***Saccharomyces cerevisiae* MPS2 Encodes a Membrane Protein Localized at the Spindle Pole Body and the Nuclear Envelope**

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The *MPS2* (monopolar spindle two) gene is one of several genes required for the proper execution of spindle pole body (SPB) duplication in the budding yeast *Saccharomyces cerevisiae* (Winey *et al.*, 1991). We report here that the *MPS2* gene encodes an essential 44-kDa protein with two putative coiled-coil regions and a hydrophobic sequence. Although *MPS2* is required for normal mitotic growth, some null strains can survive; these survivors exhibit slow growth and abnormal ploidy. The *MPS2* protein was tagged with nine copies of the myc epitope, and biochemical fractionation experiments show that it is an integral membrane protein. Visualization of a green fluorescent protein (GFP) Mps2p fusion protein in living cells and indirect immunofluorescence microscopy of 9xmyc-Mps2p revealed a perinuclear localization with one or two brighter foci of staining corresponding to the SPB. Additionally, immunoelectron microscopy shows that GFP-Mps2p localizes to the SPB. Our analysis suggests that Mps2p is required as a component of the SPB for insertion of the nascent SPB into the nuclear envelope.

INTRODUCTION

In the budding yeast, *Saccharomyces cerevisiae*, the spindle pole body (SPB) functions as the sole microtubule organizing center (Byers *et al.*, 1978; Hyams and Borisy, 1978). The SPB is embedded in the nuclear envelope (NE), which remains intact throughout the cell cycle. From its position in the NE, the SPB nucleates both cytoplasmic and nuclear microtubules from its cytoplasmic and nuclear faces, respectively. To set up a proper bipolar mitotic spindle, the SPB must be precisely duplicated during G1 of the cell cycle (Byers and Goetsch, 1975). This process of SPB duplication is thought to occur through a conservative mechanism (Vallen *et al.*, 1992).

Several genes required for SPB duplication have been identified (Winey and Byers, 1993). Among them, the *MPS2* (monopolar spindle two) gene is required for a late step in SPB duplication. At the restrictive temperature, cells mutant for *MPS2* (*mps2-1*) contain duplicated SPBs, but the nascent SPB is not inserted into the NE (Winey *et al.*, 1991). The defective SPB lies on the cytoplasmic face of the NE, unable to nucleate nuclear microtubules. As a consequence, the cells arrest in G2 of the cell cycle with large buds and unsegregated DNA, which is associated with the functional SPB. After continued incubation at the restrictive temperature, *mps2-1* cells overcome the mitotic arrest and proceed through the cell cycle; however, the nuclear DNA is asymmetrically divided: one cell receives all of the nuclear DNA and the other (daughter) cell receives no DNA. Consequently, cells that contain a mutant *MPS2* gene display an increase in ploidy. The *NDC1* gene also functions at this late step in SPB duplication: *ndc1-1* mutant strains exhibit phenotypes

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Abbreviations used: APC, anaphase-promoting complex; GFP, green fluorescent protein; NE, nuclear envelope; NPC, nuclear pore complex; SPB, spindle pole body.

A

1 MSNGAFDAIFEYAWGQIDKPISGDFIYGKDLPKLIEII**E**NIFQKAQKSGSYELRLPLFSE
 61 INKDLFRTFSNTKTFKIHKEEFDDIFFNLVNHPLREILENAFIGVDSIPSDFIIVSMNLN
 121 SPSKFLVENKNKNTEGAGIST**TPRK**KL**TESPI**KL³⁹LSRNNIGKALEVQVEELKRELTAQSL
 181 LQENERQVSELKIRLETYQEKYASIQQRFSDLOKARQVEDNQNSSRTSDPGSPLVTGIDQ
 241 KAILEEFRRRLQRQTDITISFLKDQIRREGLNCSNDKVSHSKRKHATTDGDGTFKNFISA
 301 VPSNIWVKATIRIIVCFALLAGVLPYIRKYVYAHD**TPSQ**NSRLQLSWWENSGILSKIIVWF
 361 FEDQTDLETEYRSNANVDDAYSRVFGI

B

Figure 1. Features of the *MPS2* encoded protein. (A) Deduced amino acid sequence of the *MPS2* encoded protein. The putative transmembrane segment (residues 311–327) is boxed. Potential Cdc28p phosphorylation sites are indicated in bold letters, and potential destruction box sequences are underlined. The point mutation found in the *mps2-1* allele at amino acid 39 is highlighted (E to K). (B) Coiled-coil probability plot. The x-axis is the position of Mps2p amino acids and the y-axis is the probability (p) of being in a coiled-coil using the COILS program (http://www.ch.embnet.org/software/COILS_form.html), based on the Lupas algorithm (Lupas *et al.*, 1991).

that are indistinguishable from *mps2-1* strains at the nonpermissive temperature (Thomas and Botstein, 1986; Winey *et al.*, 1993).

The SPB of *S. cerevisiae* is a disk-like structure composed of six major layers, with the central layer, or plaque, in the same plane as the NE (Bullitt *et al.*, 1997). The defective SPB in *mps2-1* and *ndc1-1* mutant cells appears to lack the inner (nuclear) plaque. It was originally proposed that Mps2p and Ndc1p function to insert the nascent SPB into the NE, allowing the inner plaque to form and nucleate microtubules on the nuclear face (Winey and Byers, 1993). Recently, Ndc1p was shown to be a shared component of SPBs and nuclear pore complexes (NPCs) (Chial *et al.*, 1998), consistent with a direct role for Ndc1p in the insertion event.

In addition to the structural details of how the SPB is duplicated, it is also important to understand the regulation of such a crucial aspect of the cell cycle. A major consequence of unregulated SPB duplication is asymmetric chromosome division. Mutants that fail in SPB duplication lead to aneuploidy (Schild *et al.*, 1981; Rose and Fink, 1987; Winey *et al.*, 1991), which is associated with some human cancer cells (Lengauer *et*

al., 1997). The regulation of cell cycle events by post-translational modifications such as protein phosphorylation and proteolysis is well documented (for reviews see King *et al.*, 1996; Lew and Kornbluth, 1996). Indeed, phosphorylation and ubiquitin-mediated proteolysis have been implicated in the regulation of the SPB duplication cycle. The Mps1p kinase is essential for proper SPB duplication (Winey *et al.*, 1991; Lauze *et al.*, 1995). Interestingly, two components of the SPB, Spc110p (Friedman *et al.*, 1996) and Spc98p (Pereira *et al.*, 1998), are phosphorylated in a cell cycle-dependent manner, and there is evidence that Mps1p may be involved in the phosphorylation of Spc98p (Pereira *et al.*, 1998).

There is also evidence that SPB duplication is regulated by the ubiquitin-proteasome pathway (McDonald and Byers, 1997). In this pathway, specific proteolysis is mediated by the covalent attachment of ubiquitin to substrate proteins, followed by targeting to the proteasome where they are degraded (Hershko and Ciechanover, 1992). The first in vivo evidence supporting a role for the proteasome in a major cell cycle transition came from the finding that some 26S proteasome mutants arrest at the G2/M stage in the

Table 1. Yeast strains used in this study

Yeast strains	Genotypes
CMY763	<i>MATα ura3-52 leu2Δ1 cim3-1</i>
CMY765	<i>MATα ura3-52 leu2Δ1 his3Δ200 cim5-1</i>
CMY826	<i>MATα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 bar1::HIS3</i>
ILM2	<i>MATα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 bar1::HIS3 PDS1::PDS1-HA-URA3</i>
MCL29	<i>MATα ura3-52/ura3-52, leu2Δ1/+, lys2-801/lys2-801, his3Δ200/his3Δ200, trp1Δ1/trp1Δ1, ade2-101/ade2-101 MPS2/mps2Δ::HIS3</i>
MCL44	<i>MATα ura3-52 trp1Δ1 his3Δ200 leu2Δ1 lys2-801 ade2-10 mps2Δ::HIS3 plus pRS316(URA3)-pGAL1-MPS2</i>
MCL94	<i>MATα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 bar1::HIS3 plus pCM190(URA3)-pTet-9mycN-MPS2</i>
MCL120	<i>MATα ura3-52 trp1Δ1 his3Δ200 LEU2 lys2-801 ade2-101 mps2Δ::HIS3 plus pRS306(URA3)-9mycN-MPS2</i>
MCL123	<i>MATα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 bar1::HIS3 PDS1::URA3-PDS1-HA plus pRS304(TRP1)-9mycN-MPS2</i>
MCL175	<i>MATα ura3-52 trp1Δ1 his3Δ200 LEU2 lys2-801 ade2-101 mps2Δ::HIS3 plus pRS306(URA3)-GFP-3mycN-MPS2</i>
SC55	<i>MATα ura3-52/ura3-52, leu2Δ1/+ lys2-801/lys2-801 his3Δ200/his3Δ200 trp1Δ1/trp1Δ1 ade2-101/ade2-101</i>
AM610	<i>MATα MPS2/mps2Δ(116-387)::HIS3 ura3-52/ura3-52 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>
D8BX5Ca	<i>MATα ura3-52/ura3-52 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>
WX178-1a	<i>MATα mps2-1 trp1Δ1 ura3-52</i>
SMY28-2b	<i>MATα mps2Δ::KanMX/mps2Δ::KanMX URA3::9xmycC-MPS2/URA3::9xmycC-MPS2 spc42Δ::LEU2/spc42Δ::LEU2 TRP1::SPC42-GFP(3x)/TRP1::SPC42-GFP(3x) trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112</i>
SMY22-5a	<i>MATα mps2Δ::KanMX URA3::9xmycC-MPS2 trp1Δ1 his3Δ200 ura3-52 leu2-3,112</i>
HC32-1C	<i>MATα spc42Δ::LEU2 TRP1::SPC42-GFP(3x) trp1Δ1 his3Δ200 ura3-52 leu2-3,112 ade2</i>
SMY1892	<i>MATα mps2Δ::KanMX/mps2Δ::KanMX URA3::9xmycC-MPS2/URA3::9xmycC-MPS2 nup49-1::URA3/nup49-1::URA3 nup49ΔGLFG::GFP-S65T-TRP1/nup49Δ::GFP-S65T-TRP1 ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>
SMY11-13c	<i>MATα nup49-1::URA3 nup49Δ::GFP-S65T-TRP1 ura3-52 his3Δ200 leu2-3,112</i>

cell cycle (Ghislain *et al.*, 1993; Gordon *et al.*, 1993). Recently, a proteasome subunit encoded by the PCS1 gene was shown to be required for SPB duplication (Russell *et al.*, 1996; McDonald and Byers, 1997). A temperature-sensitive *pcs1* strain arrests in G2 of the cell cycle with large buds and unsegregated DNA, and electron microscopy revealed that the arrested cells had a single, unduplicated SPB. On the basis of this finding, it was postulated that Pcs1p-containing proteasomes may play a role in the degradation of a particular protein(s) that is specifically required for SPB duplication.

We report here the isolation of the *MPS2* gene. Interestingly, we identified *MPS2* in a genetic screen with *CIM5*, a gene that encodes a proteasome subunit required for the G2/M transition (Ghislain *et al.*, 1993), thus linking *MPS2* to the ubiquitin-proteasome pathway. In addition, we show that *MPS2* encodes an integral membrane protein localized to SPBs and the NE. We propose that Mps2p is directly required for insertion of the nascent SPB into the NE.

MATERIALS AND METHODS

Yeast Strains and General Methods

The yeast strains used in this study are listed in Table 1. Yeast media, growth conditions, and genetic and molecular techniques were as described previously (Sambrook *et al.*, 1989; Guthrie and Fink, 1991). Yeast shuttle vectors and yeast strains used in this study are congenic with S288C (Sikorski and Hieter, 1989). ILM2 was made by transforming CMY826 with the *Clal-Sacl* fragment from pOC52 containing *PDS1-HA* (Cohen-Fix *et al.*, 1996). MCL120 and

MCL175 strains were made by integration at the *URA3* locus of the BsmI-linearized plasmid p20 or p30, respectively, in a *mps2 Δ ::HIS3* strain, followed by loss of the complementing plasmid in nonselective medium. MCL123 was made by transforming strain ILM2 with the *EcoRI*-linearized plasmid p23 to direct integration at the *MPS2* locus. SMY22-5a was made by integrating *9xmycC-MPS2* at the *URA3* locus in the strain *mps2 Δ ::KanMX/MPS2*, *ura3-52/ura3-52*, sporulating the diploid, and isolating *KanMX*⁺, *URA3*⁺ meiotic products. SMY28-2b is the product of a cross between HC32-1C (*spc42 Δ ::LEU2*, *TRP1::SPC42-GFP*, original *spc42 Δ ::LEU2*, *TRP1::SPC42-GFP* strain provided by Ian Adams and John Kilmartin (Medical Research Council, Cambridge, UK) and SMY22-5a (*mps2 Δ ::KanMX,URA3:9xmycC-MPS2*). SMY1892 was derived from SMY11-13c (original strain, SWY809, provided by Susan Wente, Washington University, St. Louis, MO), and SMY22-5a. Yeast strains harboring *URA3*-containing plasmids were counterselected by growth on media containing 5-FOA (United States Biological, Swampscott, MA) as described previously (Boeke *et al.*, 1987). Yeast strains harboring the *KanMX4* gene were selected by growth on plates containing 25 μ g/ml geneticin (G418) (Wach *et al.*, 1994).

MPS2 Isolation

The genomic plasmid bank used to clone *MPS2* was a *URA3*-marked genomic library (Rose and Fink, 1987). Two clones with overlapping inserts, designated E27 and E28, were able to rescue the temperature-sensitive phenotype of *mps2-1* cells. Plasmid E13 was created by excising a 6-kb insert from E27 with *Sall* and *XhoI*, then ligating it into the *Sall* site of pRS313. The E13 plasmid was cut with *Bam*HI, excising a 2.6-kb *Bam*HI fragment and was then religated to create E14. Plasmid E150 was constructed by digesting E14 with *XhoI* and *XbaI* and ligating this fragment into the same sites of pRS316. To define the boundaries of the complementing ORF, the E150 insert was digested from either side to generate a nested set of partial deletions.

The *MPS2* gene was sequenced with the Sequenase Version 2.0 DNA Sequencing Kit (Amersham International, Cleveland, OH) according to the manufacturer. Our sequence is identical to that for

YGL075c (*Saccharomyces* Genome Database). For sequencing of the *mps2-1* mutant lesion, genomic DNA was prepared from strain Wx178-1a as described previously (Hoffman and Winston, 1987). Primers to *MPS2* were used to generate a series of PCR fragments, which were then used as templates for sequencing using the Sequenase PCR Product Sequencing Kit (Amersham International).

Genetic Screen with *cim5*

MPS2 was also identified in a screen for genes whose overexpression is toxic in a *cim5* proteasome mutant at a semipermissive temperature, but not in the wild-type strain. A *cim5-1* temperature-sensitive mutant (strain CMY765) was transformed with a *GAL1*-regulated *S. cerevisiae* genomic DNA expression library (Ramer *et al.*, 1992) in the centromeric (*URA3*) pYES vector, (Elledge *et al.*, 1991). Cells were plated to synthetic medium containing glucose but lacking uracil (–Ura) and grown at the semipermissive temperature of 30°C. Approximately 30,000 colonies were then replica-plated to selective media (–Ura) plates containing either glucose (no library expression) or galactose plus raffinose (gal-raff) (overexpression conditions) at the same temperature. Raffinose was added to facilitate yeast growth on galactose. Colonies able to grow on the glucose plates but showing little or no growth on the gal-raff plates were selected. Only *rho*⁺ colonies showing the inhibitory effect on the gal-raff plates in a *URA3* plasmid-dependent manner were selected for plasmid recovery in *Escherichia coli*. CMY765 (*cim5-1*), CMY763 (*cim3-1*), and CMY826 (isogenic wild-type) strains were then transformed with the selected plasmids, and growth inhibition on the gal-raff plates at 30°C was retested. Fourteen independent plasmids contained yeast DNA fragments whose overexpression caused a specific inhibitory effect on growth of the *cim* mutants. One of these DNA fragments corresponded to the *MPS2* gene.

Disruption of *MPS2*

Two different null alleles of *MPS2* were constructed using a PCR-based method (Baudin *et al.*, 1993). One allele was made by replacing amino acids 116–387 with the *HIS3* gene using the one-step method (Baudin *et al.*, 1993). The other deletion was made by replacing the entire *MPS2* ORF with the Kanamycin resistance gene (Wach *et al.*, 1994; Brachmann *et al.*, 1998), *KanMX4*, using a two-step gene replacement technique (Rothstein, 1991). Both the *mps2Δ::KanMX*-containing fragment and the *mps2Δ116–387::HIS3* PCR product were used to transform a wild-type diploid strain (D8BX5CA) as described previously (Schiestl and Gietz, 1989). *HIS3* prototrophs or G418-resistant isolates, respectively, were examined by PCR for the correct recombination event.

Plasmids

p17: pCM190 (2 μ, *URA3*)-pTet-9mycN-MPS2. Nine copies of the myc epitope (EQKLISEEDL) were introduced after the initiation codon by “gene SOEing” or overlap extension (Horton, 1995). Oligonucleotides TG1 (5′-CCCAGCTTTGTTTAAACATGCGCGGTG-GCGGCCGCTCTAGA-3′) and TG2 (5′-ATCAAACGCACCGT-TACTCATCAGCCCGGGGATCCACTAGT-3′) were used to amplify by PCR a DNA fragment containing the nine-myc epitope sequence from a plasmid kindly provided by Kim Nasmyth (Institute of Molecular Pathology, Vienna, Austria). Oligonucleotide TG1 introduces an initiation codon in-frame with the first myc epitope, and TG2 contains a 3′ 21-bp overlapping region with the 5′ end of *MPS2* ORF. The 444-bp PCR-amplified fragment was called PCR-TG-A. The *MPS2* ORF was PCR amplified from a *MPS2*-containing plasmid (p2) using oligonucleotides TG3 (5′-ATGAGTAACGGT-GCGTTTGAT-3′) and TG4 (5′-CCAAACTGCAGGGCCAAGGTT-TAAAT-3′), and the 1185-bp PCR fragment was called PCR-TG-B. In a subsequent PCR reaction, the overlap between PCR-TG-A and PCR-TG-B served as a primer for extension using oligonucleotides TG1 and TG4, creating a recombinant molecule containing the

9xmyc tag after the initiation codon in-frame with the *MPS2* coding sequence. The *PmeI*–*PstI* fragment from the final PCR product was cloned into pCM190 (2 μ, *URA3*, *pTet*) (Gari *et al.*, 1997) to obtain p17. The function of the 9xmycN-MPS2 gene was verified by its ability to suppress the *mps2* temperature-sensitive mutant. In p17, 9mycN-MPS2 is expressed from a tetracycline-repressible promoter.

p20: pRS306 (integrative, *URA3*)-9mycN-MPS2 and p23: pRS304 (integrative, *TRP1*)-9mycN-MPS2. A recombinant DNA molecule was generated by “gene SOEing” containing the endogenous *MPS2* promoter and the 9mycN-MPS2 ORF. Oligonucleotides SOE1 (5′-GG-TACCGGGCCCCCTCGAGG-3′) and SOE2 (5′-TCTAGAGCGGC-CGCCACCGCGCATACCTACGTTGTCAAAGACAG-AATT-3′) were used to amplify by PCR a 671-bp fragment (PCR1) containing the endogenous *MPS2* promoter from a *MPS2*-carrying plasmid. The 9mycN-MPS2 ORF and terminator sequences were PCR amplified from pCM190-pTet-9mycN-MPS2 (p17) using oligonucleotides SOE3 (5′-ATGCGCGGTGGCGGCCGCTCTAGA-3′) and SOE4 (5′-ATGT-CATGGGAGCTCTGGTTAGTCACTCAT-TAG-3′) (PCR2). The recombinant DNA fragment, containing the endogenous *MPS2* promoter and the 9mycN-MPS2 ORF, was amplified using the overlap between PCR1 and PCR2 as a primer and oligonucleotides SOE1 and SOE4. The *SacI*–*XhoI* fragment from the final PCR product was cloned into pRS306 (integrative, *URA3*) or pRS304 (integrative, *TRP1*) vectors yielding p20 and p23, respectively. In p20 and p23, 9xmycN-MPS2 is expressed from its endogenous promoter.

p30: pRS306-GFP-3xmycN-MPS2. The GFP3 sequence from plasmid pYGF3 (kindly provided by Brendan Cormack, Johns Hopkins Medical School, Baltimore, MD) was amplified by PCR using oligonucleotides GFP1 (5′-CCCAGCTTACTAGTATGTCTAAAGGT-GAAGAATTATTC-3′) and GFP2 (5′-CCAAAAAGCACTAGTTTGTACAATTCATCCATACCATG-3′). The *SpeI* PCR fragment was cloned into p20 digested with *XbaI*.

pRS306-9xmycC-MPS2. Nine copies of the myc tag were introduced at the termination codon of *MPS2*. The 9xmyc sequence from plasmid 9xmycFZ01 (a gift from G. Hermann and Janet Shaw, University of Utah, Salt Lake City, UT) was amplified by PCR with primers 9xMycNXbaI and 9xMycCXbaI. The PCR fragment was digested with *XbaI* and ligated into a unique *SpeI* site created by PCR immediately before the termination codon in *MPS2* (plasmid pRS314.B). A 2.1-kb *SacI*/*KpnI* fragment from pRS314 was subcloned into pRS306. The function of the fusion protein was verified by the ability of pRS314-9xmycC-MPS2 to rescue the *mps2-1* temperature-sensitive phenotype and the *mps2Δ::KanMX4* strain.

Western Blot Analysis

Protein extracts were made from 25-ml aliquots of exponentially growing cells (see Figure legends for strains). Cells were pelleted and transferred to an Eppendorf tube in 150 μl of cold lysis buffer (50 mM Tris-HCl, pH 8, 0.3% Na deoxycholate, 1% Triton X-100, 0.2% SDS, 50 mM NaCl, 5 mM EDTA, 2 mg/ml each of pepstatin A, aprotinin, leupeptin, chymostatin, and Pfablock plus 1 mM PMSF). An equal volume of glass beads was added, and the cells were lysed by vortexing. Extracts were then clarified by 15 min of centrifugation at 4°C, and the supernatant was collected. Total cell protein (40 μg) was denatured by boiling in SDS sample buffer and then separated on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham). 9xmycN-Mps2p was detected using the 9E10 anti-myc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 0.1 μg/ml, and Pds1p-HA was detected using a 1:1000 dilution of 12CA5 anti-HA ascites fluid. Cdc28p, Sec61p, and hexokinase were identified using polyclonal antibodies (van Tuinen and Riezman, 1987; Wilkinson *et al.*, 1996). To detect Dpm1p, the 5C5-A7 monoclonal antibody (Molecular Probes, Eugene, OR) was used at a concentration of 4 μg/ml.

Subcellular Fractionation

Subcellular fractionation was performed essentially as described (Harris and Waters, 1996). MCL120 cells expressing 9xmycN-Mps2p from the *MPS2* promoter were grown in 250 ml of YPD at 24°C to an OD_{600 nm} of 0.5–0.8, harvested by centrifugation, and washed two times in spheroplast buffer (50 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 10 mM NaN₃, 40 mM β -mercaptoethanol). Cells were resuspended in spheroplast buffer at 40 OD U/ml. Zymolyase 100T (ICN, Costa Mesa, CA) was added to 300 μ g/ml, and cells were incubated at 30°C for 30 min with occasional mixing. The spheroplasts were washed two times in spheroplast buffer and resuspended in 0°C lysis buffer (20 mM HEPES/KOH, pH 7.4, 100 mM K-acetate, 5 mM Mg-acetate, 1 mM EDTA 1 mM DTT) plus a protease inhibitor mixture (2 μ g/ml each of pepstatin A, aprotinin, leupeptin, chymostatin, and Pablock plus 1 mM PMSF) to the same volume in which they were spheroplasted. Cells were lysed in a 5-cm³ Dounce homogenizer (25–30 strokes on ice), and then the lysates were centrifuged at 4°C for 3 min at 1000 $\times$ g. The resulting supernatant (crude extract) was centrifuged at 4°C for 15 min at 13,000 $\times$ g to generate pellet (P13) and supernatant (S13) fractions. The S13 fraction was centrifuged at 4°C for 60 min at 100,000 $\times$ g in a Beckman (Palo Alto, CA) TL-100 centrifuge generating pellet (P100) and supernatant (S100) fractions. Protein concentrations were determined using the Bradford assay (Bradford, 1976).

Mps2p was solubilized from the P13 fraction essentially as described (Ruohola and Ferro-Novick, 1987). The pellet was homogenized in lysis buffer, divided into aliquots, and centrifuged at 13,000 $\times$ g for 15 min. The pellet for each sample was resuspended in lysis buffer (control) or in lysis buffer containing either 1 M NaCl, 50 mM Tris (pH 7.5)-10 mM EDTA, 0.2 M Na₂CO₃ (pH 11), 1% Triton X-100, or 6 M urea (other combined treatments are indicated in the Figure legends). The mixtures were incubated on ice for 10 min and then centrifuged at 13,000 $\times$ g for 15 min. The supernatant was removed, and the pellet was resuspended in an equal volume of lysis buffer. Equivalent samples of the supernatant and pellet fractions were analyzed by immunoblotting.

Cytological Techniques

Flow cytometry analysis of cells was performed as described using the DNA stain propidium iodide (Winey *et al.*, 1991). Stained cells were analyzed using a Becton Dickinson FACScan flow cytometer using CELL QUEST software packages to collect and analyze the data (BDIS, San Jose, CA).

Whole-cell fluorescence microscopy (see Figure 6) was carried out essentially as described by Chial *et al.* (1998), developed from Rout and Kilmartin (1990). Briefly, cells were grown at 24°C to an OD₆₀₀ between 0.2 and 0.8. Cells were pelleted and fixed in 3.7% formaldehyde for 5 min. Cells were spheroplasted in Solution A (1.2 M sorbitol, 100 mM KPO₄, pH 7.5) containing 4.8 μ g/ml Zymolyase 100T (ICN) for 45 min at 30°C. Cells were rinsed with PBSA (10 mg/ml NaCl, 0.2 mg/ml KCl, 1.43 mg/ml KH₂PO₄), then resuspended in 50–100 μ l of Solution A. Cells (10 μ l) were spotted onto a polylysine-treated multi-well slide, then submerged in methanol followed by acetone. Slides were treated with blocker (PBSA, 10 mg/ml BSA, 0.1% Tween-20) for 5 min, followed by incubation in a 1:400 dilution of α -myc antibody overnight at 4°C. The myc antibody was detected using either a Texas Red-conjugated sheep anti-mouse antibody diluted 1:400 or an FITC-conjugated sheep anti-mouse antibody diluted 1:200 (both from Amersham), and the DNA was stained with DAPI. Standard fluorescence microscopy was performed using a Leica DMRXA/RF4/V automated microscope equipped with a digital camera (SensiCam CCD camera; Cooke, Tonawanda, NY). Images were acquired and deconvolved using the Slidebook software package (Intelligent Imaging Innovations, Denver, CO).

For visualizing GFP-Mps2p in living cells, a 2 ml aliquot of cells grown in YPD at an OD₆₀₀ of 0.5–1.0 was collected and washed in water. Cells were resuspended in 50 μ l of Dabco solution (24.5

mg/ml diazabicyclo 2–2–2 octane in PBS, 75% glycerol). The DNA was visualized with Hoechst at a final concentration of 5 μ g/ml for 5 min.

Immunoelectron microscopy was performed as described previously (Winey *et al.*, 1995; Ding *et al.*, 1997; Chial *et al.*, 1998). Cells were high-pressure frozen in a Balzers HPM-010 High Pressure Freezer (Bal-Tec, Middebury, CT). Thin sections were labeled with an affinity-purified anti-GFP rabbit polyclonal antibody (a kind gift from Jason Kahana and Pam Silver, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA) and reacted with goat anti-rabbit 15-nm colloidal gold conjugate (Ted Pella, Redding, CA), followed by rinses and staining with uranyl acetate and lead citrate.

RESULTS

Isolation of the *MPS2* Gene

The *MPS2* gene was isolated from a yeast genomic library constructed in the centromeric plasmid YCp50 (Rose and Fink, 1987) by screening transformants of *mps2–1* (Wx178–1a; Table 1) for those able to grow at the restrictive temperature of 37°C. The insert in one of the two rescuing plasmids was subcloned, identifying a region of 3.4 kb required for complementation. A nested deletion series was used for sequence analysis of the 3.4-kb insert, and the minimal complementing fragments were found to contain a single ORF that has been identified as YGL075c by the Yeast Genome Project.

To confirm that YGL075c was truly the gene mutated in *mps2–1* strains, the original 3.4-kb insert was placed in the integrative plasmid pRS306 (*URA3*). The insert in pRS306-*MPS2* directed integration at the *MPS2* locus as determined by the meiotic linkage of the integrated marker (*URA3*) to *mps2–1*. To further verify that YGL075c was indeed *MPS2*, the gene was amplified from the *mps2–1* strain (WX178–1a) and sequenced. A single base change was detected at nucleotide 114 leading to an amino acid change of a glutamic acid to lysine residue at position 39 in the protein (Figure 1A). We confirmed that the base change found in the *mps2–1* strain was the cause of the temperature-sensitive phenotype, and not a polymorphism, by cloning the region containing the mutation into a yeast integrative vector to create pRS306-*mps2–1*. Targeted integration at the *MPS2* locus, with excision of the wild-type copy of the gene, conferred temperature sensitivity that is complemented by wild-type *MPS2* on a plasmid, but not by a cross to the *mps2–1* strain (our unpublished results). These results indicate that YGL075c is the gene mutated in the *mps2–1* strain.

The *MPS2* gene was also identified in a search for proteins involved in the onset of anaphase. The *cim3–1* and *cim5–1* mutations affect two different regulatory subunits of the 26S proteasome (Ghislain *et al.*, 1993). Both mutants arrest division at the G2/M transition with unsegregated chromosomes at the restrictive temperature of 37°C, suggesting that one or more proteins must be proteolyzed for cells to enter into anaphase. We searched for proteins involved in this

proteasome-mediated metaphase/anaphase transition by screening for yeast genes whose overexpression would inhibit growth in the *cim5-1* mutant, but not the wild-type strain, at the *cim5-1* semipermissive temperature of 30°C (see MATERIALS AND METHODS). At this temperature, the *cim5* mutant population of cells contains a higher than normal fraction of cells containing a 2N DNA content, suggesting that inefficient degradation of mitotic substrates of the proteasome was limiting the rate of cell division (Ghislain *et al.*, 1993). We reasoned that the overexpression of a gene encoding such a substrate, or a protein involved in this process, might inhibit the growth of cells containing these mutant proteasomes. The overexpression of the *MPS2* protein was toxic in these cells, suggesting that Mps2p may fit these criteria.

The predicted Mps2p sequence is 387 amino acids long and shows no strong sequence similarity to other proteins in the available databases (Figure 1A). Mps2p has a predicted molecular mass of 44,587 Da and a theoretical pI of 8.4. The Mps2p sequence contains a 17 amino acid hydrophobic stretch (residues 311–327) as a putative transmembrane domain (Kyte and Doolittle, 1982; Klein *et al.*, 1985), three potential Cdc28 phosphorylation sites S/TPXK/R/Q (Songyang *et al.*, 1994), and four potential destruction box sequences RXXLXXXN/I (Glotzer *et al.*, 1991). In addition, the Mps2p sequence reveals two regions capable of forming coiled-coils by the algorithm of Lupas (Lupas *et al.*, 1991) (Figure 1B).

MPS2 Is an Essential Gene

To test whether *MPS2* encodes an essential gene product, part of the chromosomal copy of *MPS2* was replaced by homologous recombination with the *HIS3* gene (see MATERIALS AND METHODS). Sporulated diploid yeast strains heterozygous for the null allele of *MPS2* segregated a lethal mutation in tetrads inspected a few days after tetrad dissection (AM610, Table 1). Furthermore, the *HIS3* marker for the null allele was not recovered in these tetrads, showing that it cosegregated with the lethal phenotype. The null allele could be recovered from sporulated diploids only when they contained a plasmid-borne copy of *MPS2* (pRS316-*MPS2*). Furthermore, the *mps2* null strains that contained a *URA3*-marked, plasmid-borne copy of *MPS2* could not grow in the presence of 5-FOA, which induces plasmid loss, indicating that *MPS2* is essential; however, on continued incubation (10–14 d) at 23°C, the dissection plates of diploids heterozygous for the null allele of *MPS2* would contain very slow growing “pinhead” colonies that were found to contain the nutritional marker for the null allele (Figure 2A). The appearance of these colonies was variable from strain to strain and cross to cross, but approximately half of the spore clones containing

Figure 2. Deletion of *MPS2* results in abnormal ploidy. (A) A representative tetrad from the *mps2*Δ(116–387)::*HIS3*/*MPS2* strain (AM610) grown for 14 d at 23°C. (B) Histogram of relative DNA content from each of the spore clones shown in A.

a null allele eventually formed one of these very small colonies. On microscopic inspection of the dissected spores that did not form a small visible colony, most were found to have germinated and accumulated tens or hundreds of cells before ceasing to grow further. Some of the null-containing spore clones could be cultured, and PCR analysis of genomic DNA from these cells confirmed that they only contained the null allele of *MPS2* and had not acquired a wild-type copy of the *MPS2* gene (our unpublished results). Other null alleles of *MPS2*, in which the entire gene was replaced, were constructed throughout the course of this work, and all behaved identically.

The ability to culture some of the strains containing the null allele of *MPS2* allowed partial characterization of these strains. They were found to grow very slowly, with doubling times of 10–20 h. In addition, *mps2* null strains are temperature-sensitive at 34°C. Unlike strains containing the *mps2-1* mutation, which arrest as large budded cells with good viability on return to permissive temperature (Winey *et al.*, 1991), strains containing a *mps2* null allele did not show a uniform cell cycle arrest and rapidly lose viability at the nonpermissive temperature (our unpublished results). In the course of this analysis, we found by analyzing their DNA content that the surviving null

strains were all apparent tetraploids (Figure 2B), with the exception of one diploid strain. There is also a peak at <1N that may correspond to either hypoploid cells or to debris from dead cells. The survival mechanism for the strains containing a null allele of *MPS2* is not known.

Mps2p Is Present throughout the Cell Cycle

As described previously, Mps2p contains four potential destruction boxes (Figure 1A). The destruction box motif is required for the ubiquitination and subsequent degradation of several proteins, including Pds1p, Ase1p, and the mitotic cyclins, in mitosis and in the G1 phase of the cell cycle by the anaphase-promoting complex (APC)-proteasome pathway (Amon *et al.*, 1994; Cohen-Fix *et al.*, 1996; Juang *et al.*, 1997). Degradation of these proteins is important for the initiation of chromosome segregation and for the exit from mitosis. Because Mps2p is toxic when overexpressed in a proteasome mutant, we reasoned that Mps2p could be a substrate for the proteasome. We tested whether Mps2p, like other APC substrates, is an unstable protein in α -factor-arrested cells. Although *MPS2* mRNA levels are relatively constant through the cell cycle (Cho *et al.*, 1998), we placed *MPS2*, tagged with nine copies of the myc epitope, under the control of a tetracycline-repressible promoter (Gari *et al.*, 1997), whose expression is negligible in the presence of high antibiotic concentrations in the growth medium (5 μ g/ml) and low in the presence of lower antibiotic concentrations (50 or 100 ng/ml). An exponentially growing culture of cells expressing this low, constitutive level of 9xmycN-Mps2p was treated with α -factor to arrest cells in G1 phase before Start, a stage of the cell cycle in which the APC is very active (Amon *et al.*, 1994). The quantity of 9xmycN-Mps2p was compared in the exponentially growing and α -factor-arrested cells by immunoblotting of total cell extracts (Figure 3A). No decrease in 9xmycN-Mps2p levels was seen in the G1-arrested cells, suggesting that Mps2p is unlikely to be degraded by the APC-proteasome pathway.

We also tested whether the level of Mps2p oscillates during the cell cycle. For this experiment, 9xmycN-*MPS2* was placed under the control of its endogenous promoter and integrated at the *URA3* locus in a *mps2 Δ ::HIS3* strain that initially contained a plasmid-borne copy of *MPS2*. To follow Pds1p levels under the same conditions, the wild-type *PDS1* gene was replaced with a construct encoding a hemagglutinin epitope-tagged version of Pds1p, *PDS1-HA* (Cohen-Fix *et al.*, 1996). Cells simultaneously expressing 9xmycN-Mps2p and Pds1-HAp were released synchronously from α -factor arrest. Cell morphology and 9xmycN-Mps2p, Pds1-HAp, and Cdc28p levels were determined at various timepoints after release from

Figure 3. Mps2p levels show little fluctuation during the cell cycle. (A) An overnight culture of MCL 94 cells containing *pTet-9xmycN-MPS2* grown in the presence of 5 μ g/ml doxycycline (repressing conditions) was diluted in the same selective medium containing 50 or 100 ng/ml doxycycline to allow a low constitutive expression from the tet promoter. Cells were grown overnight to early log phase at 30°C (asynch.) and then divided into two separate cultures: one culture was left untreated (–) and the other was arrested in the G1 phase with α -factor (0.2 μ M final concentration) for 2 h (+). Equal quantities of total cell protein were then subjected to SDS-PAGE, and proteins were transferred to membranes to determine the levels of 9xmycN-Mps2p by immunoblot analysis. 9xmycN-Mps2p is absent when cells are grown in the presence of fully repressing concentrations of doxycycline (5 μ g/ml) or from cells that do not contain the 9xmycN-*MPS2* plasmid. The G1 arrest was verified by flow cytometric analysis. (B) Wild-type cells expressing both 9xmycN-Mps2p and Pds1-HAp (MCL123) were grown to early log phase at 24°C (asynch.) and then divided into two separate cultures: one culture was left untreated (exp) and the other was arrested in the G1 phase with α -factor (0.2 μ M) for 2 or 5 h. After 2 h, >90% of the cells were already arrested in G1 phase as determined by flow cytometry analysis, as were cells treated with α -factor for 5 h. After this time, cells were released from the arrest by washing out the α -factor. Samples were taken at the indicated time points and analyzed for 9xmycN-Mps2p, Cdc28p, and Pds1-HAp by immunoblot analysis. Cell and nuclear morphology and DNA content were analyzed by propidium iodide staining.

the α -factor arrest (Figure 3B). Levels of 9xmycN-Mps2p were roughly constant compared with the unvarying Cdc28p, except during the G1-S phases, in which 9xmycN-Mps2p appeared to decrease slightly. In contrast, Pds1-HAp completely disappeared from G1-arrested cells, as described previously (Cohen-Fix *et al.*, 1996). We conclude that the level of 9xmycN-Mps2p does not oscillate significantly in the cell cycle of *S. cerevisiae*; however, we cannot exclude the possi-

bility that the tagged version of Mps2p is somehow resistant to this type of regulation.

Mps2p Is an Integral Membrane Protein

Mps2p contains a 17 amino acid hydrophobic segment that could correspond to a transmembrane domain. Biochemical fractionation experiments were undertaken in cells expressing 9xmycN-Mps2p at its normal endogenous levels (MCL120) to test whether Mps2p is a membrane protein. Extracts obtained by gentle spheroplast lysis were fractionated by differential centrifugation at $13,000 \times g$ for 15 min followed by 60 min at $100,000 \times g$ (see MATERIALS AND METHODS). At each stage, the supernatants and pellets were collected and analyzed by immunoblotting. Mps2p fractionated with nuclei and endoplasmic reticulum (ER) membranes in the $13,000 \times g$ pellet (P13) and was undetectable in the supernatant or in the pellet obtained after $100,000 \times g$ centrifugation (S100 or P100 fractions, respectively) (Figure 4A). To control for the purity of the different fractions, the same nitrocellulose membrane was reincubated with antibodies directed against Sec61p and Dpm1p, both of which are ER integral membrane proteins (Beck *et al.*, 1990; Wilkinson *et al.*, 1996), and with antibodies directed against the cytosolic hexokinase (van Tuinen and Riezman, 1987). Sec61p and Dpm1p cofractionated with Mps2p in the P13 fraction; hexokinase did not cofractionate.

To determine the nature of the association of 9xmycN-Mps2p with the nuclear/ER membrane fraction, the $13,000 \times g$ pellet was extracted with various buffers and evaluated for the quantity of solubilized 9xmycN-Mps2p by immunoblotting. Agents that release peripheral membrane proteins (associated with membranes through electrostatic or hydrophobic interactions with integral membrane proteins) and soluble intranuclear proteins, such as 1 M NaCl or 6 M urea, did not solubilize 9xmycN-Mps2p from the P13 (Figure 4B). 9xmycN-Mps2p was partially solubilized by a detergent treatment (1% Triton X-100) or by an alkali treatment (0.2 M Na_2CO_3 , pH 11). As an internal control for integral membrane protein behavior, we used Sec61p, which was completely solubilized by 1% Triton X-100 but was not solubilized by the sodium carbonate (Figure 4B). Overall, the fractionation characteristics of 9xmycN-Mps2p suggest that it is an integral membrane protein. The unusual partial extraction by alkaline sodium carbonate may be due to the rather small hydrophobic stretch of 17 amino acids containing Tyr³²⁶ in the Mps2p sequence. A negative charge would in principle be introduced on this residue at pH 11, and this ionization might be important for stripping Mps2p from the membrane. Similar extraction behavior was seen for Sss1p, an integral ER membrane protein containing a 16 amino acid hydro-

phobic stretch including a tyrosine residue (Esnault *et al.*, 1994).

9xmycN-Mps2p was partially solubilized by 1% Triton X-100, whereas Sec61p, which spans the ER membrane multiple times, was completely extracted using the same conditions (Figure 4B). Partial extraction might indicate that the protein is trapped within multilayered membrane-detergent aggregates, which are destroyed by sonication; however, partial solubilization of 9xmycN-Mps2p from the P13 fraction was still observed after sonication in the presence of 1% Triton X-100 (our unpublished results). One possibility to explain this behavior is that 9xmycN-Mps2p could be physically associated with other proteins that prevent its full solubilization from membranes in the presence of detergent. To test this possibility, 9xmycN-Mps2p in the P13 was extracted with 1% Triton X-100 containing 6 M urea (to weaken hydrophobic interactions) or Triton X-100 containing 1 M NaCl (to neutralize electrostatic bonds). As shown in Figure 4C, 9xmycN-Mps2p was completely solubilized with both double treatments, suggesting that hydrophobic and electrostatic interactions could be preventing total solubilization of 9xmycN-Mps2p by detergent treatment alone.

Mps2p Is Localized at the SPB and the NE/ER

We localized tagged Mps2p in whole cells to confirm and potentially extend the 9xmycN-Mps2p fractionation data. To visualize Mps2p in living cells, Mps2p was tagged at the amino terminus with the green fluorescent protein (GFP). As shown in Figure 5, one or two spots of strong autofluorescence were visible that were coincident with the nuclear DNA (Figure 5, a–c). Additionally, we often observed a weaker, perinuclear GFP-Mps2p signal (Figure 5, b and c, arrows). The number, intensity, and location of the brighter spots of fluorescence suggested that they correspond to SPBs. We confirmed this localization by two methods. First, we used whole-cell indirect immunofluorescence deconvolution microscopy to determine whether Mps2p colocalized with a known SPB component, Spc42p (Donaldson and Kilmartin, 1996). We used a strain that contains both Mps2p epitope tagged at the C terminus with 9xmyc (9xmycC-Mps2p; see MATERIALS AND METHODS) and Spc42p-GFP (Chial *et al.*, 1998; Schutz and Winey, 1998). Similar to the GFP-Mps2p, 9xmycC-Mps2p localized in one or two brighter spots around the nucleus and also at the nuclear periphery (Figure 6A, 9xmycC-Mps2p). In these cells, the brighter spots of 9xmycC-Mps2p localization (red) and the Spc42-GFP (green) signals were coincident (Figure 6A, combined; overlap is yellow). It should be noted that the genetic behavior of 9xmycC-MPS2 is somewhat different from that of wild-type MPS2. Cells expressing 9xmycC-Mps2p can increase in ploidy spontaneously (haploid

Figure 4. 9xmycN-Mps2p fractionates as an integral membrane protein. A whole-cell extract was prepared from cells expressing 9xmycN-Mps2p from its normal promoter (MCL120) by gentle spheroplast lysis (see MATERIALS AND METHODS) and then fractionated as described in MATERIALS AND METHODS. (A) Cell fractionation: the same amount of protein from the whole-cell extract and S13, P13, P100, and S100 fractions were subjected to SDS-PAGE and immunoblotted with anti-myc, anti-Sec61p, anti-Dpm1p, and anti-hexokinase antibodies successively. (B) Solubilization of Mps2p from the P13 fraction prepared from MCL120. The pellet was extracted with lysis buffer (control) or lysis buffer containing 1 M NaCl, 50 mM Tris (pH 7.5)-10 mM EDTA, 0.2 M Na₂CO₃ (pH 11), 1% Triton X-100, or 6 M urea as described in MATERIALS AND METHODS. The mixtures were incubated on ice for 10 min and then centrifuged to produce a pellet fraction (P) and a supernatant fraction (S). Equivalent samples of the pellet and supernatant were analyzed by immunoblotting with anti-myc or anti-Sec61 antibodies. (C) Total solubilization of Mps2p protein from the P13. The P13 pellet was extracted with lysis buffer (control) or lysis buffer containing 1% Triton X-100, 1% Triton X-100 plus 6 M urea, or 1% Triton X-100 plus 1 M NaCl. Pellet (P) and supernatant (S) were separated and analyzed by immunoblotting.

Figure 5. Localization of GFP-Mps2p in living cells. Several cells are shown in a, in which GFP-Mps2p (strain MCL175) localizes in one or two discrete foci coincident with the nuclear DNA. The cell with two distinct spots of fluorescence is a small budded cell. The cells shown in b reveal that GFP-Mps2p also localizes to the nuclear periphery. Several cells are shown with one bright spot and a fainter signal at the nuclear periphery (arrows). A higher magnification of a budded cell is shown in c with two bright spots and a perinuclear localization. The GFP-Mps2p was visualized via autofluorescence of GFP, and the DNA was visualized with Hoechst. A single plane of focus is shown. Bar, 1 μ m.

to diploid, an *mps2-1* phenotype) but are not temperature sensitive (our unpublished results), suggesting a very mild defect in Mps2p function. Nonetheless, the localization pattern is similar to that of GFP-Mps2p, for which cells stably remain haploid. Furthermore, we observe Mps2p at the SPB during various stages of

Figure 6. 9xmycC-Mps2p colocalizes with Spc42p-GFP, but not Nup49p-GFP. (A) A strain expressing both 9xmycC-Mps2p and Spc42p-GFP (SMY28-2b) was prepared for indirect immunofluorescence microscopy (see MATERIALS AND METHODS). The Spc42p-GFP was visualized via autofluorescence of GFP, the 9xmycC-Mps2p was detected using a Texas Red-conjugated 2° antibody, and the DNA was visualized with DAPI. Four different cells are shown at a high magnification to show the detail of colocalization. The bright spots of 9xmycC-Mps2p (red) and the Spc42p-GFP (green) signals overlap, as seen in the combined image (yellow). The Spc42p-GFP and 9xmycC-Mps2p signals at the lower SPBs in images b and d do not completely overlap in these focal planes; however, Spc42p and Mps2p are not expected to be in the same exact position in the organelle. In addition, the amount of overlap depends on the ability to indirectly label Mps2p at any given SPB. (B) Two different cells from a strain expressing both 9xmycC-Mps2p and Nup49p-GFP (SMY1892) are shown at a high magnification. There are bright spots of 9xmycC-Mps2p fluorescence observed along the nuclear periphery that are devoid of Nup49p-GFP signal. All panels in this Figure represent a single optical plane that was deconvolved using the Slidebook software package (see MATERIALS AND METHODS). Bar, 1 μ m in both panels.

the cell cycle, suggesting that Mps2p is present at the SPB throughout the cell cycle.

We also explored whether the perinuclear localization of 9xmycC-Mps2p corresponded to NPCs. For this experiment, we used a strain expressing both

9xmycC-Mps2p and Nup49p-GFP. Nup49p is a well characterized NPC protein, or nucleoporin, that exhibits a characteristic punctate, nuclear rim localization pattern by whole-cell immunofluorescence microscopy (Grandi *et al.*, 1995; Bucci and Wentz, 1997). We

Figure 7. GFP-Mps2p localizes to the SPB. Cells expressing GFP-Mps2p were prepared for immunoelectron microscopy using anti-GFP antibodies and colloidal gold-conjugated secondary antibodies. Thin sections of four different cells are shown. C, cytoplasm; N, nucleoplasm; NE, nuclear envelope; MTs, microtubules; SPB, spindle pole body. Arrowheads show the localization of individual gold particles in b and d. Gold particles were observed in all 12 SPBs examined. Bar, 0.1 μ m.

observed very little overlap between 9xmycC-Mps2p and Nup49-GFP in any of the cells that we examined. An example of this is shown in Figure 6B. There are discrete areas of colocalization between the two proteins (yellow), but their localization patterns are quite distinct, suggesting that Mps2p is not a nucleoporin. In addition, there are bright spots of 9xmycC-Mps2p at the nuclear periphery that are devoid of Nup49p signal, indicative of where the SPB is located. The signal of 9xmycC-Mps2p and GFP-Mps2p at the nuclear periphery is weaker than at the SPB. On the basis of this observation and the fractionation of 9xmycN-Mps2p with membranes, we propose that Mps2p is a minor membrane component of the NE.

The second method that was used to confirm the SPB localization was immunoelectron microscopy of cells expressing GFP-Mps2p. As shown in Figure 7, the SPBs from four different cells were decorated with one (b) to five (c) gold particles, with little to no background (of 12 SPBs examined, all were decorated with ≥ 1 gold particle). We did not observe a significant number of gold particles in the NE; however, this

could be due to lower levels of Mps2p in the NE than at the SPB (see DISCUSSION). We conclude from these experiments that Mps2p tagged with either GFP or 9xmyc is a component of the SPB and the NE.

DISCUSSION

We report here that the *MPS2* gene encodes a novel 44-kDa integral membrane protein localized at SPBs and the NE. *MPS2* has been previously reported to be required for SPB duplication (Winey *et al.*, 1991). Here, we provide evidence that *MPS2* is linked to the ubiquitin-proteasome pathway.

Although we consider the *MPS2* gene to be essential, some of the spore clones containing a null allele were able to give rise to a colony containing very slow growing cells with abnormal ploidy. The fact that defects in SPB duplication can lead to increases in ploidy via a monopolar mitosis is well documented (Winey *et al.*, 1991). This event has also been observed at the permissive temperature for strains containing conditional alleles of *CDC31* and *KAR1* (Schild *et al.*,

1981; Rose and Fink, 1987). It seems possible that the surviving *mps2* null strains are not perfect tetraploids, but are aneuploid in a manner that increases or decreases the dosage of some gene(s), which allows for the nulls to survive.

The fact that *MPS2* was identified in a screen for possible regulatory or substrate proteins in the proteasome pathway is intriguing. On the basis of the genetic interaction between *CIM5* and *MPS2*, we sought to determine whether Mps2p was a potential substrate for the ubiquitin–proteasome pathway by examining protein levels throughout the cell cycle; however, with Mps2p tagged at its amino terminus with nine copies of the myc epitope and expressed from its normal promoter (Figure 3), we were unable to see any major fluctuations in Mps2p levels during the cell cycle, nor were we able to see any proteolysis of the protein in cells arrested in the G1 phase of the cell cycle with α -factor, a situation in which destruction box proteins are rapidly degraded.

At the present time, the mechanism of how *cim5-1* cells die in the presence of overexpressed Mps2p remains unknown. Interestingly, it was reported recently that GFP-Cim5p-labeled proteasomes are enriched in the NE/ER in living yeast cells, indicating that proteasomal degradation is concentrated at this compartment in yeast (Enenkel *et al.*, 1998). Because Mps2p is also localized at the NE, it is possible that Mps2p may interact with the proteasome, although it does not appear that Mps2p is a proteasome substrate. If so, overexpression of Mps2p may inhibit the normal function or localization of the proteasome such that the overexpression becomes toxic in *cim5-1* cells containing a poorly functioning proteasome. In addition to the interaction between *MPS2* and *CIM5*, the only other known gene that links SPB duplication to the ubiquitin–proteasome pathway is *PCS1* (McDonald and Byers, 1997). Like *CIM5*, *PCS1* encodes a proteasome 19S cap subunit (Russell *et al.*, 1996). *PCS1* function in SPB duplication appears to be required before *MPS2* function, in that SPB duplication is blocked at an earlier stage in *pcs1* mutants cells than in *mps2-1* cells (Winey *et al.*, 1991; McDonald and Byers, 1997). Taken together, these observations strengthen the idea that SPB duplication may be regulated by the ubiquitin–proteasome pathway.

It is clear that Mps2p has a primary role in insertion of the nascent SPB into the NE. First, *mps2-1* cells exhibit a monopolar spindle because of a defective SPB. The nascent, defective SPB is not properly inserted into the NE and lies on the cytoplasmic side of the envelope. Moreover, the SPB appears to lack the inner (nuclear) plaque and its associated nuclear microtubules, which are observed in functional SPBs. Second, tagged Mps2p localizes to the SPB as demonstrated by fluorescence microscopy and immunoelectron microscopy. Third, YGL075c/Mps2p was identi-

fied by mass spectrometry in highly purified preparations of SPBs (Wigge *et al.*, 1998), although these authors did not cytologically localize the YGL075c protein in this study. Taken together, these data suggest that Mps2p is directly involved in insertion of the nascent SPB into the NE, as originally proposed (Winey and Byers, 1993).

We have suggested previously that the *NDC1* protein also carries out this insertion event (Winey *et al.*, 1993). The *NDC1* gene function appears to be required at the same step in SPB duplication based on the execution point and the same terminal SPB morphology. Ndc1p is a membrane protein and is a shared component of NPCs and SPBs (Chial *et al.*, 1998). The work presented here extends the parallels between *NDC1* and *MPS2*. Both genes encode essential membrane proteins that localize to SPBs; however, unlike Ndc1p, Mps2p does not appear to be a component of NPCs. The caveat to this is that Mps2p is expressed at a much lower level than Ndc1p (compare Figures 5–7 with Chial *et al.*, 1998). This is most evident in the weak nuclear periphery signal (Figures 5 and 6) and a lack of gold particles found in the NE (Figure 7). Although we cannot rule out the possibility that Mps2p could be transiently associated with NPCs, Mps2p fails all criteria used to show that Ndc1p is a component of NPCs.

On the basis of the SPB localization of Mps2p and Ndc1p, one possible scenario could be that Mps2p and Ndc1p are required as membrane components for inserting the nascent SPB into the NE. There is only one other known SPB component with a putative transmembrane domain, Spc105p, which appears to localize to the nucleoplasmic face of the SPB and to the nuclear microtubules (Wigge *et al.*, 1998). It is not known whether mutations in this protein cause an SPB duplication defect. In the future, the ultrastructural details of assembling a new SPB will be important for a more complete understanding of Mps2p and Ndc1p function. In summary, *MPS2* encodes an integral membrane protein that localizes to SPBs and the NE. Future experiments with *MPS2* and the genes with which it interacts may reveal the specific function of Mps2p and provide us with new insights into the role of membrane proteins in SPB assembly.

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