MDP1, a *Saccharomyces cerevisiae* Gene Involved in Mitochondrial/Cytoplasmic Protein Distribution, Is Identical to the Ubiquitin-Protein Ligase Gene *RSP5*

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ABSTRACT

Alteration of the subcellular distribution of Mod5p-I, a tRNA modification enzyme, member of the sorting isozyme family, affects tRNA-mediated nonsense suppression. Altered suppression efficiency was used to identify *MDP* genes, which, when mutant, change the mitochondrial/cytosolic distribution of Mod5p-I,KR6. *MDP2* is the previously identified *VRP1*, which encodes verprolin, required for proper organization of the actin cytoskeleton. *MDP3* is identical to *PAN1*, which encodes a protein involved in initiation of translation and actin cytoskeleton organization. We report here the cloning and characterization of wild-type and mutant *MDP1* alleles and the isolation and characterization of a multicopy suppressor of *mdp1* mutations. *MDP1* is identical to *RSP5*, which encodes ubiquitin-protein ligase, and *mdp1* mutations are suppressed by high copy expression of ubiquitin. All four characterized *mdp1* mutations cause missense changes located in the hect domain of Rsp5p that is highly conserved among ubiquitin-protein ligases. In addition to its well-known function in protein turnover, ubiquitination has been proposed to play roles in subcellular sorting of proteins via endocytosis and in delivery of proteins to peroxisomes, the endoplasmic reticulum and mitochondria. *mdp1*, as well as *mdp2/vrp1* and *mdp3/pan1* mutations, affect endocytosis. Further, *mdp1* mutations show synthetic interactions with *mdp2/vrp1* and *mdp3/pan1*. Identification of *MDP1* as *RSP5*, along with our previous identification of *MDP2/VRP1* and *MDP3/PAN1*, implicate interactions of the ubiquitin system, the actin cytoskeleton and protein synthesis in the subcellular distribution of proteins.

SORTING of proteins to appropriate subcellular locations requires information encoded on the targeted proteins and receptors on the respective organelles as well as cytoplasmic factors that aid the targeting and import processes. Proteins delivered to mitochondria usually have amino-terminal sequences composed of basic and hydrophobic amino acids able to form an amphipathic structure (review: VON HEIJNE 1986; VERNER and SCHATZ 1988). The sequences are necessary and sufficient for mitochondrial targeting and they interact with mitochondrial membrane receptors that are hetero-oligomeric protein complexes located in the mitochondrial outer membrane (for a recent summary, see HAUCKE *et al.* 1996 and references therein). In addition to the translocases of the outer membrane (Tom proteins), import requires translocases of the inner mitochondrial membrane (Tim proteins) (PFANNER *et al.* 1996). Further, cytoplasmic chaperones are essential for the process of mitochondrial delivery. These chaperones are thought to function in generating appropriate conformations of the targeted proteins (review: MIHARA and OMURA 1996).

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To learn whether other cytoplasmic proteins play roles in delivery of proteins to mitochondria, we selected for yeast mutants that caused altered mitochondrial/cytosolic distribution of Mod5p-I,KR6, a protein involved in modification of mitochondrial and cytosolic tRNAs (ŻOŁĄDEK *et al.* 1995). Our selection for mutants (called *mdp* for mitochondrial distribution of proteins) was based on the hypothesis that tRNA-mediated suppression of the nonsense mutations will be enhanced if the cytosolic level of Mod5p-I,KR6 increases as a result of decreased mitochondrial import. We found that mutations located in four complementation groups, *mdp1–mdp4*, resulted in an increased functional cytosolic pool of Mod5p-I,KR6. Further studies of three of these groups (*mdp1–mdp3*) showed that the mutations caused depletion of the mitochondrial pools of Mod5p-I,KR6. *MDP2* and *MDP3* were cloned and sequenced and shown to be identical to *VRP1*, involved in the organization of the actin cytoskeleton (DONNELLY *et al.* 1993), and *PAN1*, involved in the initiation of protein synthesis and actin cytoskeleton organization (SACHS and DEARDOFF 1992; TANG and CAI 1996). These studies implicate the actin cytoskeleton and protein synthesis in the process of delivery of proteins to mitochondria (ŻOŁĄDEK *et al.* 1995).

Here we report the identification of *MDP1* as *RSP5*,

which encodes ubiquitin protein ligase (HUIBREGTSE *et al.* 1995) and show that *mdp1/rsp5* mutations located in the 3′ end of the *RSP5* coding sequence can be suppressed by overproduction of ubiquitin. A role for ubiquitin in mitochondrial protein import has been suggested previously by experiments which demonstrated that depletion of ubiquitin prevents insertion of a mitochondrial protein into rat liver mitochondrial membranes *in vitro* (ZHUANG and MCCAULEY 1989; ZHUANG *et al.* 1992). Our studies provide a genetic system for studies of the role of ubiquitination in mitochondrial protein delivery.

MATERIALS AND METHODS

Strains, media and transformation: The yeast strains used are listed in Table 1. Media were prepared and standard genetic manipulations were performed as previously described (SHERMAN 1991). Yeast strains were transformed by the method of CHEN *et al.* (1992). Preparation of genomic yeast DNA was done using the protocol of SHERMAN (SHERMAN *et al.* 1986) *Escherichia coli* RR1 and DH5α (SAMBROOK *et al.* 1989) were used for propagation of recombinant DNA. HB101 F *lac pro* and HB101 Tn5::PyrA(F^-) were used for γδ transposon mutagenesis. Standard methods for growth and transformation of *E. coli* were employed (SAMBROOK *et al.* 1989).

Cloning of wild-type *MDP1* gene and a multicopy suppressor: The wild-type *MDP1* gene was isolated from both YCp50 low copy (ROSE *et al.* 1987) and YEp24 multicopy (CARLSON and BOTSTEIN 1982) libraries by complementation of the temperature-sensitive (ts) growth defect of *mdp1-1* (TZ11, Table 1). Four plasmids, pC7 (YCp50 derivative), RB1, RB2 and RB4 (YEp24 derivatives) were found. Plasmids pC7 and RB1 contained genomic fragments of 16 and 5 kb, respectively, and had similar restriction patterns. A 6-kb *Hin*dIII fragment of RB1 was inserted into the integrating vector YIp5 (STRUHL *et al.* 1979) resulting in YIp-RB1. YIp-RB1 was cut with *Xho*I enzyme and used for integrative transformation of strain T8-1D (Table 1) and the *mdp1* mutant strains TZ11, TZ12, TZ23 and T85-10A. Plasmids RB2 and RB4 contained overlapping genomic sequences (5 and 4 kb) that differed from the sequences in pC7 and RB1. Plasmid RB2 was analyzed further. A 5-kb *Sal*I-*Sma*I fragment of RB2 was inserted into vector YIp5 (digested with *Nru*I and *Sal*I), and the resulting plasmid YIp-RB2 was cut with *Kpn*I and used for integrative transformation of strain T8-1D. The *Hin*dIII fragment of RB2 was transferred to YEp181 resulting in RB2-H. The *Eco*RI-*Sal*I fragment of RB2 was transferred to YEp181 resulting in YEp-RB2. Transposon mutagenesis of RB1 and RB2-H was performed employing γδ transposition as described previously (ŻOŁĄDEK *et al.* 1995).

Physical mapping of cloned sequences: A ~2.4-kb *Bam*HI fragment of RB1 and a 2-kb *Hin*dIII fragment of RB2 were radiolabeled by random priming and hybridized to a filter containing yeast chromosomes separated by pulse-field gel electrophoresis and filters containing the *Saccharomyces cerevisiae* genome in cosmid and λ clones (RILES *et al.* 1993).

Characterization of *mdp1* mutant alleles: The gap-repair method (ORR-WEAVER *et al.* 1981) was used to determine the general location of *mdp1* alleles in the *RSP5* gene. This method is based on the observation that when double-strand gapped plasmid integrates into the host genome, the plasmid-borne deletion is repaired from homologous chromosomal DNA. Deletion alleles of *RSP5* were constructed by digestion of plasmid YIp-RB1 with *Kpn*I or *Bst*EII followed by ligation. The resulting plasmids YIp-RB1-K and YIp-RB1-B, respectively, were propagated in bacterial strains, isolated and digested with *Kpn*I or *Bst*EII before transformation. Genomic DNA was prepared from strains TZ11, TZ12, TZ23 and T85-10A (Table 1) that contain *mdp1-1, mdp1-2, mdp1-13* and *mdp1-14* mutations, respectively. The 862-bp fragments from *mdp1* alleles starting from codon 686 and extending 487 bp beyond the stop codon were amplified by PCR using 1μg of genomic DNA, 5′-CTATTGATCGGTGGTATTGC-3′ and 5′-GTACCTCACGAGAGCAATTTC-3′ oligonucleotides as primers, *Taq* polymerase (Amersham) and a Air Thermo-Cycler (Idaho Technology). After 30 cycles of 93° for 1 sec, 50° for 1 sec and 72° for 16 sec, the amplification products were gel purified and cloned into vector T-pUC19 that was obtained by digestion of pUC19 with *Sma*I and incubation with *Taq* polymerase to add 3′ T-overhangs (MARCHUK *et al.* 1991). Four independent clones for each mutant allele were sequenced.

DNA sequence analysis: A 1.7-kb *Hin*dIII fragment of RB2 was subcloned into the Bluescript KS(+) (Stratagene) and the DNA sequence obtained using T3 and T7 primers. The site of γδ transposon insertion into plasmids RB1 and RB2-H was determined by sequencing the junction sequences using forward 5′-CAACGAATTATCTCCTT-3′ and reverse 5′-GGGACCTTTGTATACTG-3′ primers complementary to terminal sequences of the transposon. The DNA sequences were determined by using a Sequenase 2.0 DNA sequencing kit (U.S. Biochemicals) and ^{35}S-dATP or with an automatic sequencer (ALF, Pharmacia) and fluorescein labeled dATP. Two universal primers and one specific primer, 5′-GCTGCTTTCCATGTTGCTG-3′, were used for sequencing reactions. BLAST was used to compare the sequences with other genes (ALTSHUL 1990).

Endocytosis assay: Endocytosis assays were carried out in duplicate as described by DULIC *et al.* (1991). Briefly, 2 ml logarithmic phase (OD_{600} = 0.8) YPD cultures were harvested by centrifugation and suspended in 180 μl of fresh YPD. Ten microliters of a Lucifer yellow CH (lithium salt, Sigma) solution in water (40 mg/ml) was added. Cells were incubated at 23° for 2 hr, harvested, washed three times with ice-cold succinate buffer (50 mM succinate-NaOH, 20 mM NaN3, pH 5) and suspended in 10 μl of the same buffer. Ten microliters of a 1.6% low melting agarose solution (precooled at 45°) was added and cells were mounted on a microscope slide. A Nikon Microphot-FX fluorescence microscope was used to observe Lucifer yellow accumulation and vacuolar morphology was analyzed using DIC.

RESULTS

***MDP1* is identical to the ubiquitin-protein ligase gene, *RSP5*:** The *mdp1* mutants were isolated in a genetic selection designed to identify genes involved in the mitochondrial/cytosolic distribution of Mod5p-I,KR6 (ŻOŁĄDEK *et al.* 1995). We selected for mutations that caused an increase in the cytosolic pool of Mod5p-I,KR6 and screened among these for a subset that also caused a defect in respiration at 34° and were ts for growth on all media at 37°. Genetic analysis of the mutants revealed four complementation groups *mdp1, mdp2, mdp3* and *mdp4*, but most of the mutations were in the *mdp1* complementation group (ŻOŁĄDEK *et al.* 1995). Sixteen strains carrying *mdp1* recessive alleles were crossed to wild-type strains T8-1D or MB105-6A (Table 1) to study the segregation of the ts phenotype. Four appeared to have double mutations and are not considered further here.

The *MDP1* gene was cloned by complementation of

TABLE 1

List of *S. cerevisiae* strains

Strain	Genotype	Source
T8-1D	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519;*	ŻOŁĄDEK *et al.* (1995)
MB105-6A	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4;*	ŻOŁĄDEK *et al.* (1995)
TZ11	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp1-1*	This study
T7-7B	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp1-1*	ŻOŁĄDEK *et al.* (1995)
T100-2D	*MATa SUP11 ade2-1 mod5-1 ura3 leu2 lys2 trp1 mdp1-1*	This study
TZ12	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp1-2*	This study
TZ13	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp1-3*	This study
TZ19	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp1-9*	This study
TZ21	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp1-11*	This study
T61-4A	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 trp5 mdp1-11*	This study
TZ23	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp1-13*	This study
T85-10A	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 trp5 mdp1-14*	This study
TZ25	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp1-15*	This study
TZ26	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp1-16*	This study
TZ33	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-1, 112 his4-519 mdp2-1/vrp1*	ŻOŁĄDEK *et al.* (1995)
T27-1D	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp2-1/vrp1*	ŻOŁĄDEK *et al.* (1995)
T65-1A	*MATa leu2-3, 112 ade1 ura3-52 ile MEL1 mdp2::LEU2*	ŻOŁĄDEK *et al.* (1995)
TZ34	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp3-8/pan1-8* [rho^-]	ŻOŁĄDEK *et al.* (1995)
T37-2A	*MATa SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp3-8/pan1-8*	ŻOŁĄDEK *et al.* (1995)
TZ81	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp3-9/pan1-9*	ŻOŁĄDEK *et al.* (1995)
TZ35	*MATα SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519 mdp3-10/pan1-10*	This study

the ts phenotype of the *mdp1-1*. Clones RB1, RB2, RB4 from a multicopy library and pC7 from a single copy library were found to correct the ts growth defect. As assessed by restriction enzyme digestion patterns, RB1 and pC7 appeared to contain one genomic sequence while RB2 and RB4 appeared to contain a different sequence. Cells with pC7 grew better at the restrictive temperature than cells containing RB1. To determine whether the poor growth observed in the RB1-containing cells also affects wild-type cells, this plasmid was transformed into T8-1D cells (Table 1). Reproducibly, a very low transformation efficiency was obtained and transformants grew poorly. The data suggests that overexpression of the gene(s) encoded by RB1 is detrimental.

It was possible that the complementing DNA fragment carried by RB1 and pC7 allowed growth at the restrictive temperature by suppressing the *mdp1-1* defect rather than by providing true allelic complementation. To test this, an integrating plasmid YIp-RB1, containing the *URA3* gene and the same genomic fragment found in RB1 was linearized in the *MDP1* sequence to direct integration to *MDP1* locus and used to transform T8-1D. A stable transformant was crossed to strain TZ11 bearing the *mdp1-1* allele. The segregation of the ts phenotype with respect to the *URA3* locus was followed by tetrad analysis. All 22 tetrads showed a parental ditype (PD) segregation pattern. That is, the *URA3* marker and ts phenotype segregated as alleles, 2 ts Ura^-:2 tr Ura^+. Thus, the sequences included in the

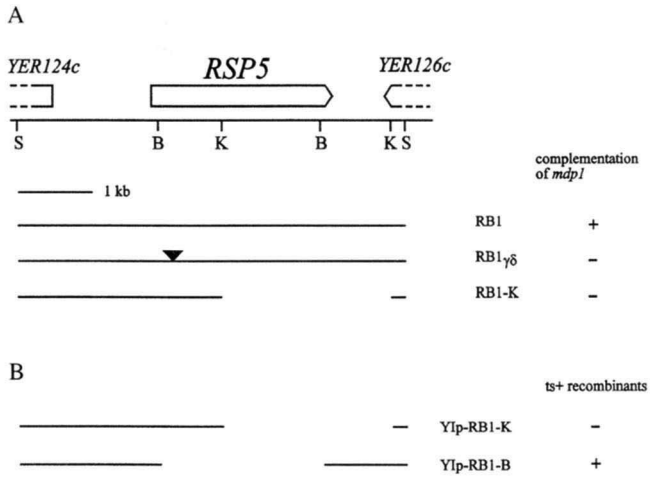

FIGURE 1.—(A) Restriction map, transposon mutagenesis and deletion analysis of the ~5.5-kb DNA fragment containing *RSP5*. Restriction endonucleases sites are labeled as follows: S, *Sau*3A; B, *Bst*EII; K, *Kpn*I. Site of transposon insertion is indicated as an arrowhead. (B) Mapping of *mdp1/rsp5* mutations by gap repair. Deletions introduced within the sequence of plasmid YIp-RB1 are represented by interrupted lines.

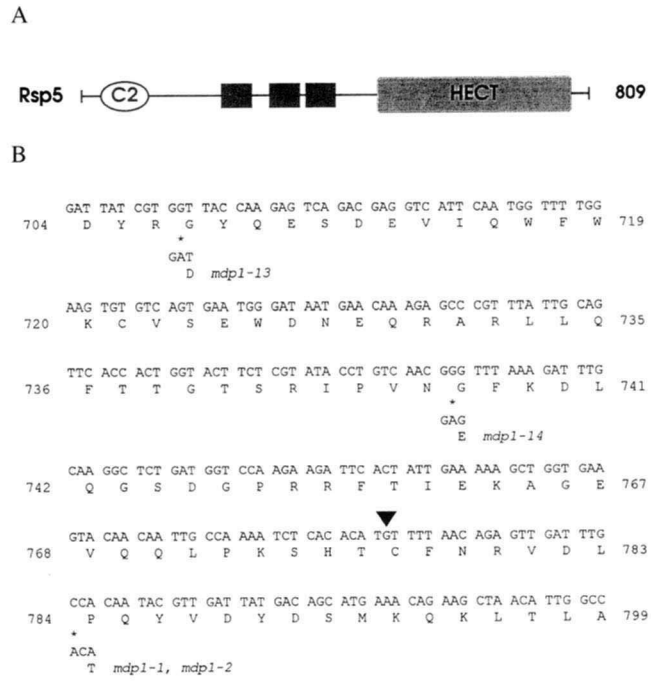

FIGURE 2.—(A) Rsp5p. C2 domain homologous to protein kinase C in white, WW domains mediating protein-protein interaction in black and hect domain homologous to E6-AP carboxyl terminus in gray. (B) Sequence of *mdp1* ts alleles. The upper line is the nucleotide sequence of the 3′ terminal part of the wild-type *RSP5*. The lower line is the deduced amino acid sequence. Amino acids are numbered from the methionine specified by the ATG initiator codon. The positions of nucleotide changes in the four *mdp1* ts alleles are indicated (*). The nucleotides and amino acid substitutions corresponding to the ts mutations are shown below the wild-type sequence. The conserved cysteine is indicated with an arrowhead.

RB1 plasmid are derived from a region of the yeast genome tightly linked to *MDP1* and most likely contain the authentic *MDP1* gene.

A fragment of RB1 was hybridized to filters containing λ and cosmid clones and mapped on chromosome *V* (ATTC No. 70833) close to *GLO3* (IRELAND *et al.* 1994). This location has now been confirmed through the yeast genome sequencing project (GenBank, unpublished data).

Transposon mutagenesis and deletion analysis of RB1 were used to localize the *MDP1* gene on the plasmid. The sequence of the region surrounding an inserted γδ element that destroyed complementation matched the sequence of *RSP5*, the gene encoding ubiquitin-protein ligase (F. S. DIETRICH, GenBank L11119, unpublished results; HUIBREGTSE *et al.* 1995). The genomic insert in plasmid RB1 contains the complete *RSP5* gene. Deletion of the 3′-terminal half (starting from the *Kpn*I site; Figure 1) of the *RSP5* gene abolished complementation of *mdp1-1*. The same deletion destroyed the ability of the cloned sequence to inhibit growth of wild-type cells. Therefore, *mdp1-1* complementation and the toxic effect are due to expression of *RSP5*. Presumably the latter is caused by overexpression of Rsp5p.

Physical location of mutations that affect Rsp5p function: The mutations in alleles *mdp1-1*, *mdp1-2*, *mdp1-13* and *mdp1-14* were localized to the 3′ end of *RSP5* by gap repair (ORR-WEAVER *et al.* 1981). Gaps were introduced into the *RSP5* sequence of YIp-RB1 by restriction enzyme digestion as described in MATERIALS AND METHODS. Strains TZ11, TZ12, TZ23 and T85-10A (Table 1) were transformed with derivatives of the integrating plasmid YIp-RB1 containing the wild-type *RSP5* gene or deletion alleles of *RSP5*. All of the transformants resulting from integration of YIp-RB1 were temperature resistant whereas those resulting from integration of YIp-RB1-K were ts, indicating that the mutations are located in 3′ terminal region of *RSP5*. In contrast, integration of plasmid YIp-RB1-B into the genome of the mutant strains resulted in temperature-resistance in ~32% of integrants. Thus, all four of the mutations reside in the region 3′ of the *Bst*EII site found in *RSP5* (Figure 1B).

The nature and precise location of the four mutations were determined by DNA sequencing (Figure 2B). For *mdp1-1* and *mdp1-2* mutants, the first nucleotide of codon 784 is changed from C to A (CCA to ACA) resulting in a proline to threonine replacement. For *mdp1-13*, there is a G to A change in codon 707 (GGT to GAT) resulting in a substitution of glycine by aspartic acid. For *mdp1-14*, there is a G to A mutation in codon 747 (GGG to GAG), which changes glycine to glutamic acid. Therefore all of the amino acid changes are localized in the highly conserved hect domain close to C-terminus of the Rsp5p (Figure 2A).

***UBI1* is a multicopy suppressor of *mdp1/rsp5* mutations:** Two other plasmids, RB2 and RB4, were isolated in the effort to clone *MDP1* (Figure 3A). They had overlapping inserts with restriction enzyme maps which differed from pC7 and RB1 (Figure 3B). Plasmid YIp-

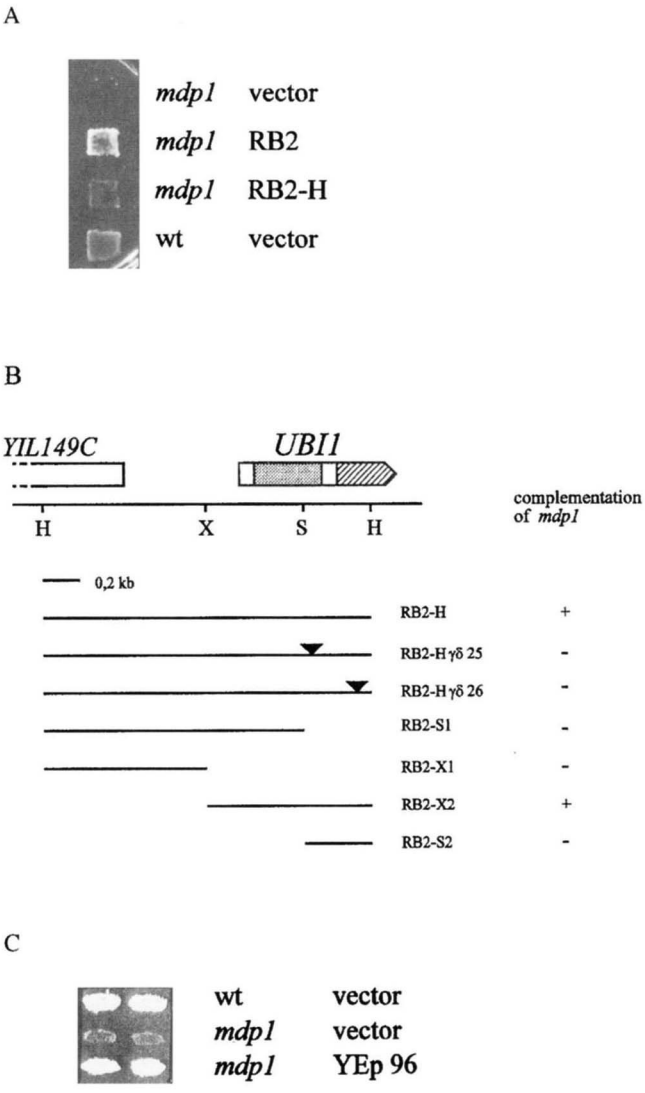

FIGURE 3.—Cloning and analysis of the multicopy suppressor of *mdp1-1*. (A) Growth of *mdp1-1* mutant strain harboring RB2 suppressing plasmid or RB2-H subclone on glucose rich medium at 37°. (B) Transposon mutagenesis and deletion analysis of RB2-H suppressing subclone. Restriction endonuclease sites are labeled as follows: H, *Hin*dIII; X, *Xba*I; S, *Sac*I. The arrowhead is site of transposon insertion; gray indicates intron inside ubiquitin encoding sequence, striped indicates the fused gene, *CEP52*, encoding a ribosomal protein. (C) Suppression of *mdp1-1* by plasmid YEp96 containing a synthetic gene encoding ubiquitin. Strains were grown on YPD medium at 37° for 2 days.

RB2 was used for integrative transformation of strain T8-1D and the resulting transformant crossed to strain TZ11 (relevant genotype *mdp1-1*). The resulting diploid was sporulated and the progeny analyzed. In 16 tetrads, the *URA3* marker and ts growth segregated independently (PD:NPD:TT was 1:2:13). All tetrads contained 2 ts:2 tr progeny, indicating that sequences from plasmid RB2 do not complement *mdp1-1* when in a single integrated copy. The data show that the yeast genomic sequences contained on plasmid RB2 are not located near *RSP5*. Therefore, RB2 sequences act as a multicopy suppressor of *mdp1-1*.

As *mdp* mutants were selected for increased levels of Mod5p-I,KR6 in the cytosol, resulting in efficient nonsense suppression of *lys2-1* (ŻOŁĄDEK *et al.* 1995), we tested the effect of plasmid RB2 on *lys2-1* suppression in a *mdp1-1* strain and compared it with a *mdp1-1* strain transformed with YEp24 as a control. Suppression of *lys2-1* requires simultaneous expression of Mod5p-I from a single copy *LEU2* based plasmid. Growth of transformants on -lys-leu-ura medium revealed that RB2 partially inhibited growth that depends on *lys2-1* suppression (not shown).

The region of RB2 able to confer multicopy suppression of *mdp1-1* was mapped. Plasmid RB2-H (MATERIALS AND METHODS) complemented *mdp1-1* less efficiently than RB2 (Figure 3A). Sequencing revealed that it contains most of the *UBI1* gene that encodes a UBI-CEP52 ribosomal protein fusion (ÖZKAYNAK *et al.* 1987; FINLEY *et al.* 1990) and the 5′-terminal portion (~1/5) of *YIL149C*, a gene encoding a protein similar to the myosin heavy chain (C. CHURCHER, GenBank Z38059, unpublished results, Figure 3B). This identification was consistent with results of a previous mapping experiment in which we used a fragment of RB2 to hybridize to filters containing λ and cosmid clones and located it on chromosome IX (ATTC No. 70542) close to *SUC2* (TAUSSIG and CARLSON 1983). Transposon mutagenesis and deletion analysis of RB2-H were used to determine whether suppression was due to the ubiquitin-ribosomal fusion protein or to the myosin-like protein. Transposons inserted into *UBI1* destroyed suppression (Figure 3B), indicating that suppression was due to expression of *UBI1* rather than *YIL149C*. Similarly, deletions removing portions of *UBI1* destroyed suppression (Figure 3B, RB2-S1;RB2-X1), but deletions of the upstream ORF did not (Figure 3B, RB2-X2).

To determine which portion of the ubiquitin-ribosomal protein fusion was responsible for suppression, we used plasmid YEp96 (ECKER *et al.* 1987) that contains a synthetic ubiquitin gene under regulation of the copper inducible promoter. Overexpression of ubiquitin alone suppressed the ts phenotype of *mdp1-1* (Figure 3C). Therefore, the ribosomal protein is not required for suppression and suppression is due to overexpression of ubiquitin. The ts growth defect of *mdp1-1* was not suppressed by a multicopy plasmid harboring *UBA1*, a gene encoding an ubiquitin activating enzyme (MCGRATH *et al.* 1991). Thus, not all components of the ubiquitin pathway function as suppressors.

Genetic interactions among *mdp1/rsp5*, *mdp2/vrp1* and *mdp3/pan1*: The *MDP1*, *MDP2* and *MDP3* genes identified in a selection for mutations that alter mitochondrial/cytoplasmic distribution of Mod5p-I,KR6, play diverse cellular functions. To determine whether products of these genes interact, we analyzed the phenotypes of *mdp* double mutants. Strains containing various *mdp1/rsp5* mutations were mated to strains containing *mdp2/vrp1* and *mdp3/pan1* mutations. The

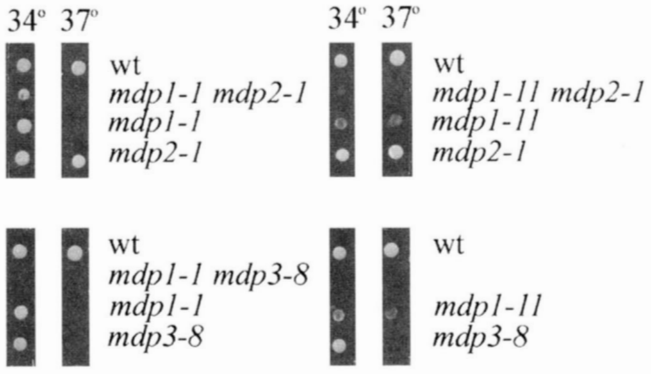

FIGURE 4.—Genetic interaction of *mdp* mutants. Spore clones from crosses *mdp1-1* × *mdp2-1*, *mdp1-1* × *mdp3-8*, *mdp1-11* × *mdp2-1* and *mdp1-11* × *mdp3-8* are shown. Growth was on YPD medium for 1 day of incubation at temperatures indicated. Mutant *mdp1-11 mdp3-8* was not viable and was not spotted on plate.

resulting diploids were sporulated and growth phenotypes of the progeny were analyzed.

The double mutant *mdp1-1 mdp2-1* shows an additive growth defect only at 34° on YPD. *mdp1-11 mdp2-1* double mutants were viable but grew significantly slower on YPD medium at 28° than the single mutants and did not grow on glycerol medium at 28°. The extreme slow growth of this double mutant on YPG at 23° indicates, however, that the cells maintain intact mitochondrial DNA. The *mdp1-11* mutation also shows a synthetic growth defect with the *mdp2::LEU2* disruption allele (not shown). Thus, *RSP5* and *VRP1* show allele-specific synthetic growth defects.

Alleles *mdp1-3, mdp1-9, mdp1-13, mdp1-14, mdp1-15* and *mdp1-16* have additive effects with *mdp3-8*. Double mutants grow significantly slower on YPD and YPG at 28° than either single mutant and they do not grow on YPD and YPG at 34° (not shown). For *mdp1-1*, the effect of combination with *mdp3-8* is less pronounced than for *mdp1-3, mdp1-9, mdp1-13, mdp1-14* and *mdp1-15* and double mutants grow similarly to the single mutants at 28° but do not grow at 34° on YPD (Figure 4). In contrast, the double mutant *mdp1-11 mdp3-8* is not viable as shown from analysis of 37 tetrads resulting from a cross of strain TZ21 (*mdp1-11*) to strain T37-2A (*mdp3-8*). *mdp1-11* shows a synthetic lethal effect with the other two *mdp3/pan1* alleles: *mdp3-9* and *mdp3-10*. Thus, *RSP5* and *PAN1* show allele-specific synthetic lethality.

***mdp1/rsp5, mdp2/vrp1* and *mdp3/pan1* mutations affect endocytosis:** Recent studies by HEIN *et al.* (1995), GALAN *et al.* 1996 and MUNN *et al.* 1995 implicated a role of Rsp5p and Vrp1p in endocytosis. To determine whether the *mdp* alleles of these genes and also *mdp3/pan1* affect endocytosis, we assayed fluid phase endocytosis in *mdp* mutants using the fluorescent dye, Lucifer yellow. Accumulation of endocytosed material in the vacuole was observed in parental strain T8-1D (Figure 5). Even at permissive temperature (23°), none of the three *mdp* mutants, *mdp1-1, mdp2-1, mdp3-9*, accumulate Lucifer yellow in the vacuole (Figure 5). The defect in endocytosis is most severe for the *mdp3-9* mutant and least severe for the *mdp1-1* mutant. All mutants and the parental strain have wild type vacuole morphology as observed by Nomarski optics (Figure 5). Therefore, not only do the *mdp* mutants missort Mod5p-I,KR6, they all cause defects in endocytosis.

DISCUSSION

We report that the yeast *mdp1* mutations that alter the distribution of the mitochondrial/cytoplasmic pool of Mod5p-I,KR6 (ŻOŁĄDEK *et al.* 1995) are located in the *RSP5* gene that encodes ubiquitin-protein ligase (HUIBREGTSE *et al.* 1995), an E3 enzyme of the ubiquitination pathway. Ubiquitin is a 76-aa polypeptide that can be covalently attached to target proteins, through an isopeptide bond formation between the carboxyl terminus of ubiquitin and the ϵ-amino group of lysine residues on the substrate. Additional ubiquitin molecules can be attached to lysine groups of ubiquitin itself forming multi-ubiquitinated proteins. Protein ubiquitination involves three classes of enzymes, the Ub-activating enzyme (E1), Ub-conjugating enzyme (E2) and, in some cases, Ub-protein ligases (E3) that play roles in substrate recognition (CIECHANOVER 1994). *RSP5* is an essential gene in yeast (HEIN *et al.* 1995). Its product is a member of the E6-AP ubiquitin-protein ligase family of proteins which share a C-terminal hect-domain that is essential for enzyme activity (HUIBREGTSE *et al.* 1995). Four members of this family, Rsp5p, Pub1 (NEFSKY and BEACH 1996), human protein hORF3 (N. NOMURA, GenBank D42055, unpublished data) and mouse NEDD-4 (KUMAR *et al.* 1992) also share N-terminal sequence similarities. They contain three or four copies of an ~30 amino acid stretch referred to as WW (BORK and SUDOL 1994) or WWP (ANDRÉ and SPRINGAEL 1994) (Figure 2A). WW domains are also found in several other proteins of yeast, nematodes and vertebrates that are not related to ubiquitination pathway. This domain was recently shown to be involved in protein-protein interactions (CHEN and SUDOL 1995). N-terminal to the WW domain, Rsp5p, Pub1 and hORF3 each contain a single C2 domain that has some similarity to protein kinase C (AZZI *et al.* 1992) and other proteins and could mediate an interaction with phospholipids (CLARK *et al.* 1991).

All of the mutations, spontaneous and UV-induced that we found and characterized that affect Rsp5p activity were located in the fragment of *RSP5* sequence encoding hect domain (Figure 2). The proline 784, which is altered in *mdp1-1* and *mdp1-2*, is located seven amino acids C-terminal of the essential cysteine at position 777, which falls in the active site of the enzyme (HUIBREGTSE *et al.* 1995). The G (707) to D(707) and G (747) to E (747) substitutions found in *mdp1-13* and *mdp1-14* are also located in a hect domain, N-terminal to the essential cysteine. The replaced proline and both glycines are highly conserved in the E6-AP-related fam-

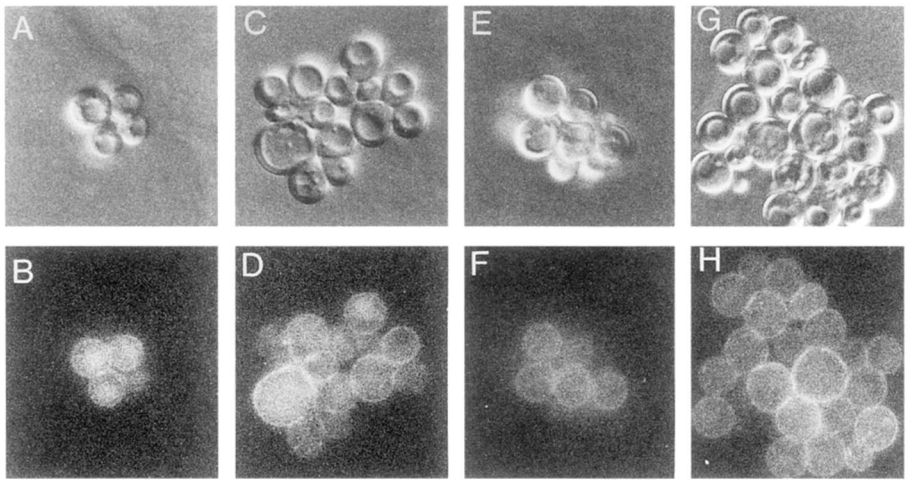

FIGURE 5.— *mdp* mutants are defective in fluid phase endocytosis. The Lucifer yellow endocytosis assay was performed on the parent strain T8-1D (A and B); *mdp1-1* mutant T7-7B (C and D); *mdp2-1* mutant TZ33 (E and F); *mdp3-9* mutant TZ81 (G and H). The same fields were viewed for epi-fluorescence (B, D, F, and H) and by DIC microscopy (A, C, E, and G).

ily (HUIBREGTSE *et al.* 1995). The three-dimensional structures of Rsp5p ubiquitin-protein ligase or homologous ligases from other organisms are not known. Therefore, predictions as to the possible effects of the replacements on the protein structure can not be made. Multicopy suppression of *mdp1* alleles located in the hect domain by ubiquitin could be explained if ubiquitin, due to its increased pool, is transferred from E2 enzymes to ubiquitin protein ligase (E3) more efficiently to form a covalent ubiquitin-ubiquitin protein ligase complex. Alternatively, ubiquitin may interact noncovalently with ubiquitin-protein ligase. Possibly both mechanisms operate simultaneously as was demonstrated for suppression of *cdc34* mutants by overexpression of ubiquitin (PRENDERGAST *et al.* 1995).

A well-recognized function of ubiquitin modification of proteins is to direct short-lived and abnormal proteins for degradation by the 26S proteasome (reviews: FINLEY 1992; CIECHANOVER 1994). However, not all proteins that receive the ubiquitin modification demonstrate rapid turnover (review: JENTSCH 1992). Ubiquitin can affect the subcellular distribution of proteins via processes that only indirectly involve protein degradation (review: HOCHSTRASSER 1996). For example, unmasking of the nuclear localization sequence and subsequent nuclear localization of the transcription factor, NFκB depends upon phosphorylation followed by ubiquitin-dependent proteasome degradation of IκBα, which anchors NFκB in the cytosol (DIDONATO *et al.* 1996). To act on IκBα, the kinase that phosphorylates this protein must itself be ubiquitinated, thus in this case ubiquitination serves to alter the function of the kinase (CHEN *et al.* 1996). In another example of ubiquitin affecting turnover indirectly, endocytosis of the yeast Ste2p mating pheromone receptor is induced upon ligand binding, which also induces Ste2p ubiquitination, necessary for its endocytosis; however, Ste2p turnover happens in vacuoles and not via the proteasome (HICKE and RIEZMAN 1996). There have been many earlier studies that implicated ubiquitin in subcellular distribution of proteins. Ubiquitin has been implicated in endocytosis (KÖLLING and HOLLENBERG 1994; HEIN *et al.* 1995; EGNER and KUCHLER 1996), in delivery of proteins to peroxisomes (WIEBEL and KUNAU 1992; CRANE *et al.* 1994), to endoplasmic reticulum (SOMMER and JENTSH 1993) and to mitochondria (ZHAUNG and MCCAULEY 1989; ZHUANG *et al.* 1992). It remains to be determined whether ubiquitination in each of these cases directly involves proteasome function or whether like for Ste2p it serves as a "tag" for the distribution processes.

How does ubiquitination affect the distribution of Mod5p-I to the mitochondria? Two models seem feasible. For the first involving proteasome-mediated turnover, the *mdp1/rsp5* mutations could affect the ubiquitination and subsequent proteasome-mediated degradation of negative regulators of mitochondrial distribution such that failure to ubiquitinate the regulators would result in stabilization, ultimately decreasing mitochondrial import. Alternatively, ubiquitin could serve as tag, altering the conformation/function of proteins, ultimately altering their distribution. In this case, either Mod5p-I or regulators of mitochondrial import could be the targets of ubiquitin. We have no evidence for ubiquitination of Mod5p-I as this protein migrates to the same position of SDS PAGE whether isolated from wild-type or *mdp1/rsp5* mutant cells. However, we have noticed about a twofold increase in the steady state levels of Mod5p-I,KR6 in *mdp1/rsp5* mutants (ŻOŁĄDEK *et al.* 1995), which could indicate that there may be ubiquitinated forms of this protein. Although a decrease in Mod5p-I turnover could explain the increase in the cytoplasmic pools evidenced in *mdp1/rsp5* mutants, it can not readily explain the depletion of the mitochondrial pool of the enzyme nor the defects in mitochondrial metabolism. If Mod5p-I is ubiquitinated, this modification probably affects its distribution by a mechanism separate from a direct effect on its stability. Further studies are necessary to identify the substrates for ubiquitin-protein ligase that affect the distribution of Mod5p-I and perhaps other proteins delivered to mitochondria.

Mutations of *RSP5* have been isolated numerous times in other laboratories and shown to affect several cellular processes, including transcription, protein

turnover and subcellular distribution of proteins. Mutations of this gene have been isolated as a suppressor of *spt3* null mutations (B. BERG, A. HAPPEL and F. WINSTON, personal communication). *SPT3* encodes a protein involved in initiation of transcription (EISENMANN *et al.* 1992). *pip1* mutations that are located in *RSP5* affect the expression of the peroxisomal catalase activity, presumably also at the level of transcription (P. PAVLIK and H. RUIS, personal communication). Isolated as *npi1* mutations, lesions of *RSP5*, block inactivation of the general amino acid permease, Gap1p (JAUNIAUX and GRENSON 1990; HEIN *et al.* 1995). Rsp5p was also found to be required for basal and stress-induced degradation of uracil permease encoded by *FUR4* (GALAN *et al.* 1994; HEIN *et al.* 1995) due to Fur4p ubiquitination (GALAN *et al.* 1996). Finally, our studies of *mdp1* mutations show Rsp5p to affect fluid phase endocytosis and the subcellular distribution of proteins to mitochondria. At least the effects on protein turnover and subcellular distribution of proteins to mitochondria appear to result from defective ubiquitin-protein ligase activity per se because we found that the temperature sensitivity caused by *mdp1/rsp5* mutations can be suppressed by overproduction of ubiquitin and overexpression of ubiquitin also affects Mod5p-I-dependent nonsense suppression.

How does mutation *mdp1/rsp5* have all these effects? As ubiquitin has been shown to be important in protein turnover as well as subcellular distribution of proteins to mitochondria, nuclei and peroxisomes and in endocytosis, it is not difficult to imagine how mutations of a single gene encoding a component of ubiquitination pathway could affect the various nuclear, mitochondrial and cell surface phenomena that have been reported for *RSP5*. It is of interest, however, to consider how Rsp5p could interact with multiple targets. Rsp5p has three WW domains and WW domains have been shown to bind to proteins that contain the core sequence XPPXY (review: EINBOND and SUDOL 1996). It has been proposed that different WW domains may recognize different proline-rich motifs, imposing specificity in the protein-protein interactions (EINBOND and SUDOL 1996). It is possible that each of the three Rsp5p WW domains is able to interact with individual proteins, directing Rsp5p function to multiple targets. As all of the mutations we characterized affect the catalytic activity of Rsp5p, by this model they would be expected to have pleiotropic consequences; however, if this model is correct, then alteration of individual WW binding domains could affect single processes such as endocytosis, or protein delivery to mitochondria, or protein delivery to nuclei. We intend to test this model in future experiments.

We have also shown that the various *MDP* gene products interact genetically as *mdp1/rsp5* mutations are synthetically lethal in combination with *mdp3/pan1*. *mdp1/rsp5* in combination with *mdp2/vrp1* also negatively affects cell growth. Further, all *mdp* mutants have a defect in fluid phase endocytosis. In fact, the *end5-1* mutant that is defective in internalization of plasma membranes was found recently to be allelic to *VRP1* (MUNN *et al.* 1995). Also it was recently found that *mdp1/rsp5* mutations prevent endocytosis of uracil permease from the plasma membrane at the restrictive temperature (GALAN *et al.* 1996). Synthetic genetic interactions could be the result of protein-protein interactions. We were able to show interactions between particular fragments of Rsp5 and Pan1 proteins (B. GAJEWSKA, unpublished results) in two-hybrid system but not between Rsp5p and Vrp1p. Another mechanism for synthetic genetic interactions includes the function of the relevant gene products in parallel or dependent pathways. Our studies implicate interactions of the actin cytoskeleton, protein synthesis, and ubiquitination in delivery of proteins to mitochondria. We now have a set of genetic and molecular tools that should allow dissection of these important and complicated interactions.

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