Hieter, 1989) to generate plasmid pKB35. Plasmid pJR1265, the source of c-myc epitope, consists of six tandem repeats of the epitope sequences in the polylinker of plasmid pBluescript KS (+) and was a gift from R. Hampton (University of California, San Diego). Sequences corresponding to five tandem copies of the c-myc epitope were purified from plasmid pJR1265 after digestion with Clal and Ncol and treatment with Klenow and were ligated into the unique SmaBI site within the MDM12 gene in pKB35 to yield pKB36. The correct orientation and fusion junctions of the c-myc–MDM12 construction were confirmed with restriction endonuclease and DNA sequence analysis.

Fluorescence Microscopy

Staining of live cells with the mitochondria-specific dye DASPMI (2-[4-dimethylaminostryl]-1-methylpyridinium iodide) and fluorescence microscopy were as previously described (Yaffe, 1995). Indirect immunofluorescence microscopy was performed as described previously (McConnell et al., 1990). The 9E10 antibodies (Evan et al., 1985) used for immunodetection of c-myc-Mdm12p were purchased from Berkeley Antibody Co. (Richmond, CA).

Cell Fractionation

Yeast cells were grown in semisynthetic lactate medium (Daum et al., 1982), homogenized, and subjected to differential centrifugation to isolate mitochondria and other subcellular fractions as previously described (Yaffe, 1991; Schauer et al., 1985). Mitochondrial subfractions were isolated as described by Daum et al. (1982). Extraction of mitochondria with sodium carbonate solution was performed as described (Sogo and Yaffe, 1994). SDS-PAGE and immunoblotting were performed by standard procedures (Douglas and Butow, 1976; Towbin et al., 1979).

Results

The mdm12-1 Mutation Causes Abnormal Mitochondrial Morphology and Distribution

The mdm12-1 mutant was identified in a collection of S. cerevisiae temperature-sensitive strains by microscopic screening of cells stained with the mitochondria-specific vital dye DASPMI (McConnell et al., 1990; Sogo and Yaffe, 1994). When visualized by fluorescence microscopy, wild-type mitochondria appeared as extended tubules and tubular networks peripherally distributed in both mother and bud portions of the cell (Fig. 1). In contrast, mdm12-1 mutant cells typically displayed one or two large round mitochondria per cell (Fig. 1). These giant mitochondria were frequently localized exclusively to the mother portion of cells, with mitochondrial staining largely absent from daughter buds. The mdm12-1 mutant displayed giant mitochondria and defective mitochondrial distribution at both permissive (23°C) and nonpermissive (37°C) temperatures (see Fig. 2), and mdm12-1 cells grew slowly at the permissive temperature. Large round mitochondria were present in mdm12-1 cells cultured on both fermentable (glucose) and nonfermentable (glycerol) carbon sources. Despite their grossly altered morphology, the mdm12-1 mutant mitochondria appeared to be competent for respiration because mdm12-1 cells grew, although very slowly, at 23°C on media containing glycerol. In addition, DASPMI uptake by mdm12-1 mitochondria indicated the presence of a significant membrane potential (Bereiter-Hahn, 1976). The defects in mitochondrial distribution and morphology in mdm12-1 mutant cells were confirmed by indirect immunofluorescence microscopy using antibodies specific for OM14, a major protein of the mitochondrial outer membrane (Fig. 2 B). Nuclear division and distribution appeared unaffected by the mdm12-1 mutation based on DAPI (4,6-diamidino-2-phenylindole) staining of nuclear DNA (Fig. 2 C).

The phenotype of the mdm12-1 mutant closely resembled that of another mutant, mdm10, previously characterized by our laboratory (Sogo and Yaffe, 1994), as well as that of a different mutant, mmm1 (Burgess et al., 1994). The mdm12-1 mutation was recessive and genetically distinct from mdam10 based on complementation analysis and allelism tests. In addition, the cloned MDM10 gene did not complement the mdm12-1 mutation. Haploid mdm10-null mdm12-1 double mutants were viable at 23°C and displayed temperature-sensitive growth, mitochondrial distribution, and mitochondrial morphology phenotypes similar to those of the single mutant (parental) strains (data not shown). The MDM12 gene was not identical to MMM1 (see below), and the mdm12-null mmm1-null double mutant displayed no synthetic phenotypes (data not shown). The mdm12 mutant was also complemented by other available mdm mutants tested. The mdm12-1 mutation therefore appeared to define a new MDM gene.

MDM12 Encodes a Novel Protein with a Homolog in Fission Yeast

To identify the molecular basis of the mdm12 mutant defects, the MDM12 gene was isolated from a wild-type S. cerevisiae genomic DNA library by complementation of the mdm12-1 temperature-sensitive growth defect. Nucleotide sequence analysis of a 1.3-kb DNA region (Fig. 3), which fully complemented the mutant phenotype, re-
revealed a single open reading frame of 813 bp encoding a putative polypeptide of \( \sim 31 \text{kD} \). An analysis of the predicted \( MDM12 \) product failed to reveal any known sequence motifs (Bairoch, 1992); however, the region from amino acid 24 to residue 40 is uncharged and of sufficient length to comprise a potential membrane-spanning domain. Subsequent to our analysis, the \( MDM12 \) open reading frame was independently identified as part of DNA sequence analysis by the \( S.\) \( \text{cerevisiae} \) Genome Database and has been designated YOL009c.

The cloned \( MDM12 \) gene was physically mapped to a region near the centromere on the left arm of chromosome XV (as described in Materials and Methods). The \( mdm12-1 \) mutation was mapped via genetic linkage analysis to the same region. These mapping data indicated that the mutant represented the authentic \( MDM12 \) gene rather than an extragenic suppressor.

The SWISSPROT and GENBANK data bases were searched for proteins homologous to Mdm12p using the BLAST sequence comparison program (Altschul et al., 1990). This search identified a single homologous protein: an uncharacterized, hypothetical gene product in the fission yeast \( S.\) \( \text{pombe} \). Because the DNA sequence of the \( S.\) \( \text{pombe} \) gene reported in the database contained a number of ambiguities, the gene was cloned by PCR, and its complete nucleotide sequence was determined. This analysis revealed a longer open reading frame than that previously reported in the database, with the gene encoding a potential product of 273 amino acids (Fig. 4 A). Alignment of the predicted \( S.\) \( \text{cerevisiae} \) Mdm12p with its \( S.\) \( \text{pombe} \) homolog indicated that the proteins shared significant homology over their entire lengths, with 32% sequence identity and 50% sequence similarity (Fig. 4 A). In addition to homology with the \( S.\) \( \text{pombe} \) protein, Mdm12p exhibited one region of sequence similarity to another protein, Mmm1p (Fig. 4 B). Although the level of identity between these regions of Mdm12p and Mmm1p was low (23%), the resemblance was noteworthy because loss of Mmm1p function produces a phenotype similar to that caused by an \( mdm12 \) mutation (Burgess et al., 1994).

**Fig. 2.** Indirect immunofluorescence confirms the mitochondrial inheritance and morphology defects of the \( mdm12-1 \) mutant. \( MDM12 \) (wild type; MYY290) and \( mdm12-1 \) (MYY621) cells were grown in YPD medium at 23°C, incubated at 37°C for 2 h, fixed with formaldehyde, and processed for immunofluorescence. Mitochondria were detected with antibodies specific for the mitochondrial outer membrane protein OM14 (B), and mitochondrial and nuclear DNAs were visualized by DAPI staining (C). Brightfield images of the corresponding cells are shown in A. Bar, 2 \( \mu m \).

**Fig. 3.** Restriction map of \( MDM12 \). Represented is a 1,283-bp KpnI–XbaI DNA fragment containing \( MDM12 \) complementing activity. The \( MDM12 \) open reading frame (solid black arrow) extends from nucleotide 284 to nucleotide 1099. In the \( mdm12::URA3 \) gene replacement, the portion of the \( MDM12 \) coding region between the SnaBI and MscI sites, corresponding to most of the \( MDM12 \) gene, is replaced with \( URA3 \) coding sequences (shaded gray arrow). Restriction sites: \( H \), HindIII; \( K \), KpnI; \( M \), MscI; \( S \), SnaBI; \( Sp \), SphI; \( X \), XbaI.

**Fig. 4.** Alignment of the amino acid sequences of \( MDM12 \) and \( URA3 \) genes. A, Alignment of the predicted \( S.\) \( \text{cerevisiae} \) Mdm12p with its \( S.\) \( \text{pombe} \) homolog. \( B \), Alignment of the predicted \( S.\) \( \text{pombe} \) Mdm12p with its \( S.\) \( \text{pombe} \) homolog. The shaded region indicates regions of sequence similarity. The Mdm12p and Mmm1p sequences share significant homology over their entire lengths, with 32% sequence identity and 50% sequence similarity. (A) Alignment of the amino acid sequences of \( MDM12 \) and \( URA3 \) genes. (B) Alignment of the predicted \( S.\) \( \text{pombe} \) Mdm12p with its \( S.\) \( \text{pombe} \) homolog. The shaded region indicates regions of sequence similarity. The Mdm12p and Mmm1p sequences share significant homology over their entire lengths, with 32% sequence identity and 50% sequence similarity.
The deficient mitochondrial inheritance caused by loss of MDM12 function was apparent at both permissive (23°C) and nonpermissive (37°C) temperatures. To quantify these inheritance defects, mitochondrial distribution in populations of mutant or wild-type cells was examined by fluorescence microscopy after incubation at 23°C or 37°C. At both temperatures, 100% of wild-type (MDM12) cells exhibited normal mitochondrial inheritance with mitochondria distributed in both mother and bud. In contrast, 84% (235/281) of mdm12-null cells grown at 23°C displayed a defect in mitochondrial inheritance. The fraction of mdm12-null cells with mitochondrial distribution defects increased to 89% (114/128) after incubation for 4 h at 37°C.

One difference between the mdm12-1 and mdm12-null mutants was that cells harboring the latter mutation were defective for sporulation. Diploid cells homozygous for the mdm12-null mutation did not form spores or asci when plated on sporulation medium, whereas mdm12-1/mdm12-1 homozygous diploids were capable of sporulation (data not shown).

Although mdm12 cells were able to grow (albeit very slowly) on nonfermentable carbon sources, mdm12 and mdm10 mutant cells readily produced respiration-deficient (rho− or rho0) cells at rates much higher than that of the wild-type parental strain. During overnight growth on glucose-containing medium, 59 ± 6% of mdm12-null cells and 98 ± 2% of mdm10-null cells lost mitochondrial respiratory function, whereas only 4 ± 2% of wild-type cells became respiration deficient during the same treatment.
but were largely absent when cells were incubated at 37°C (data not shown). To determine whether high-level expression of \textit{S. cerevisiae} MDM12 might produce a comparable mutant phenotype, the MDM12 gene was cloned behind the \textit{ADH1} promoter and expressed in \textit{S. cerevisiae} cells. The \textit{ADH1–MDM12} construct fully complemented the \textit{mdm12}-null mutant and did not produce any apparent phenotype in wild-type cells. The similarity of the mitochondrial distribution and morphology defects caused by expression of the \textit{S. pombe} homolog to the phenotype of \textit{mdm12} mutant cells suggests a partial conservation of function between the \textit{S. cerevisiae} and \textit{S. pombe} homologs of Mdm12p.

**Identification of SOT1, a Suppressor of Both mdm12 and mdm10**

To characterize further the \textit{mdm10} and \textit{mdm12} mutant defects, mutations able to bypass the cellular requirement for \textit{MDM10} or \textit{MDM12} were identified by selecting for growth of the mutant cells at 37°C. One such mutation, designated \textit{SOT1} (suppressor of \textit{mdm10/mdm12}), was initially identified as a suppressor of the \textit{mdm10-null} mutation but was also found to suppress the temperature-sensitive growth defect of \textit{mdm12-null} cells (Fig. 6 A). \textit{SOT1} also suppressed the growth defects of the \textit{mdm12-1} mutant and the \textit{mdm10 mdm12} double mutant (data not shown). Microscopic analysis of \textit{mdm10-null} and \textit{mdm12-null} cells harboring \textit{SOT1} revealed mitochondrial distribution and morphology at 23°C nearly identical to those found in wild-type cells (Fig. 6 B). Suppression of defects in growth (Fig. 6 A) and mitochondrial morphology (data not shown) was less effective at 37°C. In addition, although \textit{SOT1}-suppressed haploid cells could grow at the elevated temperature, \textit{SOT1} failed to suppress many of the \textit{mdm12} mutant defects in diploid cells. \textit{mdm12/mdm12} cells that were either heterozygous (\textit{SOT1/sot1}) or homozygous (\textit{SOT1/SOT1}) for the \textit{SOT1} (mutant) allele failed to grow at 37°C and did not exhibit suppressed (normal) mitochondrial morphology at either 23°C or 37°C. This lack of suppression in diploid cells prevented a determination of the dominant or recessive character of \textit{SOT1} with respect to the mitochondrial morphology and high temperature growth traits. A single copy of \textit{SOT1} was able to suppress fully the sporulation defect of the \textit{mdm12-null} homozygous diploid strain, which indicated that the \textit{SOT1} mutation was dominant (or codominant) to the wild-type allele (here designated \textit{sot1}).

In the absence of \textit{mdm10} or \textit{mdm12}, the \textit{SOT1} mutation did not confer any apparent mutant phenotype. \textit{SOT1} cells grew normally at both low and high temperatures on a variety of media and displayed normal mitochondrial distribution and morphology. In addition, \textit{SOT1} was unable to suppress the defects in mitochondrial morphology caused by expression of the \textit{S. pombe} Mdm12p homolog in \textit{S. cerevisiae} cells (data not shown). \textit{SOT1} showed no genetic linkage to \textit{mdm12} or \textit{mdm10} and appears to define a novel gene.

**Mdm12p Is a Component of the Mitochondrial Outer Membrane**

Two proteins previously shown to be required for normal mitochondrial morphology, Mdm10p and Mmm1p, are constituents of the mitochondrial outer membrane (Sogo and Yaffe, 1994; Burgess et al., 1994). To determine whether Mdm12p occupied a similar subcellular location, the protein was localized by indirect immunofluorescence microscopy and subcellular fractionation. To facilitate this analysis, antiserum was raised against a peptide whose sequence corresponded to the COOH terminus of Mdm12p, and antibodies specific to Mdm12p were purified on an affinity column (as described in Materials and Methods).

Indirect immunofluorescence microscopy of wild-type cells using affinity-purified anti-Mdm12p antibodies revealed that Mdm12p is associated with mitochondria (Fig. 7 A). The antibody failed to localize to mitochondria in \textit{mdm12-null} cells (which harbored \textit{SOT1} to maintain normal mitochondrial morphology) (Fig. 7 B). Mdm12p was also detected in mitochondria in wild-type cells harboring the \textit{SOT1} mutation (Fig. 7 C) and in \textit{SOT1}-suppressed \textit{mdm10-null} cells (Fig. 7 D).
To identify the protein species corresponding to Mdm12p, subcellular fractions isolated from wild-type (MDM12) and mdm12-null cells were analyzed by immunoblotting. Both MDM12 and mdm12-null strains harbored the SOT1 suppressor mutation. A protein species of ~31 kD, the predicted molecular size of Mdm12p, was detected in wild-type but not in mutant cell extracts (Fig. 8A). This protein species was enriched in the mitochondrial fraction. In addition, the antibodies detected two additional protein species of ~47 and 85 kD that appeared specific to MDM12 (Fig. 8A). All three protein species were enriched in the mitochondrial fraction and deficient in fractions depleted of mitochondria (Fig. 8A). Mdm12p showed a similar distribution in wild-type cells that did not carry the SOT1 mutation (data not shown). The relationship between the 31-kD protein and the two species of higher molecular mass is unclear. Various chemical treatments of mitochondria failed to change the relative amounts of the polypeptides (data not shown), and a specific consensus sequence for covalent modification (that might generate the higher molecular mass species) is absent from the predicted protein sequence of Mdm12p. The 47- and 85-kD species may represent Mdm12p-containing complexes or modified forms of Mdm12p. Alternatively, these species may be distinct from Mdm12p but share an antigenic epitope and, coincidentally, display decreased abundance in the absence of Mdm12p.

As an independent approach to investigating the MDM12 gene product, the localization of Mdm12p tagged with the c-myc epitope (described in Materials and Methods) was examined. Subcellular fractionation and Western blot analysis detected a polypeptide of ~40 kD, corresponding to the expected size of c-myc–Mdm12p, which was enriched in the mitochondrial fraction (Fig. 8B). Two other protein species of ~60 and 83 kD, which were likewise specific to cells harboring the c-myc–tagged construct, were also detected. These larger species were reminiscent of the higher molecular mass bands detected with antibodies specific to the native Mdm12p (discussed above). The 83-kD species was enriched in the mitochondrial fraction, whereas the 60-kD species was more evenly distributed in all subcellular fractions and represented the major species detected in total cellular extracts (Fig. 8B). One caveat to these results is that the c-myc–MDM12 construct only partially complemented the temperature-sensitive growth and mitochondrial morphology defects of mdm12 cells, and therefore the distribution and molecular forms of the epitope-tagged Mdm12p may not accurately reflect the properties of native Mdm12p.
Submitochondrial fractionation and immunoblot analysis of subcellular fractions from wild-type cells (strain MYY290) expressing c-myc–Mdm12p. Fractions are labeled as in A. Large arrow indicates species of predicted size of c-myc–Mdm12p, and small arrows indicate two additional protein species specific to cells harboring c-myc–MDM12. (C) Western blot of wild-type mitochondria (M), purified mitochondrial outer membrane (O), and inner membrane (I) fractions. 20 μg of protein was resolved in each lane. Fractions were analyzed using antibodies to Mdm12p (top), OM45 (middle), and F₁β (bottom). (D) Mitochondria (M) were resuspended in 0.1 M Na₂CO₃. Supernatant (S) and membrane pellet (P) fractions were recovered by centrifugation for 1 h at 100,000 g. Protein samples were analyzed by immunoblotting with antibodies to Mdm12p or Mdm10p.

Discussion

Mdm12p is essential for normal mitochondrial morphology and inheritance. Loss of this protein leads to the appearance of giant mitochondria and defective transfer of mitochondria into daughter buds. This role for Mdm12p is very similar to that of Mdm10p, and several additional observations suggest that these two proteins function at the same step in mediating mitochondrial behavior. First, mdm10 mdm12 double mutant cells display a phenotype identical to that of either single mutant. Second, Mdm10p and Mdm12p share a similar subcellular and submitochondrial localization. Third, the SOT1 mutation suppresses loss of either Mdm10p or Mdm12p or the loss of both proteins. Finally, two recently identified multicopy suppressors each provide partial suppression (i.e., slow growth at 37°C and partial restoration of tubular mitochondrial morphology) of mdm10, mdm12, or the double mutant (Berger, K.H., and M.P. Yaffe, unpublished results).

Cells lacking Mdm10p, Mdm12p, or both proteins exhibited aberrant mitochondrial morphology at all temperatures, yet they were viable at 23°C. Even at this permissive temperature, the mdm12 mutant displayed defective mitochondrial inheritance and appeared to give rise to a large proportion of unbudded cells lacking mitochondria. This mitochondrial inheritance defect is likely to be responsible for the mutant’s slow growth at permissive temperature.
because cells that failed to receive mitochondria would be inviable (Gebelska et al., 1983; Yaffe and Schatz, 1984). Growth of mdml2-deficient cells at permissive temperature suggests the existence of a backup or bypass pathway for mitochondrial distribution that operates at lower temperatures. At high temperatures, such a bypass pathway might fail to function or be insufficient for the metabolic needs of the cell. Although the molecular basis for a bypass pathway is unknown, the SOT1 mutation might define a component of this pathway and act to stimulate its activity.

Mdm12p is the third S. cerevisiae protein of the mitochondrial outer membrane to be identified whose loss causes temperature-sensitive growth and the appearance of enlarged, spherical mitochondria defective for distribution to buds at all temperatures. A potential role for these proteins—Mdm10p, Mmm1p, and Mdm12p—is as hooks or handles for attachment of mitochondria to cytoskeletal elements (Burgess et al., 1994; Sogo and Yaffe, 1994). Alternatively, these polypeptides might function primarily to alter properties of the mitochondrial outer membrane that are necessary for the maintenance of tubular mitochondrial morphology, and the mitochondrial inheritance defects might be a secondary consequence of the aberrant morphology. Given the similar phenotype caused by loss of Mdm12p, Mdm10p, or Mmm1p and the shared location of these three proteins, they may well function together in a complex.

The identification of a homolog of Mdm12p in fission yeast, together with the effect of the cloned homolog on three proteins, they may well function together in a complex. Mdm10p, or Mmm1p and the shared location of these proteins—Mdm10p, Mmm1p, and Mdm12p—is as hooks or handles for attachment of mitochondria to cytoskeletal elements (Burgess et al., 1994; Sogo and Yaffe, 1994). Alternatively, these polypeptides might function primarily to alter properties of the mitochondrial outer membrane that are necessary for the maintenance of tubular mitochondrial morphology, and the mitochondrial inheritance defects might be a secondary consequence of the aberrant morphology. Given the similar phenotype caused by loss of Mdm12p, Mdm10p, or Mmm1p and the shared location of these three proteins, they may well function together in a complex.

The identification of a homolog of Mdm12p in fission yeast, together with the effect of the cloned homolog on three proteins, they may well function together in a complex. Mdm10p, or Mmm1p and the shared location of these proteins—Mdm10p, Mmm1p, and Mdm12p—is as hooks or handles for attachment of mitochondria to cytoskeletal elements (Burgess et al., 1994; Sogo and Yaffe, 1994). Alternatively, these polypeptides might function primarily to alter properties of the mitochondrial outer membrane that are necessary for the maintenance of tubular mitochondrial morphology, and the mitochondrial inheritance defects might be a secondary consequence of the aberrant morphology. Given the similar phenotype caused by loss of Mdm12p, Mdm10p, or Mmm1p and the shared location of these three proteins, they may well function together in a complex.

The identification of a homolog of Mdm12p in fission yeast, together with the effect of the cloned homolog on three proteins, they may well function together in a complex. Mdm10p, or Mmm1p and the shared location of these proteins—Mdm10p, Mmm1p, and Mdm12p—is as hooks or handles for attachment of mitochondria to cytoskeletal elements (Burgess et al., 1994; Sogo and Yaffe, 1994). Alternatively, these polypeptides might function primarily to alter properties of the mitochondrial outer membrane that are necessary for the maintenance of tubular mitochondrial morphology, and the mitochondrial inheritance defects might be a secondary consequence of the aberrant morphology. Given the similar phenotype caused by loss of Mdm12p, Mdm10p, or Mmm1p and the shared location of these three proteins, they may well function together in a complex.

The identification of a homolog of Mdm12p in fission yeast, together with the effect of the cloned homolog on three proteins, they may well function together in a complex. Mdm10p, or Mmm1p and the shared location of these proteins—Mdm10p, Mmm1p, and Mdm12p—is as hooks or handles for attachment of mitochondria to cytoskeletal elements (Burgess et al., 1994; Sogo and Yaffe, 1994). Alternatively, these polypeptides might function primarily to alter properties of the mitochondrial outer membrane that are necessary for the maintenance of tubular mitochondrial morphology, and the mitochondrial inheritance defects might be a secondary consequence of the aberrant morphology. Given the similar phenotype caused by loss of Mdm12p, Mdm10p, or Mmm1p and the shared location of these three proteins, they may well function together in a complex.