**MHP1, an Essential Gene in *Saccharomyces cerevisiae***

**Required for Microtubule Function**

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**Abstract.** The gene for a microtubule-associated protein (MAP), termed MHP1 (MAP-Homologous Protein 1), was isolated from *Saccharomyces cerevisiae* by expression cloning using antibodies specific for the *Drosophila* 205K MAP. MHP1 encodes an essential protein of 1,398 amino acids that contains near its COOH-terminal end a sequence homologous to the microtubule-binding domain of MAP2, MAP4, and tau. While total disruptions are lethal, NH2-terminal deletion mutations of MHP1 are viable, and the expression of the COOH-terminal two-thirds of the protein is sufficient for vegetative growth. Nonviable deletion-disruption mutations of MHP1 can be partially complemented by the expression of the *Drosophila* 205K MAP. Mhp1p binds to microtubules in vitro, and it is the COOH-terminal region containing the tau-homologous motif that mediates microtubule binding. Antibodies directed against a COOH-terminal peptide of Mhp1p decorate cytoplasmic microtubules and mitotic spindles as revealed by immunofluorescence microscopy. The overexpression of an NH2-terminal deletion mutation of MHP1 results in an accumulation of large-budded cells with short spindles and disturbed nuclear migration. In asynchronously growing cells that overexpress MHP1 from a multicopy plasmid, the length and number of cytoplasmic microtubules is increased and the proportion of mitotic cells is decreased, while haploid cells in which the expression of MHP1 has been silenced exhibit few microtubules. These results suggest that MHP1 is essential for the formation and/or stabilization of microtubules.

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**Microtubules (MTs)** are involved in a number of essential cellular processes such as cell morphogenesis, intracellular transport, cell motility, and mitosis. Important potential regulators of MT dynamics are the MT-associated proteins (MAPs) (Lee, 1993; Wiche et al., 1991). Initially MAPs were isolated from neural tissues rich in MTs by means of copurification with polymerized MTs. These neural MAPs, including MAP 1A (Schoenfeld et al., 1989), MAP 1B (Noble et al., 1989), MAP2 (Lewis et al., 1988), and tau (Lee et al., 1988), were shown to stimulate MT assembly from purified tubulin in vitro (Cleveland et al., 1977; Murphy et al., 1977; Bulinski and Borisy, 1979). Although these MAPs have been characterized biochemically, their specific functions have not been determined unambiguously.

A group of ubiquitously expressed, thermostable MAPs has also been described as phosphorylated proteins of ~200 kD (MAP4-type proteins) comprising (as in the case of MAP1, MAP2, and tau) multiple protein isoforms. Immunocytochemistry has shown that these MAPs colocalize with mitotic spindle and interphase MTs (Bulinski and Borisy, 1980; Iznat et al., 1993). Cloning and sequencing of mouse, human (Chapin and Bulinski, 1991; West et al., 1991), and bovine (Aizawa et al., 1990) MAP4 have revealed that the MAP4 proteins share an MT-binding domain homologous to the MT-binding motif first discovered in tau and MAP2 (Lee et al., 1988; Lewis et al., 1988). The common sequence motif, the assembly promoting (AP) sequence (Aizawa et al., 1989), consists of three or four basic repeats of 31 amino acid residues within the COOH-terminal domain of the proteins. In several species MAPs of ~200 kD have been discovered that are immunologically related to the *Drosophila* 205K MAP but not to MAP4-type proteins (Kimble et al., 1992). The *Drosophila* 205K MAP was analyzed and found to contain no sequence homologies to other MAPs. Moreover, it possesses an MT-binding region without homology to tau, MAP2, or MAP4 (Irminger-Finger et al., 1990). However, the 205K MAP is similar to MAP4 and MAP2 in terms of an extensive NH2-terminal acidic region...
and, as in the case of tau, a short, acidic COOH-terminal region that is preceded by a basic region containing the MT-binding domain.

The in vivo functions of all the MAPs identified so far remain uncertain, mainly because of the absence of genetic studies. In the isolated case of the Drosophila 205K MAP where such studies were possible, the protein was found not to be essential (Pereira et al., 1992). A powerful tool to more fully investigate the functions of MAPs is the yeast Saccharomyces cerevisiae, since it is possible to establish genetic interactions between MAPs and tubulin or other cytoskeletal components. A number of laboratories have attempted to identify MAPs in yeast. Their approaches have involved purifying proteins by MT-affinity columns (Barnes et al., 1992) or from cellular structures comprising MTs such as the spindle pole body (SPB) (Kilmartin et al., 1993) and the centromere-binding complex (Jiang et al., 1993), identifying yeast proteins that cross-react with antibodies against mammalian MAPs (Hasek et al., 1991), cloning genes complementing cellular defects involving MTs (Hoyt et al., 1990; Sternars et al., 1990; McMillan and Tatchell, 1994), and cloning yeast sequences using PCR with degenerated primers derived from homologous sequences of different species (Hyman et al., 1992). These strategies led to the identification of CPFS, a component of the yeast centromere-binding complex, a homologue of mammalian MAP1A and B (Jiang et al., 1993), and a number of genes encoding proteins that share similarities with motor proteins (Berlin et al., 1990; Meluh and Rose, 1990; Hoyt et al., 1992; Lillie and Brown, 1992; Roof et al., 1992; Li et al., 1993). In a biochemical approach, Sep1 has been identified as an accessory protein in MT function in yeast (Interthal et al., 1995). A genetic screen for complementation of a temperature-sensitive allele of the 13-tubulin gene led to the identification of STU1, a gene encoding a protein with a predicted size of 174 kD and no significant homology to known proteins (Pasqualone and Huffaker, 1994).

We chose to search for yeast proteins that cross-react with antibodies directed against the Drosophila 205K MAP, leading to the discovery of a novel essential MAP gene, MHP1 (MAP Homologous Protein 1). We present evidence that the product of this gene, Mhp1p, interacts with MTs and that mutations in MHP1 influence MT structure and function during interphase and mitosis.

Materials and Methods

Strains and Microbiological Techniques

All yeast strains used in this study are listed in Table 1. Genetic manipulations and growth media were standard (Sherman et al., 1986; Rose et al., 1990).

Cloning of MHP1

A partial sequence of the MHP1 gene was cloned by screening a λgt11 cDNA expression library from S. cerevisiae (kindly provided by Dr. B. Altman, University of Bern, Switzerland). 20,000 plaques were screened and plated on Y1090r- host cells to a plaque density of 1,000-2,000 plaques per plate. Plates were incubated at 42°C for 4 h, or until very small plaques were visible, then overlaid with nitrocellulose filters previously soaked in 10 mM isopropyl β-D-thiogalactopyranoside (IPTG), and incubated for 4 h at 37°C. Filters were blocked with 3% BSA in TBS for 30 min, washed with TBS, and incubated with a polyclonal anti-205K MAP antibody (Goldstein et al., 1986) in a 1:500 dilution for 2 h at room temperature. After washing with TBS, the secondary antibody, a mouse anti-IgG coupled to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), was applied in a 1:5,000 dilution. Positive plaques were visualized with 5-bromo-4-chloro-3-indoylphosphate-4-nitro blue tetrazolium salt (BCIP) and nitro blue tetrazolium (NBT) (Sigma Chemical Co.). Plaques in the region of the signal were resuspended in phage buffer (10 mM Tris, pH 7.5, 10 mM MgCl2), replated, and rescreened with the same procedures. Three positive plaques were obtained and tested for the expression of the immunoreactive protein on Western blots. The insert of one phage was subcloned into the Bluescript-vector (pBS) and sequenced. The 740-bp insert was used as a probe to screen a library of genomic DNA partially digested by Sau3A (kindly provided by Dr. B. Dujon, Institute Pasteur, Paris, France), and the clone F2B8 was isolated and sequenced. General molecular cloning techniques were as described (Maniatis et al., 1989).

DNA Sequence Analysis

DNA sequencing was performed with the dideoxy chain termination method (Sanger et al., 1977), using the Sequenase kit (UBS; United States Biochemical Corp., Cleveland, OH). Both strands of the genomic region containing the MHP1 gene were sequenced on average twice. Sequence data were assembled and analyzed with the Genetics Computer Group programs (Devereux et al., 1984).

Computer Analysis of the MHP1 Sequence

Secondary structure analysis of the amino acid sequence of MHP1 was performed using the algorithms of Garnier et al. (1978) and Chou and Fasman (1978). The protein databases PIR and Swissprot were searched with the entire Mhp1p sequence using FASTA (Pearson and Lipman, 1988). The sequence of Mhp1p was compared directly to sequences of known MAPs using FASTA and PILEUP (Devereux et al., 1984).

In Vitro Transcription–Translation

Coupled in vitro transcription and translation were performed with a transcription–translation kit (Promega Corp., Madison, WI) and [35S]methionine, according to the manufacturer’s instructions. For each reaction 2 μg of plasmid DNA were used. N1300 was a subclone in pBS containing the region from bp 656-3,662, and N1211 was generated by restriction digestion. Construct C1188 was generated by subcloning the PCR-amplified region from bp 3,562-4,368 into pBS.

MT-binding Assay

MT-binding assays were performed using porcine brain tubulin, purified by phosphocellulose chromatography. MTs were preassembled and stabilized in the presence of 2 mM GTP and 20 μm taxol as described (Vallee, 1982). To test the MT-binding activity of the in vitro–translated proteins, the reaction mixture was centrifuged to precipitate nonsoluble material, protease inhibitors were added to the assembly reaction, and 4 μl of the in vitro translation reactions was added to assembled MTs in a total volume of 25 μl. When Mhp1p from yeast cells was tested, protease inhibitors were added to the assembly reaction before addition of 15 μl total yeast protein extract in a total of 50 μl. Yeast cells (100 μg) were lysed in 100 μl lysis buffer (100 mM Pipes-KOH, pH 6.9, 2 mM EDTA, 1 mM MgCl2) by vigorous vortexing (30 s) with glass beads and immediately used for the binding assay to minimize the degradation of Mhp1p. Assembly reactions were centrifuged, pellets were rinsed with assembly buffer and recentrifuged, and supernatants and pellets were analyzed by PAGE.

Antibody Production and Purification

The COOH-terminal region of MHP1 from bp 3,562-4,368 was amplified by PCR, generating terminal NdeI sites, and cloned into the NdeI site of the His-tag expression vector pET-15b (Studier et al., 1990), generating the plasmid pET-C1188, which was expressed in Escherichia coli. The apparent molecular mass of the His-tagged protein expressed by pET-C1188 was 36-kD on SDS gels. The 36-kD protein, termed C1188p, was purified on “His-bind” resin (Novagen, Madison, WI), and 100 μg was injected subcutaneously into rabbits. The collected sera were centrifuged, and the supernatant was immunopurified on membrane-bound antigen as described (Olmdsted, 1986).
Table I. Saccharomyces cerevisiae Strains Used in This Study

| Name  | Genotype                                      | Sources         |
|-------|-----------------------------------------------|-----------------|
| Y501  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | P. Hieter       |
| Y502  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y503  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y504  | n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63    | This work       |
| Y505  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y506  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y507  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y508  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y509  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y521  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y531  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y561  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y562  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y563  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y564  | n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y565  | 2n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |
| Y566  | n a/ura3-32 lys2-801, ade2-101, trpl-1Δ63  | This work       |

Tubulin-binding Assay

Crude bacterial lysates from 200 ml cultures of E. coli expressing C1188p from plasmid pET-C1188 or the His-tagged NH2-terminal region of MHP1, encoding amino acids 1–440, from plasmid pET-N440, were loaded onto His-bind resin columns. PET-N440 had been generated by cloning the PCR-amplified coding region of MHP1 with terminal NdeI sites into the NdeI site of pET-15. Deletion of the 3' XhoI fragment and religation allowed the creation of pET-N440. The Ni-columns were washed extensively with Pipes and eluted with His-bind elution buffer. Column fractions were analyzed by PAGE and Western blotting.

Western Blots

Blotting procedures were performed as described (Ortega Perez et al., 1994). The membranes were incubated at room temperature for 2 h with anti-MHP1 in a 1:200 dilution, or anti-205K MAP in a 1:500 dilution. The secondary antibody was alkaline phosphatase–conjugated anti-rabbit IgG (Sigma Chemical Co.), in a 1:5,000 dilution, and the reaction was visualized by BCIP and NBT (Sigma Chemical Co.). When a chemiluminescence detection method was chosen, anti-MHP1 was used in a 1:1,000 dilution, and goat peroxidase-coupled secondary antibody was used in a 1:5,000 dilution. Signal detection was carried out with the ECL kit (New England Nuclear, Boston, MA).

Indirect Immunofluorescence Microscopy

Yeast cells were harvested during exponential growth phase and prepared for immunofluorescence staining as described (Kilmartin and Adams, 1984). The anti-α-tubulin antibody YOL1/34 (Kilmartin et al., 1982) was used at a 1:20 dilution, and a 1:200 dilution was used for the polyclonal anti-MAP antibodies. To visualize MTs and Mhplp, TRITC-labeled secondary antibodies (Socochim, Pully, Switzerland) were used for tubulin, and FITC-labeled anti-rabbit IgG (Socochim) were used for the polyclonal anti-MHP1 antibodies. The DNA staining dye 4',6-diamidino-2-phenylindole (DAPI) and 0.1% p-phenylenediamine were added to the mounting medium to visualize the nuclear region. An alternative procedure was applied to better preserve the cytoplasmic localization of Mhp1p; the fixation and digestion of cells was 30 min each, and the time of antibody incubation was reduced to 50–60 min.

Epitope Tagging of Mhp1p

The antigenic myc-tag (Munro and Pelham, 1984) was PCR amplified, generating terminal BamHI sites, and cloned into the BamHI site located between the GAL promoter and the MHP1 sequence on plasmid pMAC-Pst (Table II), generating pMACmyc-Pst. Constructs were sequenced to confirm correct insertion and conservation of the open reading frame (ORF). Y507 cells, transformed with plasmid pMACmyc-Pst (Tables I and II), were grown in selective medium overnight and for another 10 h in selective medium containing 1% galactose or 1% glucose. For immunofluorescence microscopy, the tagged epitope was detected with monoclonal...
were transformed with pMAC-MHP or pMAC-Pst (Table II), and Leu +
generated by excising the PstI-EcoRI fragment (from position 1667 to
gene) was used to transform Y501 cells, and His + transformants Y531
to complement the pMAC-MHP. The region including nucleotides 1-670, downstream from
the region containing the marker gene was confirmed by PCR. The
transformants were obtained that contained the
region including nucleotides 1-670, downstream from
the region containing the marker gene. A 3.5-kb SalI-SalI fragment (one SalI site near the 5' end of
the region containing the marker gene) was excised by inserting the
marker gene between two NcoI sites (positions -2 and 1833)
within the MHP1 gene. The MHP1-TRP1 construct was excised from the plasmid
with Sall (one genomic Sall site and one site in the plasmid linker) and
transformed into the diploid Trp- strain Y501 (Rothstein, 1983). Trio +
was generated by cloning a PvuII fragment containing
the region from bp 1,769-4,339 of the
MHP1 gene between the two NcoI sites
at position 1667 and 1679, respectively (see Fig. 2 A). A 4.5-kb Sall–HindIII fragment was excised and used to generate the heterozygous Ura +
cells Y105. Insertion of the marker gene was confirmed on Southern blots.
 mhpl-Δ::TRP1 was generated by cloning a PvuII fragment containing
the TRP1 marker gene between two NcoI sites (positions –2 and 1833)
within the MHP1 gene. A 3-kb NotI–SalI fragment (both sites in the linker sequence of pBS) was excised and used to transform Y501 cells, and Trp +
transformants Y521 were selected. The correct insertion of the marker gene was confirmed by PCR. The
mhpl-Δ::HIS3 mutation was generated by excising the PstI–EcoRI fragment (from position 1667 to
3626) and replacing it with a PstI–EcoRI fragment containing the HIS3
marker gene. A 3.5-kb SalI–Sall fragment (one Sall site near the 5' end of
MHP1 and one Sall site in the linker sequence at the 3' end of the MHP1
gene) was used to transform Y501 cells, and His +
transformants Y531 were selected and analyzed for insertion of the marker gene by PCR.
 mhpl-Δ::kanr allele was generated by PCR amplifying the "kan +" gene from the plasmid pFA6-kanMX4 (Wach et al., 1994), using the primers
5' CACTTAAATACGCAAAATATACAAGATAGCCCTACAACT-
CCACTAGTGGAT 3'. The resulting DNA fragment contained terminal ends homologous to 45 bp flanking the respective translation start and
stop sites of MHP1. Transformants were selected on plates containing rich
medium and 2% galactose. In 8 out of 12 tetrads, two colonies of normal
size and two small colonies could be observed after 4–5 d of incubation at
30°C, with the normal-sized colonies unable to grow on G418. All small
colonies were Leu + and resistant to G418. Colonies growing from individual spores were followed by microscopic inspection for 7 d. Leu + and
G418-resistant cells from glucose plates were grown in liquid medium containing
either glucose or galactose, and aliquots were taken after 4 and
20 h of growth and prepared for immunofluorescence microscopy.
Overexpression of the 3' half of MHP1, designated mhpl-Δ3, was performed with the clone pMAC-Pst. The mhpl-Δ3 allele was generated by cloning the region from bp 1,769-4,339 of the
MHP1 gene between the PstI and SalI sites of pMAC-80 (Table II). The correct insertion was verified by restriction analysis. Y501 and Y502 cells were transformed, and
Leu + or Leu - Trp + lines were generated and designated Y505 and Y506,
respectively. pMAC-Bam was generated by cloning a BamHI–PstI frag-
ment (bp 657–1,684) of MHP1 between the BamHI and PstI sites of
pMAC-Pst. Y501 cells were transformed with pMAC-Bam and Leu +
transformants were designated Y508. For phenotypic analysis, the cells
were grown in selective medium overnight, and 5 to 20 h in selective
medium and 2% galactose.

Results
Cloning of MHP1
To clone genes encoding yeast MAPs, a cDNA expression library was screened with a previously produced antibody raised against the Drosophila 205K MAP (Goldstein et al., 1986). The positive cDNA phase 205.41a was used as hybridization probe to isolate the genomic clone F2B8 (Fig. 1 A). DNA fragments from different regions within F2B8 were used to determine the size of the MHP1 transcripts on Northern blots (Fig. 1 A, probes 1 and 2). Both probes hybridized to an mRNA of 4,500 nucleotides. DNA se-
quence analysis of the transcribed region, including probe 1 and 2, revealed an ORF coding for 1,398 amino acids

Complementation and Overexpression Experiments
To complement the mhpl-Δ::kanr allele with MHP1, we generated
pMAC-MHP. The region containing nucleotides 1–670, downstream from
the unique Sall site, was amplified by PCR with terminal BamHI sites and
cloned into the BamHI site of pMAC-80, a derivative of pRS315 (Sikorski and Hieter, 1989), into which we had inserted the GAL promoter (Table II). The remaining 3.6 kb of the MHP1 coding region from the Sall site at
bp 752 to the 3' end (Sall site in the multicloning site of pBS) was in-
serted into the Sall site, and correct insertion was determined by restriction
digestion analyses. Heterozygous mhpl-Δ::kanr alleles Y561 were transformed with pMAC-MHP or pMAC-Pst (Table II), and Leu +
and G418-resistant cells were sporulated and dissected on glucose- and ga-
lactose-containing plates. In 8 out of 12 tetrads, two colonies of normal
size and two small colonies could be observed after 4–5 d of incubation at
30°C, with the normal-sized colonies unable to grow on G418. All small
colonies were Leu + and resistant to G418. Colonies growing from individ-
ual spores were followed by microscopic inspection for 7 d. Leu + and
G418-resistant cells from glucose plates were grown in liquid medium contain-
ing either glucose or galactose, and aliquots were taken after 4 and
20 h of growth and prepared for immunofluorescence microscopy.

Table II. Plasmids Used in This Study

| Plasmid          | Description                                                                 | Source or Reference |
|------------------|-----------------------------------------------------------------------------|---------------------|
| pADNS            | LEU2, 2p.                                                                    | Colicelli et al., 1989 |
| pADN-205         | 205K MAP cDNA cloned into pADNS polylinker                                   | This study          |
| pBS-Bam          | 3.7-kb MHP1 fragment, containing amino acid codons 219-1,398 and 3'-untranslated region, in pBS | This study          |
| pET-15b          | T7 expression vector containing His-tag                                       | Studier et al., 1990 |
| pET-C1188        | 820-bp MHP1 fragment, containing amino acid codons 1,188-1,398 and 3'-untranslated region, in pET | This study          |
| pET-N440         | 1,320-bp MHP1 fragment, containing amino acid codons 1-440, in pET          | This study          |
| pMAC-80          | GAL4 promoter cloned between SacI and BamHI site of polylinker of the pRS315 plasmid | This study          |
| pMAC-Bam         | 3.7-kb MHP1 fragment, containing amino acid codons 219-1,398 and 3'-untranslated region, in pMAC-80 | This study          |
| pMAC-MHP         | 4.2-kb MHP1 fragment, containing amino acid codons 1-1,398 and 3'-untranslated region, in pMAC-80 | This study          |
| pMAC-PST         | 2.5-kb MHP1 fragment, containing amino acid codons 561-1,398 and 3'-untranslated region, in pMAC-80 | This study          |
| pMACMyc-Pst      | Antigenic myc-tag, cloned into BamHI site of pMAC-Pst at 5'-end of MHP1 sequence | This study          |
| prS315           | LEU2, CEN6                                                                   | Sikorski and Hieter, 1989 |
| Yep24            | URA3, 2p.                                                                    | Botstein et al., 1979 |
| Yep-MHP          | 5.5 kb, containing MHP1 gene in Yep24                                         | This study          |

The Journal of Cell Biology, Volume 135, 1996 1326

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preceded by a putative CAAT box and a TATA box at positions −52 and −27, respectively (Fig. 2 A). The coding region of \textit{MHP1} is identical with ORF YJL042W on chromosome \textit{X} and is separated by 344 noncoding bp from \textit{NSP1} (Hurt, 1988) and ~1,300 bp from \textit{GYP6} (Strohm et al., 1993).

**DNA and Protein Sequence Analysis**

No significant homologies with other proteins were found when the EMBL and Swissprot databases (release May 1996) were searched with the \textit{MHP1} sequence. From amino acid residues 128–137, nine histidines are encoded that could have a function in metal binding. With sequence analysis programs for the detection of specific motifs, a possible EF-hand motif was identified between amino acid residues 615 and 629 (Fig. 2 A). When the \textit{MHP1} protein sequence was compared directly with protein sequences of known MAPs using PILEUP, MAP4, MAP2, tau, and Mhp1p could be aligned and showed a significant similarity in a region close to the COOH terminus, including residues 1,211–1,266 in Mhp1p (Fig. 2 B). Within this region, Mhp1p has between 23 and 32% identity or between 33 and 38% similarity with the sequences of the other MAPs. The highest similarity score was found with MAP4, and the lowest with tau. Included in this region is (from residues 1,234–1,266 of Mhp1p) one repeat of the AP motif common to tau, MAP2, and MAP4 (Lee et al., 1988; Lewis et al., 1988; Aizawa et al., 1990; Chapin and Bulinski, 1991; West et al., 1991). Some 50 residues downstream from the putative MT-binding motif, a nuclear localization signal (NLS) consensus sequence (Roberts, 1989) is present (Fig. 2 A).

\textit{MHP1} encodes a hydrophilic protein, with a calculated isolectric point (pI) of 6.9, but is composed of alternating basic and acidic regions within which the calculated pI values range from 10–12 and from 4.5–5, respectively (Fig. 1 B). The highest concentration of charged amino acids is observed within acidic and basic regions near the COOH-terminal end of Mhp1p. The NH2-terminal and the COOH-terminal regions of Mhp1p are very proline rich, typical of extended protein structures, but between residues 1,070 and 1,150 a propensity for the formation of α helices is predicted (Chou and Fasman, 1978; Garnier et al., 1978).

A total of 52 potential phosphorylation sites for the cAMP-dependent kinase (Edelmann et al., 1987), the calcium-phospholipid–dependent kinase (Gonzales et al., 1989), the Ca2+-calmodulin–dependent kinase (Pearson et al., 1985), and the casein kinase II (Diaz-Nido et al., 1988) are situated within the NH2-terminal two-thirds of Mhp1p, but only two potential sites are localized within the COOH-terminal domain (residues 1,201–1,398) of the protein (Figs. 1 B and 2 A).

**Identification of Mhp1p with Anti-MHP1 Antibodies**

To obtain Mhp1p-specific antisera, clone pET-C1188 encoding amino acids 1,188–1,398 was expressed in \textit{E. coli}. The antiserum was tested on Western blots and recognized the purified samples of C1188p and C1188p in protein lysates of induced bacterial cultures. On Western blots of whole cell extracts from \textit{S. cerevisiae}, a 200-kD protein as well as smaller proteins of ≤46 kD were consistently recognized by anti-MHP1 (Fig. 3 A; Y501 and Y502) when 20 μg of total protein extract was loaded.

The amount of anti-MHP1 reactive protein is reduced in \textit{mhp1+}/*mhp1-Δ1 heterozygous cells Y502 compared with mhp1+ cells Y501 (Fig. 3 A). When an equal amount of protein from Y509 cells overproducing Mhp1p from a multicopy plasmid was loaded, the 200-kD protein was substantially increased on Western blots, but also a protein of 150 kD could be detected. The full-length \textit{MHP1} gene expressed in \textit{E. coli} also migrates at 200 kD, supporting the conclusion that full-length Mhp1p is identical with the 200-kD band (data not shown). Less intense staining was observed for proteins of 230, 90, 60, and 46 kD. After longer exposure times, the same patterns of proteins were observed in the protein extracts from Y501 and Y502 cells.

To test the specificity of the antibody reaction, competition experiments were performed using purified C1188p. The addition of 1–10 μg/ml purified C1188p was tested, and 5 μg/ml was found to be sufficient to compete the antibody recognition of Mhp1p on Western blots. When 5 μg/ml purified C1188p was added during the antibody incubation, the 200-kD as well as the other proteins observed in Y509 cells could be completely competed with C1188p (Fig. 3 A, b). These proteins presumably contain the COOH terminus against which anti-MHP1 was raised, and are either isoforms of Mhp1p and/or degradation products, although the caveat remains that anti-MHP1 might recognize an epitope that is present on other proteins. The observed size of 200 kD for Mhp1p is considerably larger than its calculated molecular mass of 154 kD, but it could result from phosphorylation and abnormal migration as a result of the acidic portions of the protein.
Figure 2. DNA sequence and predicted protein sequence of MHP1. (A) DNA sequence of the MHP1 gene and predicted amino acid sequence are presented. Bold letters indicate the TATA and CAAT sequences, a polyadenylation signal, the first methionine initiation codon, and potential initiation codons of the mhp1-Δ2: URA3 mutation (see Fig. 6). Potentially phosphorylated amino acids are indicated by an asterisk below the residue. The putative EF hand motif (beginning at residue 615), the region homologous to MAP2, MAP4, and tau (from residues 1,211–1,266), and the potential NLS (residues 1,314–1,317) are underlined. Sequence data are available from EMBL/GenBank/DDBJ under accession number X84256. (B) Protein sequence alignment of AP sequences of different MAPs. Protein sequences of Mhp1p (1,211–1,266), human (233–288) and mouse (222–277) tau, mouse MAP2 (1,709–1,764), human (1,007–1,062) and mouse (980–1,035) MAP4, and bovine MAP U (944–977) are aligned. Amino acids, identical or similar (E/D, K/R, I/L/V) in at least three proteins, are boxed. The alignment corresponding to the AP repeat is bordered with asterisks.

A 200-kD protein plus a 180-kD protein were also reactive with the anti-205K MAP antiserum originally used for the screening of the Agt11 expression library (Fig. 3 B, lanes 1 and 2), but only the 200-kD protein copurified with yeast tubulin on a DEAE column (Bellaout et al., 1992). When heat-stable yeast proteins were tested with anti-MHP1 and anti-205K MAP, the 200-kD protein was also detected by both antisera (Fig. 3 B, lanes 3 and 4).

Mhp1p Has MT-binding Activity In Vitro

To determine whether Mhp1p binds to MTs in vitro, MT-binding and cosedimentation experiments were carried out. Total yeast proteins were incubated with preformed MTs and centrifuged, and supernatant and pellet fractions were analyzed on Western blots probed with anti-MHP1 (Fig. 4 A, a). The anti-MHP1-reactive 200-kD protein was coprecipitated with MTs and could be identified on Western blots. To localize the MT-binding activity within the MHP1 sequence, deletion-bearing clones were in vitro transcribed and translated, and aliquots of the in vitro translation reactions were tested in MT-binding assays. The COOH-terminal fragment C1188 coprecipitated with preformed MTs with >80% of the counts of the transla-
Figure 3. Identification of Mhp1p on Western blots. (A) Western blot of total yeast extracts from homozygous mhpl+ cells, Y501, heterozygous cells Y502, and cells overproducing Mhp1p, Y509. (a) 7.5% SDS gels were loaded with the equivalent of 50 μg of yeast cells, blotted and probed with affinity-purified anti-MHP1. (b) An identical Western blot as in a was prepared and used for an antigen competition assay using purified Cl188p. (B) Western blot of total yeast protein extracts probed with anti-Cl188p and anti-205K MAP antibodies. 10 μg total yeast protein extract (lane 1) and 5 μg of protein copurified with tubulin on a DEAE column (lane 2) were loaded and probed with anti-205K MAP. (Arrows) Position of the 200-kD protein that is very low in abundance but reproducibly recognized by anti-205K MAP; a representative Western blot out of five independent experiments is shown. Sizes of molecular weight standards are indicated on the right side.

Figure 4. Microtubule-binding assays. (A) MT-binding assays performed with preformed MTs from purified pig tubulin. (a) The binding of wild-type Mhp1p was tested using a total protein extract from Y509 cells incubated with preformed MTs and subsequent precipitation of the macromolecules. Aliquots of the supernatant (S) and pellet (P) fractions were analyzed by PAGE and tested on Western blots with anti-MHP1: PonceauS staining (left) and antibody staining (right). The 200-kD Mhp1p (arrow) is not very abundant but could be reproducibly detected in several independent experiments. (b) The MT-binding activity of different truncation products generated by in vitro transcription and translation of MHP1 deletion-bearing clones. MT-binding reactions were precipitated, and supernatant (S) and pellet (P) fractions were analyzed by PAGE. The autoradiography of the gel is shown. (B) Tubulin-binding assay using His-tagged Cl188p. Protein extracts from bacteria transformed with pET-N440 or pET-C1188 were loaded onto Ni-columns. Total protein extracts from yeast were applied to the column to test whether tubulin could be retained. Samples were analyzed by PAGE and Western blotting, with the Coomassie-stained gel shown in a and the Western blot probed with anti-tubulin in b. Gels were loaded with aliquots from load (lane 1), flow-throw (lane 2), wash (lane 3), and elution (lane 4) fractions. (C) The relative sizes of the translation products used in the MT-binding assays are shown schematically.
but not in N1300 may be needed for efficient binding or, alternatively, that the folding of a putative binding domain may be disturbed in the N1300 deletion.

To confirm by an independent approach that the COOH-terminal domain of Mhp1p contains a region that promotes MT-binding, we took advantage of the His-tag purification procedure of proteins expressed from the pET-15b vector in *E. coli*. We used the COOH-terminal peptide Cl188p, immobilized on Ni-columns, to identify proteins that interact with this region of Mhp1p. Ni-columns containing bound Cl188p were saturated with crude yeast protein extracts in MT-binding buffer, washed, and then eluted with the standard metal-chelation buffer. Several proteins bound to the Cl188p-column. A 48-kD protein was identified as tubulin on Western blots probed with antibodies against α- or β-tubulin (Fig. 4 B). In control experiments, crude yeast protein extracts were loaded on a Ni-column that was preabsorbed with either a protein extract from bacteria not expressing exogenous genes or a protein extract from bacteria transformed with the His-
tagged NH$_2$-terminal part of Mhp1p, including amino acid residues 1–440. In both cases, some proteins were bound, but the binding of tubulin or MTs was only observed after preabsorption with the bacterial extract producing C1188p (Fig. 4 B, right). Hence, the MT-binding activity was confirmed to reside principally in the COOH-terminal region of Mhp1p containing the AP-homologous sequence.

Immunolocalization of Mhp1p

Immunolocalization of Mhp1p with anti-MHP1 was carried out in Y501 and Y509 cells that carry MHP1 from a multicopy plasmid (Table II). Less intense staining was observed in Y501 cells than in Y509 cells that are presented (Fig. 5, A–C). Cytoplasmic MTs were decorated in ~60% of interphase cells (Fig. 5, A–C). In mitotic cells, short metaphase spindles as well as elongating spindles were stained (Fig. 5, D–F). Cytoplasmic staining was also observed in Y509 cells when an altered fixation and labeling procedure was applied (see Materials and Methods). Immunolocalization of Mhp1p in cells transformed with a myc-tagged MHP1, on the plasmid pMACmyc-Pst (Table II), confirmed the localization of Mhp1p on MTs (Fig. 5, G–I). No staining was observed with anti-myc antibodies in Y501 cells (Fig. 5, J–L), demonstrating that anti-myc staining was not due to fluorescence spill-over.

MHP1 Is Essential for Cell Growth

Since Mhp1p was shown to bind to MTs in vitro, it was of interest to determine whether MT structure or function was disturbed in cells lacking MHP1. We constructed a series of disruption alleles of MHP1 by replacing different regions of the ORF with marker genes (Fig. 6 A). Deletion of the entire coding region of MHP1 (mhp1–Δ6::kan$^+$) was performed in diploid Y501 cells, and tetrads of six independent transformants were dissected. The mhp1–Δ6::kan$^+$ mutation led to lethality, and the kan$^+$/kan$^-$ phenotype segregated 2:2 in all complete tetrads, the surviving spores being unable to grow on G418. The deletion of a COOH-terminal region from amino acids 818–1,210 also resulted in lethality in haploid cells (mhp1–Δ1::TRP1), indicating

![Figure 6. MHP1 disruption mutations. (A) Restriction maps of the different disruption alleles. The relative sites of the ATG initiation codon and the TGA stop codon are indicated. Note the presumed ATG translation start codon (see Fig. 2 A), which allows translation downstream from the URA3$^+$ insertion in the mhp1–Δ2::URA3 mutation, absent in the mhp1–Δ4::TRP1 mutation as well as in the mhp1–Δ5::HIS3 mutation. The region downstream from the URA3$^+$ insertion in the mhp1–Δ2::URA3 mutation was cloned into the inducible plasmid pMAC-80 and designated mhp1–Δ3. (B) Tetrad analyses of cells heterozygous for the alleles indicated in A.](image-url)
that the NH$_2$-terminal 818 amino acids encoded on the mhpl-$A_1$::TRP1 allele are not sufficient for viability.

Surprisingly, the interruption of the ORF after amino acid 558 by the insertion of the URA3$^+$ marker gene led to viable haploid cells (Fig. 6). Tetrad analysis of the heterozygous diploid strain $mhpl^+$/mhpl-$A_2$::URA3, Y105, resulted in four viable spores, the Ura$^+$/Ura$^-$ phenotype segregating 2:2 and the mhpl-$A_2$::URA3 haploid cells Y106 growing only slightly slower than the wild-type cells. When we analyzed the MHP1 mRNA in wild-type and mhpl-$A_2$::URA3 disrupted cells, we found that the mutant cells expressed two transcripts (Fig. 7 A), a 1.8-kb mRNA covering the MHP1 5' sequences and a 2.7-kb mRNA covering MHP1 3' sequences, while wild-type cells expressed a single 4.5-kb mRNA. This 4.5-kb transcript is not observed in the disrupted haploid mhpl-$A_2$::URA3 cells. A weak hybridization of a 2.8-kb RNA is visible in Y501 cells that might be a second MHP1 transcript or a nonspecific cross-hybridization. These results suggest that MHP1 function can be obtained when NH$_2$-terminal and COOH-terminal sequences of MHP1 are expressed from separate transcripts. Analysis of the proteins expressed in wild-type and mhpl-$A_2$::URA3 cells on Western blots (Fig. 7 B) shows that in wild-type cells, a 200-kD protein and bands of 150, 90, and 46 kD react with anti-MHP1, while in mhpl-$A_2$::URA3 haploid cells, no proteins >90 kD can be detected.

To further test the hypothesis that sequences downstream of the URA3$^+$ insertion were expressed spontaneously in the mhpl-$A_2$::URA3 allele, we generated deletion alleles mhpl-$A_4$::TRP1 (deletion of the first 613 amino acids) and mhpl-$A_5$::HIS3 (deletion of the region encoding amino acids 557–1,210) that do not contain the two presumed ATG translation initiation codons at positions 1792 and 1839, respectively. Both alleles proved to be lethal.

The progeny of mhpl-$A_1$::TRP1, mhpl-$A_4$::TRP1, mhpl-$A_5$::HIS3, or mhpl-$A_6$::kan$'$ cells could germinate and go through a few cell divisions, but they died at stages of 8 to 32 cells. The time needed for germination was variable for different spores and explains the size differences of the colonies (Fig. 6 B). The heterozygous cells showed a reduced growth rate in rich medium. Analysis of asynchronously growing Y502 and Y561 cells by DAPI and anti-tubulin staining showed 12% of the cells with short or long spindles and divided nuclei, compared with 2–3% rou-

Figure 7. Analysis of the mhpl-$A_2$ mutation. (A) Northern blots were probed with a 700-bp probe from the 5' end and a 600-bp probe from the 3' end of MHP1 (see Fig. 1 A). In mhpl-$A_2$::URA3 haploid cells Y106, the 5'-end probe and the 3'-end probe detected different mRNAs (arrows). In wild-type cells Y501, a 4.5-kb RNA was detected with both probes. (B) Western blot of total protein from mhpl-$A_2$::URA3 haploid cells (Y106) and diploid Y509 cells probed with anti-MHP1. In mhpl-$A_2$::URA3 haploid cells, anti-MHP1 reacts with a 90-kD protein compared with a 200-kD protein and smaller proteins in mhpl+$A_1$ cells, Y509.

Altered MT Phenotypes Induced by MHP1 Overexpression Mutations

Since the heterozygous deletion mutations of MHP1 showed a gene dosage effect, we investigated whether overexpressed MHP1, or deletion-bearing alleles of MHP1, would lead to a dominant phenotype. Y509 cells that express MHP1 constitutively from a multicopy plasmid (pYEP-MHP) exhibit a reduced growth rate (Fig. 8 A). The analysis of MT structures in asynchronously growing Y509 cells showed that the amount of cells with elongated spindles and divided nuclei was <1%, while the parental strain Y501 grown under similar conditions contained 2–3% of cells with short or long spindles and divided nuclei. In addition, Y509 cells showed an increased cell volume (150–200% of diploid wild-type cells), many abnormally long MT filaments emanating from one SPB, increased nuclear DNA staining, and small patches of DAPI-stained material observable in the cytoplasm (Fig. 8 B, a). In 6% of the cells, bud formation occurred but no spindle was formed. The increase in length and number of MTs in Y509 cells, as a consequence of increased expression of MHP1, is compatible with the role of Mhp1p as a positive regulator of MT stability.

A different phenotype could be induced by the overexpression of the COOH-terminal portion of Mhp1p. The region encoded on pMAC-Pst (Table II), designated mhpl-$A_3$, was transformed into homozygous mhpl$^+$ cells and into mhpl$^+$/mhpl-$A_1$ heterozygous cells, generating Y505 and Y506 cells, respectively. Cells were grown in medium containing either glucose, galactose, or raffinose (a sugar derepressing the GAL promoter and leading to less expression than galactose). The induction of mhpl-$A_3$ was monitored on Western blots, and the expression of a 90-kD protein, as had been identified in mhpl-$A_2$ cells under conditions of unstimulated expression (see Fig. 7 B), could be observed in Y505 and Y506 when cells were grown in galactose or raffinose (data not shown).

The expression of mhpl-$A_3$ provoked a negative effect on growth rate. While control cells Y502 reached saturation after ~24 h with a doubling time of 3 h in media containing galactose, for transformed Y505 and Y506 cells, longer times were required to reach saturation when grown in galactose-containing media, with generation times of 4.5 and 6 h, respectively. When media containing increasing amounts of galactose and decreasing amounts of glucose, or the partial inducer raffinose, were used, the growth rates of Y505 and Y506 cells were correlated with the galactose or raffinose content in the media.

To determine the consequence of the expression of mhpl-$A_3$ on MT structure and function, we analyzed asynchronously growing cultures by immunofluorescence mi-
Figure 8. Overexpression of the MHP1 gene and of the mhp1-Δ3 allele. (A) Growth rate of Y502, Y509, and Y506 cells was monitored by measuring the OD₆₀₀ (OD₆₀₀ at time 0 was 0.05) in selective medium containing 2% galactose. (B) Analysis of MT structure in Y509 (a and b) and Y506 (c and d) cells. The formation of long cytoplasmic MTs (arrowheads) can be observed in Y509 cells overexpressing MHP1. The accumulation of cells with short spindles (arrowheads) is shown in a typical field of Y506 cells overexpressing the mhp1-Δ3 allele (c and d). Nuclei are visualized by DAPI staining (a and c), and MTs by anti-tubulin staining (b and d). Bar, 4.5 μm.

cytoscopy with anti-tubulin staining. When Y505 and Y506 cells were grown in raffinose or galactose, their progression through mitosis was slowed down in various stages of mitosis. With the activation of the GAL10 promoter, an accumulation of cells with large buds, monopolar or short bipolar spindles, unseparated nuclei within the mother cells or at the bud neck, and cytoplasmic MTs extended into the daughter cells (but very sparse or not detectable) could be observed (Fig. 8 B), while the number of cells with separated nuclei and long spindles decreased with increased expression of mhp1-Δ3. The percentage of cells exhibiting this phenotype was correlated with the level of mhp1-Δ3 expression (higher when cells were grown in galactose than when grown in raffinose) and with the time after induction (Table III). After 20 h of induction, 60% of the Y505 cells were large budded and had short or monopolar spindles formed. Only 5% of the cells were large budded with elongated spindles and nuclear migration that had proceeded into the bud. In the heterozygous Y506 cells, we observed 78% large-budded cells of which 73% had short or monopolar spindles. The comparison of the cell cycle arrest phenotype of Y506 cells after different times of mhp1-Δ3 induction demonstrates that a short bipolar spindle can be formed, but spindle elongation might
be hampered. In control cells transformed with pMAC-MHP overexpressing the entire MHP1 gene or transformed with pMAC-80 (Tables I and II), no such phenotypes were observed. The NH₂-terminal 561 amino acid residues of MHP1 therefore appear to be required for the function of Mhplp during progression through anaphase.

**MHP1 Null Allele Complemented with MHP1 and with mhp1-Δ3**

To further characterize the null phenotype of the MHP1 deletion alleles, we have performed rescue experiments with MHP1 on plasmids under the control of inducible promoters. pMAC-MHP contains full-length MHP1 under the GAL10 promoter, while pMAC-Pst contains the 3' region of the gene designated mhp1-Δ3. With both plasmids, the lethal phenotype of the haploid mhp1-Δ6::kan' cells could be partially rescued, giving rise to small colonies (Fig. 9 A). The fact that mhp1-Δ3 could also partially compensate for the null mutation of MHP1 is consistent with the finding that the insertion mutation mhp1-Δ2 is viable, and confirms that the expression of the region encoding amino acids 560-1,398 is sufficient for vegetative growth. With both plasmids, rescue could only be observed when dissections were carried out on plates containing glucose, and tetrads dissected on plates containing galactose gave rise to only two colonies of normal size. Microscopic inspection of the spores on galactose-containing plates, at intervals of 1 d, showed that all cells sporulated and divided initially with the same doubling times until they reached the ~16-32 cell stage. Two colonies slowed down in their doubling time and finally stopped growing. We presume that the overproduction of Mhplp under the GAL10 promoter was toxic to the cells, and that leaky expression of MHP1 from the GAL10 promoter allowed reduced transcription and translation of MHP1 and mhp1-Δ3 in the small-sized colonies growing on glucose plates.

**MHP1 Expression Is Required for Sufficient Formation of MTs**

To examine the MT phenotype in the MHP1 null allele, we have generated haploid mhp1-Δ6 cells Y564, complemented with inducible MHP1 on the plasmid pMAC-MHP. When Y564 cells were grown under noninduced conditions, Mhplp was below detectable levels and tetrads dissected on plates containing glucose and tetrads dissected on plates containing galactose gave rise to only two colonies of normal size. Microscopic inspection of the spores on galactose-containing plates, at intervals of 1 d, showed that all cells sporulated and divided initially with the same doubling times until they reached the ~16-32 cell stage. Two colonies slowed down in their doubling time and finally stopped growing. We presume that the overproduction of Mhplp under the GAL10 promoter was toxic to the cells, and that leaky expression of MHP1 from the GAL10 promoter allowed reduced transcription and translation of MHP1 and mhp1-Δ3 in the small-sized colonies growing on glucose plates.

**Table III. Cell Cycle Arrest of mhp1-Δ3 Mutant**

| Cell line | Time of induction* | Unbudded | Mononucleate | Monopolar spindle | Large bud | Short spindle | Elongated spindle |
|-----------|--------------------|----------|--------------|-----------------|----------|---------------|------------------|
|           | h                  |          |              |                 | % (n)    |               |                  |
| Y501      | 0                  | 97 (118) | 0            | 0               | 0        | 3 (4)         |
| Y501      | 4                  | 96 (205) | 0            | 2 (4)           | 2 (4)    |
| Y501      | 12                 | 96 (160) | 0            | 1 (2)           | 3 (5)    |
| Y501      | 20                 | 97 (174) | 0            | 1 (2)           | 2 (4)    |
| Y506      | 0                  | 91 (153) | 0            | 2 (3)           | 7 (12)   |
| Y506      | 4                  | 45 (87)  | 14 (25)      | 0               | 14 (13)  |
| Y506      | 12                 | 39 (37)  | 14 (13)      | 24 (46)         | 33 (31)  |
| Y506      | 20                 | 22 (17)  | 25 (19)      | 48 (35)         | 14 (13)  |

*Time of induction indicates hours of growth in galactose-containing medium. Numbers in other columns correspond to percentages and numbers of cells observed with the indicated phenotype.
tion, no rescue was observed. To test possible effects of the pADNS plasmid, control experiments were also performed with the pADNS plasmid alone, but no rescue was observed. These results indicate that the partial rescue of the mhp1-Δ1 and mhp1-Δ6 mutations depends specifically on the expression of 205K MAP. From the Trp'Leu' haploid cells, strain Y504 was generated and growth was analyzed under conditions that permitted or suppressed the expression of 205K MAP. After depriving the cells of glucose to silence the ADH promoter, no growth was observed for Y504 cells, while Y502 cells could grow with galactose instead of glucose as a carbon source, although initially at a slower rate (Fig. 10 A). We conclude that the viability of haploid mhp1-Δ1 cells is partially restored by the expression of the 205K MAP, and that 205K MAP and Mhp1p share some functional similarity.

Analysis of MT structures in Y504 cells displayed an abnormal increase of cytoplasmic MTs in number and length. Y504 cells also showed an accumulation of cells with elongated (25%) and short spindles (15%). The elongated spindles contained thicker bundles of MTs than comparable wild-type cells, and the number and length of cytoplasmic MTs that emanate from the SPBs were increased. The axis of the mitotic spindle was altered in some cases, and multinucleated cells with multiple spindles could be observed (Fig. 10 B). In addition, the nuclear DNA staining appeared diffuse or fragmented and in some cases, no nuclear staining could be observed. Similar phenotypes were observed in haploid mhp1-Δ6 cells, Y566, rescued with pADN-205. No such aberrant MT structures were observed in the diploid Y503 and Y565 cells, which suggests that the 205K MAP phenotype is recessive to the Mhp1p phenotype when both proteins are coexpressed, and that the phenotype observed in rescued cells is not only due to the expression of the exogenous 205K MAP, but also to the lack of a functional MHP1.

Discussion

MHP1 encodes a novel protein that has MT-binding activ-
Figure 10. Partial rescue of the mhp1-A1 mutation by the Drosophila 205K MAP. (A) Heterozygous Y503 and Y565 cells that carry the pADN-205 plasmid were dissected, and they gave rise to small colonies of rescued mhp1-A1 or mhp1-A6 haploid cells, termed Y504 and Y566, respectively. Growth curves of Y502 and rescued Y504 cells in glucose and galactose medium are shown, but they were similar for Y565 and Y566 cells. Cells were grown overnight and diluted to an OD600 of 0.05 so as to start with equivalent amounts of cells, and growth was monitored. No growth was observed for Y504 cells grown in galactose. (Inset) Typical tetrad of heterozygous mhp1/mhp1-A1 cells transformed with the pADN-205 plasmid and of cells transformed with pADNS. (B) Analysis of MT structures in Y504 cells complemented with the Drosophila 205K MAP. DAPI staining is shown in a, and anti-tubulin staining is shown in b. Abnormally long and thick cytoplasmic MTs and mitotic MTs are indicated with small arrowheads, and multinucleated cells with multiple spindles are indicated with larger arrowheads. Bar, 4.5 μm.

It is in vitro. Its subcellular localization on MTs and the phenotype of aberrant MT structures in mutant cells suggest a function for Mhp1p in MT organization, particularly during mitosis. Mhp1p shares a short region of sequence homology with MAP2, MAP4, and tau, but not with 205K MAP. However, a polyclonal antibody directed against the Drosophila 205K MAP, which we used for expression cloning of MHP1, cross-reacts with Mhp1p. This antibody was previously shown to recognize mouse and human MAP4 proteins (West, R.R., K.M. Tenbarge, M. Gorman, L.S.B. Goldstein, and J.B. Olmsted, 1988. J. Cell Biol. 107:460a), but intriguingly, neither the mouse nor the human MAP4 species shares significant sequence homologies with the Drosophila 205K MAP. It is therefore likely that the anti-205K MAP antibody recognizes conserved MAP-specific epitopes, and that Mhp1p, 205K MAP, and MAP4 are structurally related proteins resulting from a homology that cannot be detected at the level of primary sequence comparison.

Mhp1p possesses structural features in common with
other known MAPs. Its estimated size on SDS gels is 50% larger than its calculated molecular weight, an observation made for other highly charged MAPs (Irminger-Finger et al., 1990; West et al., 1991). It is possible, however, that the 230-, 200-, and 150-kD proteins that are recognized by anti-MHP1 antibodies are isoforms of Mhp1p generated by posttranslational modification. Mhp1p is highly charged and the putative MT-binding domain is followed by an acidic COOH-terminal region, which is similar to the organization of tau and 205K MAP (Lee et al., 1988; Irminger-Finger et al., 1990). In contrast with MAP2, tau, and 205K MAP consisting of an acidic NH2-terminal portion, a basic domain containing the MT-binding region, and in the case of tau and 205K MAP an acidic COOH-terminal region, Mhp1p contains alternating acidic and basic regions, and its acidity gradually increases from the NH2-terminal to the COOH-terminal end. However, the density of potential phosphorylation sites gradually decreases from the NH2-terminal to the COOH-terminal end (Fig. 1 B) and could lead to more negative charges within the NH2-terminal half of the protein.

Phosphorylation has been characterized as an important mechanism for the regulation of the interaction of MAPs with MTs. In contrast to tau, MAP2, and 205K MAP, the potential kinase target sites on the amino acid sequence of Mhp1p localize within the NH2-terminal two-thirds of the protein with fewer sites in the COOH-terminal portion containing the putative MT-binding region. It is therefore possible that MT binding in Mhp1p is less influenced by phosphorylation than by other functions residing in the NH2-terminal portion of Mhp1p. An important function for the NH2-terminal domain of Mhp1p is also suggested by the phenotype induced by the mhp1Δ3 allele (Fig. 9).

We have demonstrated that the region of Mhp1p sufficient and essential for MT binding is located within the COOH-terminal 210 amino acid residues of Mhp1p. The localization of the AP homologous sequence motif within this region is consistent with its role in MT binding. However, the region found to be homologous to the AP motif is less basic than other AP sequences, although flanked by sequences containing basic residues (Figs. 1 B and 2). It is conceivable that these adjacent basic sequences participate in MT binding in Mhp1p or that the binding is achieved with a predominantly basic charge, as was reported for binding motifs of other MAPs (Hemphill et al., 1992). In support of this idea is the finding that an interrepeat region of the AP repeats in tau has higher MT affinity than a single repeat (Goode and Feinstein, 1994). A significant contribution of the sequences outside the AP motif, which was also reported for MAP4 (Olson et al., 1995), might explain why for Mhp1p a single repeat could be sufficient for MT binding. The reduced binding activity of the deletion N1300, containing the AP-homologous sequence but not the entire COOH terminus of Mhp1p, could be due to a participation of sequences close to the COOH terminus to MT binding. It is also possible that the protein folding of the deletion N1300 is altered when the COOH terminus is missing.

The generation of Mhp1p-specific antibodies permitted the identification of Mhp1p as a 200-kD protein on Western blots comigrating with bacterially produced Mhp1p. Minor bands of 150, 90, and 60 kD also react with anti-MHP1 and are increased in YS09 cells overproducing Mhp1p. These proteins could be degradation products, isoforms, or products of differential transcription and translation. The former possibility would be consistent with the expression of a 2.8-kb mRNA observed in wildtype cells, which hybridized to the 3'-end probe of MHP1 (Fig. 7 A). The differential transcription and/or translation could be a regulatory mechanism for the expression of MHP1 gene products encoding different functions. The localization of the anti-MHP1–reactive epitope to cytoplasmic and nuclear MTs is consistent with MT-binding experiments. However, since anti-MHP1 was generated against the COOH-terminal peptide C1185p, we cannot exclude that the staining observed in immunofluorescence analyses is due to cross-reaction with isoforms of Mhp1p <200 kD, as observed on Western blots.

Indirect evidence that differential transcription and translation could lead to functional products comes from the viable mhp1Δ2 mutation, derived from an insertion in the NH2-terminal half of the protein-coding region, that disrupts the ORF after residue 557. It seems likely that mhp1Δ2 mutant cells express both NH2- and COOH-terminal truncation products of MHP1, since two separate transcripts are detected. The translation of a COOH-terminal 90-kD protein, detected with anti-MHP antibodies in mhp1Δ2 cells (Fig. 7 B), could be initiated at one of the candidate methionine initiation codons located at positions 598 and 612 (Fig. 2 A). The mhp1Δ4 mutation deleting coding sequences from residues 1–613, including the two potential translation initiation codons, is lethal in haploid cells. Also, the mhp1Δ5 mutation, deleting residues downstream of the URA3* insertion site in the mhp1Δ2 mutation, results in lethality in haploid cells, supporting the conclusion that transcription initiation can occur at positions 598 or 612. Viability of the null mutant could also be restored with plasmid pMAC-Pst (Fig. 9 A) containing the region downstream from amino acid position 561, demonstrating that the COOH-terminal region is sufficient for viability without the NH2-terminal portion of the gene expressed separately.

The results of gene disruption experiments and in vitro MT-binding experiments suggest that Mhp1p is organized in a COOH-terminal domain (residues 821–1,398) essential for MT binding, and an NH2-terminal domain (residues 1–561) important for proper function of Mhp1p, which is absent in the mhp1Δ3 mutation. Deleting the region of MHP1 that includes the domain essential for MT binding leads to lethality in haploid cells (Fig. 6), but the NH2-terminal deletion allele mhp1Δ3 can partially compensate lethality of the complete null allele, demonstrating that mhp1Δ3 provides sufficient MHP1 function for viability (Fig. 9). The overexpression of the mhp1Δ3 allele leads to reduced growth with a specific phenotype of heterogeneously arrested cells at different stages of mitosis mostly before the onset of anaphase. The number of mutant cells is correlated with the expression level of the mutant protein. In contrast with mutants effecting SPB duplication, such as kar1 and cdc31 (Vallen et al., 1994), mhp1Δ3 cells arrest at various times of spindle formation, and it is the number of cells with elongated spindles and divided nuclei that becomes more reduced when the expression of mhp1Δ3 is increased (Table III). The hetero-
A different phenotype was observed with the overexpression of the entire MHP1. The additional production of Mhp1p results in increased formation and/or stabilization of cytoplasmic MTs. It is possible that the protein encoded on mhp1Δ-Δ3 is missing a signal that retains it in the cytoplasm or that the truncated form is more efficiently transported to the nucleus interacting with nuclear MTs. Reports from a number of laboratories demonstrate that the interaction of MAP4-type proteins with MTs is regulated during the cell cycle by phosphorylation by specific kinases (Aizawa et al., 1991; Vandré et al., 1991; Ookata et al., 1995). Therefore, a possible explanation for the cell cycle block observed in mhp1Δ-Δ3 cells and the phenotype induced by overexpression of MHP1 could be deregulated phosphorylation. Increased levels of Mhp1p or the mutant protein encoded on mhp1Δ-Δ3 could expend the capacities of different specific kinases that regulate the interaction of Mhp1p with MTs. Mutational analyses and biochemical approaches should provide information on the function of the NH2-terminal domain of Mhp1p and help to identify regulatory factors that interact with Mhp1p, such as kinases.

All mutations induced by gene disruption, overexpression of the entire MHP1, or the COOH-terminal part of MHP1 affect the formation of MTs and the function of MTs during the progression through cell cycle. A reduced gene dosage in heterozygous mhp1+/-mhp1Δ-Δ1:TRP1 and mhp1+/-mhp1Δ-Δ1:kanR cells appears to affect cells at late mitosis, since in an exponentially growing culture, the number of cells at this stage is elevated. In cells overexpressing the entire MHP1 gene, we observe long cytoplasmic MTs, indicating that an increased concentration of Mhp1p promotes MT polymerization and/or increases MT stability. In complete null mutants Y564, expressing minimal amounts of Mhp1p from the “silenced” GAL10 promoter, the formation of MTs is reduced. In Y504 and Y566 cells rescued by the expression of 205K MAP, an excess of aberrant MT structures can be observed as a result of the MT-stabilizing effect of overexpressed 205K MAP. A similar phenotype had been observed in the fission yeast when Mhp1p was overexpressed (Olmsted, J.B., and J.R. McIntosh. 1994. Mol. Biol. Cell. 5:169a). These data suggest that Mhp1p regulates the fine tuning of MT stability that cannot be achieved by the expression of a truncated form of Mhp1p or by a surrogate MAP from another species. Future research will focus on the timing and nature of the essential interactions of Mhp1p with MTs by using mhp1Δ-Δ6 haploid cells complemented by mutagenized inducible MHP1 on expression plasmids. Overall, the system described here should also permit the determination of functions generally possessed by MAPs by domain-swapping experiments between Mhp1p and other MAPs, and by the testing of hybrid proteins in MHP1 null mutants.

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