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Yeast spindle pole body duplication gene *MPS1* encodes an essential dual specificity protein kinase

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The *MPS1* gene has been previously identified by a mutant allele that shows defects in spindle pole body (SPB) duplication and cell cycle control. The SPB is the centrosome-equivalent organelle in the yeast *Saccharomyces cerevisiae*, and it nucleates all the microtubules in the cell. We report the isolation of the *MPS1* gene, which encodes an essential protein kinase homolog. The *MPS1* open reading frame has been fused to those that encode the LexA protein or the GST protein and both of these constructs function in yeast. The fusion proteins have been affinity-purified from yeast extracts and the GST chimeric protein has been found to be a phosphoprotein. Both proteins have been used to demonstrate intrinsic *in vitro* protein kinase activity of Mps1p against exogenous substrates and itself (autophosphorylation). A mutation predicted to abolish kinase function not only eliminates *in vitro* protein kinase activity, but also behaves like a null mutation *in vivo*, suggesting that kinase activity contributes to the essential function of the protein. Phosphoamino acid analysis of substrates phosphorylated by Mps1p indicates that this kinase can phosphorylate serine, threonine and tyrosine residues, identifying Mps1p as a dual specificity protein kinase.
Key words: mitosis/*MPS1*/protein kinase/spindle pole body/yeast

Introduction

The yeast *Saccharomyces cerevisiae* has as its sole microtubule organizing center the spindle pole body (SPB). This organelle serves as the yeast equivalent to the centrosome of mammalian cells. Like centrosomes, the SPB is duplicated during a precise interval in the cell cycle to yield the two poles of the mitotic spindle. This process occurs during the G_1 interval of the *S.cerevisiae* cell cycle and is coordinated with other cell cycle events. SPB duplication has been characterized cytologically, and several mutants have been identified that are specifically defective in SPB duplication (reviewed by Winey and Byers, 1993). As a class, the mutants that fail in SPB duplication transit G_1 and S phases and give rise to cells with monopolar spindles. Three distinct aberrant SPB morphologies have been detected in mutants, and these morphologies appear to indicate which step of SPB duplication has failed in a given mutant (Winey *et al.*, 1991). Generally, mutants defective in SPB duplication exhibit a transient G_2 arrest in the cell cycle. At the non-permissive temperature, the mutants arrest as large budded cells, with post-S phase DNA content and monopolar spindles. The exception to this behavior is the *mps1-1* mutant, which uniquely identifies a post-mating factor arrest step in SPB duplication (Winey *et al.*, 1991). At the non-permissive temperature, *mps1-1* cells exhibit no pause in the cell cycle and commit aberrant DNA segregation and cytokinesis. Indeed, *MPS1* has been implicated in a mitotic checkpoint that arrests the cell cycle in response to failed SPB duplication (E.Weiss and M.Winey, in preparation).

Molecular analyses of genes involved in *S.cerevisiae* SPB duplication have identified several types of proteins that are involved in the control and/or execution of this process. The *CDC31* gene product is a small Ca^{2+} binding protein of the calmodulin family that has been shown to be a SPB component (Sprang *et al.*, 1993; Biggins and Rose, 1994). The *KAR1* gene product is a novel protein of 53 kDa, and may also be a SPB component (Rose and Fink, 1987; Vallen *et al.*, 1992). Mutant alleles of *KAR1* show genetic interactions with *CDC31* and its mutant alleles (Vallen *et al.*, 1994), and Kar1p binds Cdc31p *in vitro* (Biggins and Rose, 1994). Other SPB components include the product of the *NUF1/SPC110* gene, which is a coiled-coil protein (Mirzayan *et al.*, 1992; Kilmartin *et al.*, 1993), and calmodulin, which binds the *NUF1/SPC110* gene product (Geiser *et al.*, 1993). Finally, the *NDC1* gene is required for a late step in SPB duplication and it encodes a hydrophobic protein that appears to be a constituent of the nuclear envelope (Winey *et al.*, 1993).

We report here the isolation and characterization of the *MPS1* gene and its gene product. The gene encodes a 764 amino acid protein with significant homology to protein kinases. *MPS1* is the same gene as the recently reported *RPK1* kinase homolog (Poch *et al.*, 1994). These workers found *MPS1* as an interesting open reading frame (ORF) adjacent to the *ACT2* gene that they had previously identified (Schwob and Martin, 1992). Poch *et al.* (1994) report that *MPS1* is essential. They found that *MPS1* mRNA levels were constant in mitotic cells, decreased in mating factor-treated cells, and increased in meiotic cells. They also suggest that *MPS1* may encode a dual specificity protein kinase, based on significant amino acid sequence homology in the kinase domain between *MPS1* and the mammalian dual specificity protein kinases PYT/TTK (Mills *et al.*, 1992; Lindberg *et al.*, 1993) and *esk* (Douville *et al.*, 1992). We show that the *MPS1* gene product

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(Mps1p) does indeed have protein kinase activity *in vitro*, and that the kinase phosphorylates serine, threonine and tyrosine residues. Furthermore, a GST–Mps1 fusion protein is phosphorylated in yeast, suggesting that Mps1p is a phosphoprotein *in vivo*. We have implicated an essential, dual specificity protein kinase in yeast SPB duplication and cell cycle control.

Results

Isolation of the MPS1 gene

MPS1 was isolated from the yeast genomic library constructed by Rose *et al.* (1987) in the centromeric plasmid, YCp50. One plasmid that complemented the *mps1-1* mutation was isolated independently nine times, and all nine isolates could complement *mps1-1* upon reintroduction into yeast. The restriction map of the ~9 kb insert in this plasmid (YCpMPS1) is shown in Figure 1. The region required for complementation of *mps1-1* was identified by deleting regions of the genomic insert in YCpMPS1 as shown in Figure 1. This analysis showed that only a 3.5 kb fragment from one end of the insert was necessary for complementation of *mps1-1*. This data is consistent with two 'frameshift' mutations made by filling in and ligating at unique restriction sites within the complementing fragment (Figure 1).

Various methods were used to demonstrate that the complementing fragment in YCpMPS1 contains the bona fide *MPS1* gene. First, the *Eco*RI–*Sal*I fragment containing the putative *MPS1* gene was subcloned from YCpMPS1 to pRS306, an integrative plasmid (Sikorski and Hieter, 1989). The resulting plasmid (YIpMPS1) was integrated in the genome of yeast strain Wx257-5c (Table I). Transformants were crossed to the *mps1-1* mutant strain Wx241-12b (Table I). The integrated marker, *URA3*, on YIpMPS1 showed no recombination with *mps1-1* in 25 tetrads, demonstrating that the cloned fragment and the mutation are linked. Furthermore, the *URA3* integrant showed linkage to *trp*, as predicted by the previously reported map position of *mps1* (10 cM distal to *trp1*; Winey *et al.*, 1991). The second line of evidence that the bona fide *MPS1* gene had been cloned came from mapping the complementing DNA of YCpMPS1 to the yeast physical map. An *Eco*RV fragment was used to probe the overlapping set of λ phage used to construct the yeast physical map as in Riles *et al.* (1993) (see Materials and methods). The probe hybridized to the λ clone Olson #3686 (ATCC #70420) and to the overlapping cosmid Olson #9533 (ATCC #70974), which place this DNA in MERG unit 0964 on the left arm of chromosome IV in the vicinity of the *mps1-1* mutation. Finally, plasmid-to-chromosome conversion has demonstrated that the *mps1-1* mutation maps to the ORF identified as the *MPS1* gene (discussed below), and a null allele of this ORF fails to complement the *mps1-1* mutation. The finding that the recently reported gene *RPK1* (Poch *et al.*, 1994) is the same as *MPS1*, suggests that the linked genes *PRP9* and *ACT2* (Schwob and Martin, 1992) that Poch *et al.* (1994) used to find *MPS1* must also be centromere linked on chromosome IV*L*.

Characterization of the MPS1 gene

The minimal complementing region of YCpMPS1 was further characterized by Southern analysis. An *Eco*RV fragment internal to the *MPS1* ORF was labeled and used to probe genomic yeast DNA that had been cut with various restriction enzymes, and only one fragment was detected in each lane, except for two digests where the restriction enzymes are known to cut in the segment used as a probe (data not shown). This result is consistent with the physical mapping of the *MPS1* gene reported above, which identified only one locale in the genome. These results suggest that the *MPS1* gene is unique in *S.cerevisiae*.

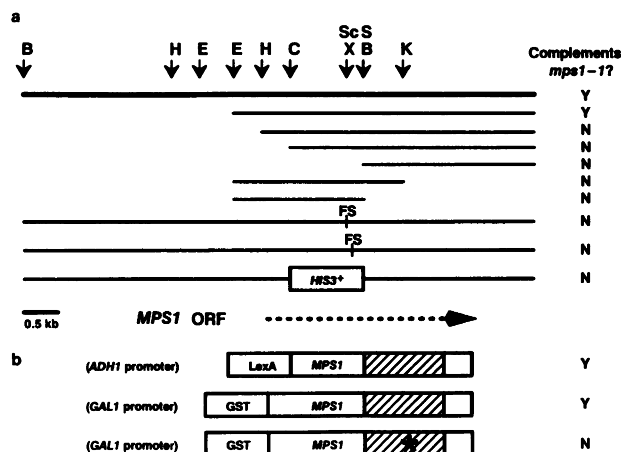

Fig. 1. Restriction map and complementation data for the identification of the *MPS1* gene (**a**) and the fusion protein constructs (**b**) used for analysis of protein kinase activity. The top line (a) depicts the original clone of *MPS1*, with restriction sites B (*Bam*HI), H (*Hin*dIII), E (*Eco*RI), C (*Cla*I), X (*Xba*I), K (*Kpn*I), Sc (*Sac*I) and S (*Sph*I). The lower lines show subclones and mutants including frameshift mutants (FS) and the insertion/deletion null allele used ($HIS3^+$, see Materials and methods). The fusion protein constructs (b) and the *MPS1* ORF are aligned with the above restriction map. The constructs are described in Materials and methods. The kinase domain in *MPS1* is shown as the cross-hatched region, and the asterisk on the bottom construct marks the position of the 'kinase-dead' mutation (see Materials and methods).

The strategy used for DNA sequencing involved cloning restriction fragments into phage M13 and the use of custom oligonucleotide primers. DNA sequence analysis of the complementing insert in YCpMPS1 revealed a single long ORF of 2292 nucleotides with the potential to encode a protein of 764 amino acids with a calculated mol. wt of 87 kDa. The derived amino acid sequence of Mps1p that we determined has one amino acid change in the N-terminus of the protein from the sequence reported by Poch *et al.* (1994). This discrepancy arises from a difference in the DNA sequence at position +436 (T→G), resulting in amino acid number 146 (a serine) changing to an alanine in our sequence. This change has been noted in the GenBank file (Accession number L08909) for *MPS1/RPK1*.

Comparison of the derived amino acid sequence of the *MPS1* gene with protein sequences in various databases revealed significant similarity between the C-terminal region of the putative *MPS1* gene product and members of the protein kinase superfamily. Closer examination revealed that amino acids 447–704 of Mps1p could be aligned with the consensus sequences for the 11 subdomains of a protein kinase domain as defined by Hanks *et al.* (1988), and that Mps1p contains all consensus amino acids in each subdomain (Figure 2). As noted by Poch *et al.* (1994), the protein kinases most similar to *MPS1*

Table I. Yeast strains

Name	Genotype	Source
Wx241-12b	α, *mps1-1, ura3-52, his3Δ200*	this study
Wx257-5c	**a**, *ura3-52, his3Δ200, leu3-3,112, trp1Δ1*	this study
D8bx5cA	**a**/α, *ura3-52/ura3-52, his3Δ200/his3Δ200, leu2-3,112/leu2-3,112, trp1Δ1/ trp1Δ1*	this study
EGY48	α, *his3, trp1, lex::leu2-3a, ura3-52,*	R.Brent
BJ2168	**a**, *leu2, pep4-3, prb1-1122, prc1-407, trp1, ura3-52*	E.W.Jones

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Domains  :        I                 II                  III                   IV
SER/THR  :  g G        V         a K                   E
MPS1     : LGRGGSSRVYKVKGSGNRVYALKRVSFDAFDDSSIDGFKGEIELL..EIERPKRVIQLLDYEMGDGLLY
esk      : I-S----K-FQ-LNEKKQIN-I-Y-NLEDA-SQT-ESYRN--AF-nkLQQHSDKI-R-Y---ITEQYI-
PYT/TTK  : .-.----.-..-.......--.-.-...E.-...L-....--.Y-.........-.-.---..-...-

         :   V                                        VI                 VII
         :   E                              h       hrD K  N          k  Dfg
MPS1     : LIMECGDHDLSQILNQRSGMPLDFNFVRFYTKEMLLCIKVVHDAGIVHSDLKPANFVLVKGILKIIDFG
esk      : MV----NI--NSW-KKKK::SINPWERKS-W-N--EAVHII-QH-------------I-D-M--L----
PYT/TTK  : ..----.--..-...::.-.......-.-.--.....-..-----------L.-.-.--.----
                                                                            A
         :                     VIII                             IX
         :                      pE                           D w  g
MPS1     : IANAVPEHTVNIYRETQIGTPNYMAPEALVAMNYTQNSENQHEGNKWKVGRPSDMWSCGCIIYQMIYGK
esk      : ---QMQPD-TS-VKDS-V--V-------IRD-::SSSR--:SKI:RT--SPR--V--L---L-Y-T--R
PYT/TTK  : ---.....-..V....-.--.---P---.K.-::....--:G.S:-S-I..K--.--.---.-.-.---

         :               X                                XI
         :                                              R        h
MPS1     : PPYGSFQGQ:NRLLAIMNPDVKIPFPEHTSNNEKIPKSAIELMKACLYRNPDKRWTVDKVLSSTF
esk      : T-FQHIIN-VSK-H--I--AHE-E--::::::-ISE-DLRDVL-C--V---KE-ISIPEL-THPY
PYT/TTK  : .-..Q...-I..-.--.D-N..-.--:::::D.P.-..Q...-.--K-D-.Q-......-A...
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Fig. 2. Alignment of the kinase domains of *MPS1* and of the mammalian genes PYT/TTK (Mills *et al.*, 1992; Lindberg *et al.*, 1993) and *esk* (Douville *et al.*, 1992). The *MPS1* amino acid sequence is shown, beginning at amino acid number 446. Dashes in the *esk* and PYT/TTK sequences indicate positions of identity with the *MPS1* sequence, and a period (.) in the *esk* sequence is a position where it is identical to PYT/TTK, but differs from *MPS1*. The two mammalian kinases are virtually identical to one another, exhibiting 96% identity over their entire length. Approximately 45% of the residues in the *MPS1* kinase domain are identical to the mammalian kinases, and 67% are similar when allowing for conservative amino acid changes. The subdomains (domains I–XI) of the kinase domain were defined by Hanks *et al.* (1988). The conserved amino acids in serine/threonine kinases (SER/THR) are after Lindberg *et al.* (1992), where the upper case amino acids are invariant, and the amino acids in lower case are nearly invariant. The italicized E in subdomain V is not part of the SER/THR consensus, but a feature of dual specificity kinases (Lindberg *et al.*, 1992). The position of the 'kinase-dead' mutation in subdomain VII is shown as the underlined A at the position of the D it replaced.

are the dual specificity kinases *esk* from mice (Douville *et al.*, 1992) and PYT/TTK from humans (Mills *et al.*, 1992; Lindberg *et al.*, 1993) (Figure 2). The product of the yeast *MPS1* gene and the mammalian gene products are similarly organized, each with the kinase domain in the C-terminal half of the protein. However, the N-terminal region of *MPS1* and the 60 amino acids C-terminal of the kinase domain show no similarity to PYT/TTK, *esk*, or any sequence in the available databases.

MPS1 is an essential gene

A deletion/insertion mutation in the *MPS1* ORF was created to determine if *MPS1* is an essential gene for yeast as suggested by the conditional-lethal *mps1-1* allele (Winey *et al.*, 1991). A *Cla*I–*Bam*HI fragment was removed from the *MPS1* gene and replaced with a *Cla*I–*Bam*HI fragment carrying the yeast $HIS3^+$ gene (Struhl, 1985; see Materials and methods). A plasmid carrying the *mps1Δ::HIS3* allele is not able to complement the *mps1-1* mutation, indicating that the *MPS1* gene function had been disrupted by the insertion/deletion mutant. An *mps1Δ::HIS3*-containing fragment was used to transform a diploid yeast strain (D8bx5cA, Table I) to histidine prototrophy in a one-step gene transplacement (Rothstein, 1991). The correct transplacement of *mps1Δ::HIS3* into the *MPS1* locus in four separate transformants was confirmed by a polymerase chain reaction (PCR) protocol (see Materials and methods). Tetrads from each of the four sporulated diploids gave rise to only two viable segregants, and all viable segregants were histidine auxotrophs (n = 37). These results indicate that the transformed diploids segregate a lethal mutation that is linked to the *HIS3* gene, and presumably the lethality is due to the loss of *MPS1* function. The *MPS1* gene on a plasmid (YCpMPS1) was found to rescue the lethal mutant, and the plasmid is required for mitotic viability as these cells will not grow on 5-fluoroorotic acid (5-FOA) plates which select against the *URA3* marker on YCpMPS1 (Boeke *et al.*, 1987). A final demonstration that the null allele disrupts the *MPS1* gene involved selecting diploids from a cell (*mps1-1, his3, LEU2*, strain Wx241-12b, Table I) to spore mating, using spores from the disruption diploid (*mps1Δ::HIS3, his3Δ200, leu2-3, 112*). These diploids (His^+, Leu^+) where found to be temperature-sensitive for growth, indicating that the disruption had abolished *MPS1* function and allowed expression of the *mps1-1* phenotype in diploids with the genotype *mps1-1/mps1Δ::HIS3*.

To determine if the putative kinase activity was import-

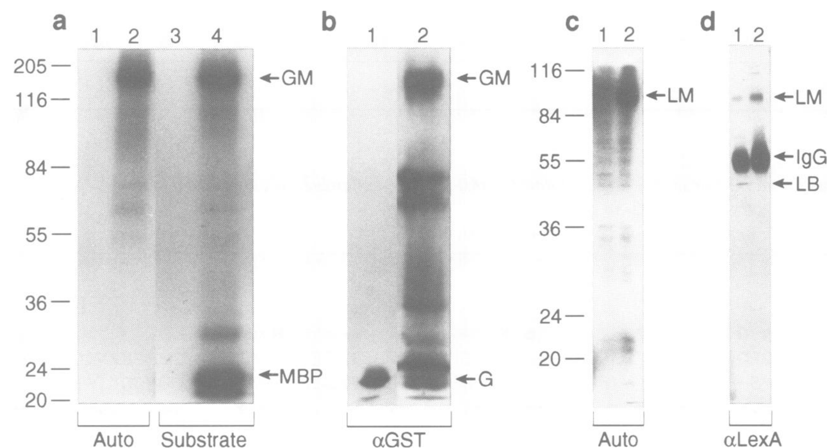

Fig. 3. Protein kinase assays using the GST (**a**) and the LexA (**c**) fusion proteins. Affinity purification of the correct fusion proteins is shown by immunoblot analysis (**b** and **d**). GST (G) (a, lanes 1 and 3) or GST−Mps1Δ2 (GM) (a, lanes 2 and 4) was affinity purified from yeast and used in kinase assays under conditions for autophosphorylation (a, lanes 1 and 2), or substrate phosphorylation (a, lanes 3 and 4) using MBP (see Materials and methods). Similarly, LexA−bicoid (LB) (Golemis and Brent, 1992) or LexA−Mps1Δ63 (LM) were immunoprecipitated and assayed for autophosphorylation activity (c, lanes 1 and 2, respectively). The positions of MBP and the autophosphorylated Mps1p fusion proteins are indicated, and are shown directly by immunoblotting using anti-GST antibody (b, lane 1 GST, lane 2 GST−Mps1Δ2), or by using anti-LexA antibody (d, lane 1 LexA−bicoid, lane 2 LexA−Mps1Δ63).

ant to *MPS1* function, a 'kinase-dead' allele was constructed using a PCR-based protocol (see Materials and methods). The particular point mutation (D_{580}→A_{580}, Figure 2) was chosen on the basis of the mutagenesis of the yeast cAMP kinase (Gibbs and Zoller, 1991). In that gene, the mutation of D_{228}→A_{228} in subdomain VII of the catalytic domain (consensus sequence DFG, see Figure 2) was the sole single amino acid change of wild-type to alanine that rendered the enzyme non-functional *in vitro* and the gene non-functional *in vivo*. Similar genetic results were found with the *mps1*D/A allele. The mutation was made in the *GST::MPS1Δ2* construct (see Materials and methods), which normally complements both the *mps1-1* mutation and the *mps1Δ::HIS3* null allele. The *GST::mps1D/A* mutant construct, however, fails to complement *mps1-1* and the *mps1Δ::HIS3* allele, indicating that protein kinase activity is required for the essential function of *MPS1*.

Mps1p has protein kinase activity

Chimeric proteins containing nearly all of Mps1p have been used to demonstrate that Mps1p has protein kinase activity *in vitro*. The *MPS1* ORF was fused to two different heterologous protein tags, GST and LexA, under the control of heterologous promoters for increased expression levels of the chimeric proteins in yeast (see Figure 1 and Materials and methods). These fusion proteins, GST−Mps1Δ2 and LexA−Mps1Δ63, were enriched from yeast lysates by affinity purification on glutathione resin or by immunoprecipitation, respectively. The material isolated from yeast extracts using glutathione agarose or anti-LexA antibodies was analyzed by immunoblotting using anti-GST or anti-LexA antibodies, respectively, (Figure 3b, lane 2; and 3d, lane 2) and proteins of slightly higher than the expected mol. wts for the two chimeric proteins were detected. These precipitated proteins were used in kinase assays (as described in Materials and methods) under conditions for autophosphorylation or for the phosphorylation of the exogenous substrate myelin basic protein (MBP). Both chimeric proteins exhibited autophosphorylation and substrate phosphorylation activity (Figure 3a, lanes 2 and 4; and 3c, lane 2; substrate phosphorylation by LexA−Mps1Δ63 not shown). In contrast, no kinase activity was detected in the immunoprecipitates of the GST protein alone or the LexA−bicoid fusion protein (Figure 3a, lanes 1 and 3; and 3c, lane 1), indicating that the activity seen was specific to the affinity purification of the Mps1p fusion protein.

The possibility exists that the protein kinase activity shown in Figure 3a is not intrinsic to Mps1p, but is due to a co-purifying kinase in both the LexA−Mps1Δ63 immunocomplexes and the GST−Mps1Δ2 complexes. The 'kinase-dead' mutation (D_{580}→A_{580}, see Materials and methods) was introduced into the *GST::MPS1Δ2* construct and the mutant protein was isolated and assayed as above to determine if the observed protein kinase activity is intrinsic to Mps1p. Figure 4b, lane 2 shows that the mutant protein can be isolated from yeast and detected by immunoblot analysis using anti-GST antibody. However, this mutant protein has no detectable protein kinase activity, either in assays of autophosphorylation or in assays for the phosphorylation of exogenous substrate (Figure 4a, lanes 2 and 4). We conclude that the observed protein kinase activity is intrinsic to Mps1p, and that it is the sole activity found in the glutathione bead/GST−Mps1Δ2 preparations as shown in Figures 3 and 4.

The immunoblot detection of GST−Mps1Δ2 and GST−Mps1Δ2D/A (kinase-dead protein) shown in Figure 4b, lanes 1 and 2, indicates that the proteins have different apparent mol. wts when isolated from yeast. The inactive GST−Mps1Δ2D/A protein migrates at the expected mol. wt of 112 kDa, whereas the active GST−Mps1Δ2 protein migrates at a higher apparent mol. wt and the electrophoretic band is less distinct. Increases in apparent mol. wt and indistinct bands can be indicative of post-translational modification of the polypeptide in question. The GST−Mps1Δ2 and GST-Mps1Δ2D/A proteins were subjected to protein phosphatase treatment with protein phosphatase 2A (PP2A, Materials and methods) to determine if the different mobilities of these proteins are caused by

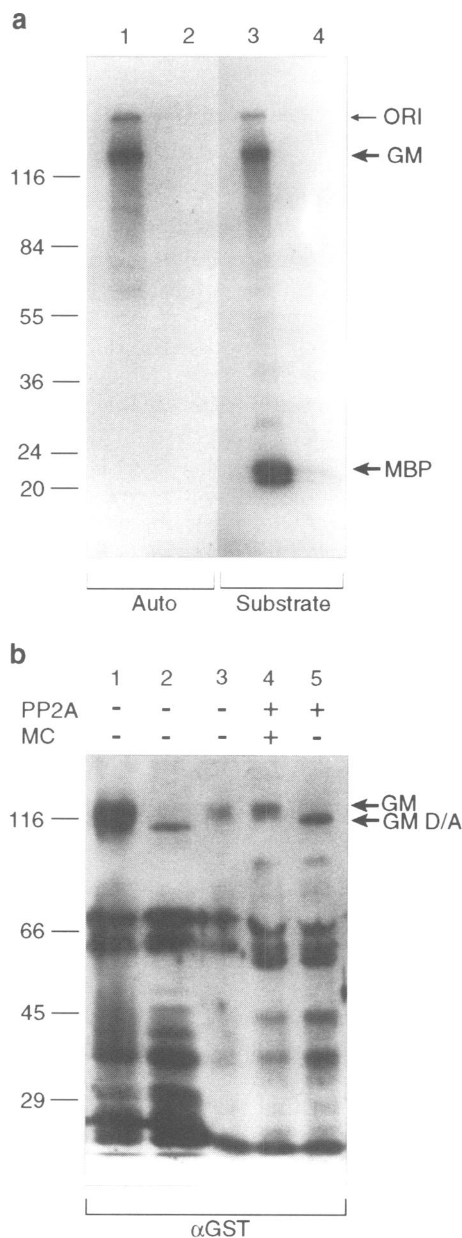

Fig. 4. Analysis of the GST−Mps1Δ2D/A (kinase-dead) mutant protein. GST−Mps1Δ2 (GM) and GST−Mps1Δ2D/A (GM D/A) proteins were affinity purified from yeast and used in assays for autophosphorylation and substrate phosphorylation (**a**), and immunoblotting with anti-GST antibodies (**b**, see Materials and methods). Lanes 1 and 3 in (a) and lane 1 in (b) were loaded with affinity-purified GST−Mps1Δ2, and lanes 2 and 4 in (a) and lane 2 in (b) were loaded with affinity-purified GST−Mps1Δ2D/A. (b) also shows immunoblot detection of phosphatase-treated GST−Mps1Δ2 using anti-GST antibodies (lanes 3–5). The proteins were incubated with nothing (b, lane 3), the inhibitor microcystin (MC) and PP2A (b, lane 4), or PP2A alone (b, lane 5) as described in Materials and methods.

phosphorylation. The mobility of the inactive GST−Mps1Δ2D/A protein is unaffected by phosphatase treatment (data not shown), whereas Figure 4b shows that PP2A treatment of the active GST−Mps1Δ2 protein does alter its mobility (lane 5), such that it has the same apparent mol. wt as the inactive GST−Mps1Δ2D/A protein. In addition, the shift in mobility appears to be due to phosphatase activity alone, as the addition of the PP2A

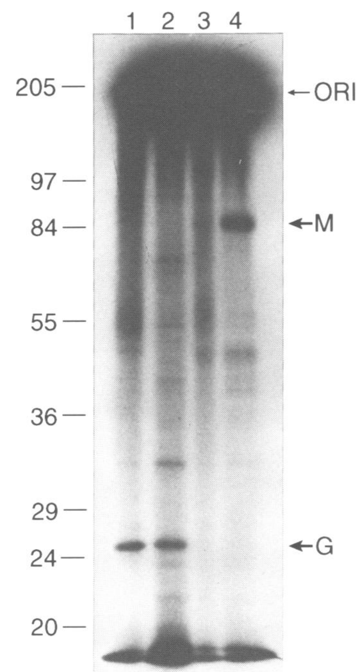

Fig. 5. Analysis of *in vivo* ^{32}P-labeled GST (G) (lanes 1 and 2) and GST−Mps1Δ2 (M) (lanes 3 and 4) proteins. Cells were labeled and the GST proteins were affinity purified as described in Materials and methods. Glutathione agarose loaded with GST or GST−Mps1Δ2 was subjected to thrombin cleavage, creating two fractions; the material remaining on the beads (lane 1, GST; lane 3, GST−Mps1Δ2) and the material liberated by the cleavage [lane 2, GST; lane 4, Mps1Δ2 (M)].

inhibitor microcystin resulted in a mobility similar to the untreated protein (Figure 4b, lanes 3 and 4). It appears that the retarded mobility of the GST−Mps1Δ2 protein is largely a result of phosphorylation, and that this may be due to autophosphorylation since the inactive GST−Mps1Δ2D/A protein appears to lack these modifications.

We wished to demonstrate independently that Mps1p is a phosphoprotein *in vivo*. Yeast cells containing a plasmid-borne *GST::MPS1Δ2* gene were labeled with [^{32}P]orthophosphate (see Materials and methods), and GST−Mps1Δ2 was isolated and analyzed by gel electrophoresis and autoradiography. We found that the GST protein itself is phosphorlylated in yeast (Figure 5, lanes 1 and 2), although the ^{32}P signal observed for the significant amount of protein present is quite low. GST−Mps1Δ2 contains a thrombin cleavage site (Miller *et al.*, 1993) that allows the cleavage of the Mps1Δ2 moiety from GST. The thrombin cleavage of GST−Mps1Δ2 isolated from ^{32}P-labeled cells was carried out as described by Miller *et al.* (1993) and the Mps1p moiety from the chimeric protein was released from the GST protein and found to be a phosphoprotein as well (Figure 5, lane 4). In this experiment, ^{32}P-labeled GST is not detected (Figure 5, lane 3), either because GST is not phosphorylated in the chimeric protein or because the level of phosphorylated GST is too low to detect. Consistent with the apparent phosphorylation of GST−Mps1Δ2 as detected by phosphatase treatment, we have found this protein to be a phosphoprotein *in vivo*.

Finally, the amino acid specificity of the Mps1p protein kinase was determined by phosphoamino acid analysis of *in vitro* labeled GST−Mps1Δ2 from autophosphorylation

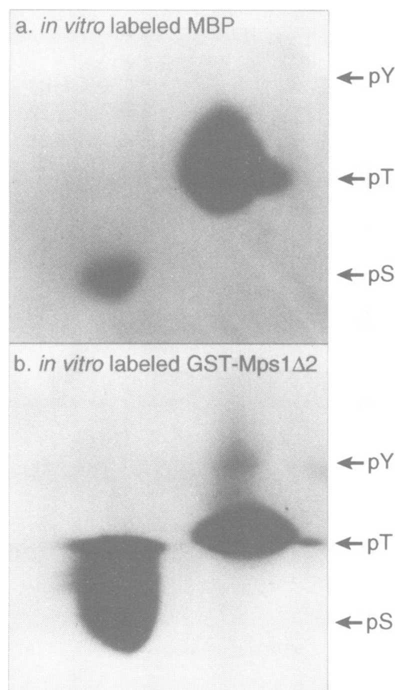

Fig. 6. Phosphoamino acid analysis of *in vitro* labeled MBP (**a**), and GST−Mps1Δ2 (**b**) as described in Materials and methods. The positions of phosphoserine (pS), phospothreonine (pT) and phosphotyrosine (pY) are indicated and were determined by the migration of unlabeled markers, as described by Kamps and Sefton (1989).

reactions and from *in vitro* labeled MBP (as in Figures 3 and 4). Figure 6a shows that phosphoserine and phosphothreonine residues were detected in *in vitro* labeled MBP, and that the major signal is from phosphothreonine. Similar analysis of autophosphorylated GST−Mps1Δ2 showed a phosphotyrosine signal as well, although phosphothreonine and phosphoserine are the predominant signals (Figure 6b). It is clear, however, that Mps1p will autophosphorylate on all three hydroxy amino acids, indicating that Mps1p can function as a dual specificity protein kinase *in vitro*.

Discussion

We have described the isolation and characterization of the *S.cerevisiae MPS1* gene. This gene was previously identified by a mutant allele that causes defects in SPB duplication and cell cycle regulation (Winey *et al.*, 1991). We have demonstrated that this gene encodes an essential, dual specificity protein kinase that is a phosphoprotein *in vivo*. The *MPS1*-encoded protein is 764 amino acids long, wherein the kinase domain is located in the C-terminal region of the protein and the N-terminal region is unique. Mps1p kinase activity is similar to several other dual specificity kinases, in that phosphorylation of tyrosine can be detected by autophosphorylation, and the level of tyrosine phosphorylation is modest (Lindberg *et al.*, 1992). Furthermore, the Mps1p kinase favors threonine phosphorylation over serine phosphorylation on the exogenous substrate MBP, which has also been observed for the mammalian *esk* kinase (Douville *et al.*, 1992). The significance of these patterns of phosphorylation by Mps1p is unknown, because *in vivo* substrates of the kinase, save Mps1p itself, have not been identified and mutations of the phosphorylation sites on Mps1p have not been examined.

The *MPS1* gene has recently been independently reported by Poch *et al.* (1994). These workers also report that *MPS1* is an essential gene using null alleles distinct from the one reported here. Poch *et al.* (1994) examined the transcript levels of *MPS1* mRNA and found that the mRNA levels were constant in mitotic cells. Interestingly, they report that *MPS1* mRNA levels fall in mating factor-treated cells. We have previously reported that *MPS1* is required for the completion of SPB duplication upon release from mating factor arrest (Winey *et al.*, 1991). The reduction in *MPS1* mRNA levels in cells exposed to mating factor may be a mechanism to block SPB duplication at the appropriate stage in mating cells. Synchronization of SPB duplication in the mating partners is required for efficient karyogamy, which is initiated by the SPBs (Byers and Goetsch, 1975; Rose *et al.*, 1986). Poch *et al.* (1994) also report an increase in *MPS1* mRNA levels in meiotic cells. We have observed that **a**/α *mps1-1/mps1-1* strains fail to form spores at the non-permissive temperature of 30°C (M.Winey, unpublished results), indicating that *MPS1* is required for meiosis.

Poch *et al.* (1994) constructed a depletion allele of *MPS1*, where the *MPS1* gene was placed under the control of the *GAL10* promoter. Yeast cells containing this allele could be cultured on galactose-containing media, but perished when transferred to glucose-containing media. Cells dying upon transfer to glucose-containing media were analyzed by flow cytometry and DAPI staining of DNA in whole cells. The authors observed failure to arrest in the cell cycle, and cells with aberrant DNA contents. They interpret these results as the cells failing to enter S phase, but carrying out mitosis. In contrast, we have reported that the *mps1-1* mutants, when released from α factor arrest at the non-permissive temperature, complete DNA synthesis and then missegregate their chromosomes (Winey *et al.*, 1991), yielding a flow cytometric profile of DNA content reminiscent of the *MPS1* depletion experiment (Poch *et al.*, 1994). We propose that the *MPS1* depletion allele reported by Poch *et al.* (1994) gives rise to failure in SPB duplication and lack of cell cycle arrest in response to the defective spindle, identical to the conditional alleles. Poch *et al.* (1994) recognized that failure of the cells to arrest and their continued aberrant cell cycles were indicative of a defect in a checkpoint control. We concur, and have demonstrated that *MPS1* is required for a checkpoint that monitors some aspect of SPB duplication or function (E.Weiss and M.Winey, in preparation).

Protein kinases have been implicated in the regulation of many cellular events, and the behavior of centrosomes is no exception. While no kinase besides *MPS1* has been directly implicated in spindle pole duplication, phosphorylation of centrosome components has been correlated with *in vitro* assembly (Felix *et al.*, 1994). Furthermore, protein kinases have been implicated in modification of centrosome activity. A variety of experiments have demonstrated that the *in vitro* microtubule nucleation capacity of isolated centrosomes is decreased by kinase inhibitors or by treatment with the MPM2 antibody, which recognizes an M phase-specific phosphorylated epitope,

and increased by treatment with cyclin-associated protein kinase complexes (Centonze and Borisy, 1990; Buendia *et al.*, 1992; Ohta *et al.*, 1993). As suggested by these results, centrosomes do contain phosphoproteins (Kalt and Schliwa, 1993). Furthermore, the level of phosphorylation at the centrosome, as detected by MPM2 antibody staining, does change during the cell cycle, peaking during mitosis. This phenomenon has been observed in both mammalian cells (Centonze and Borisy, 1990) and in *S.pombe* (Masuda *et al.*, 1992). Finally, it has been suggested that centrosomes may have protein kinases as components, but their role is unknown (Kalt and Schliwa, 1993). To date, the localization of Mps1p in the yeast cell is unknown.

The mammalian dual specificity protein kinases, *esk* and PYT/TTK, have the most amino acid sequence homology to the *MPS1*-encoded protein, but that similarity is limited to the kinase domain. The mammalian genes were thought to be involved in cell cycle control because their expression is limited to proliferating cells (both stem cells and transformed cells) that are actively going through cell cycles (Douville *et al.*, 1992; Mills *et al.*, 1992). Consistent with this idea, more recent data from Hogg *et al.* (1994) and Schmandt *et al.* (1994) have shown cell cycle periodicity for the expression of the PYT/TTK gene and for the level of kinase activity. While these fluctuations in PYT/TTK activity suggest some cell cycle role for this kinase, its function is not known. In our initial attempts to determine if *MPS1* and PYT/TTK are functional homologs, chimeric genes (the N-terminus of Mps1p with the PYT/TTK catalytic domain) have failed to rescue *mps1-1* mutant cells at the restrictive temperature. (J.Bachant and M.Winey, unpublished results).

We will need to understand the regulation of *MPS1* activity and identify the proteins that interact with Mps1p to better understand the role of this gene in SPB duplication and in cell cycle control. Regulation of Mps1p by phosphorylation seems to be a reasonable possibility since GST−Mps1Δ2 is a phosphoprotein *in vivo*. Regulation of mRNA is also a possibility, since mRNA abundance is altered in certain parts of the yeast life cycle (Poch *et al.*, 1994). Identification of gene products that interact with Mps1p will be crucial to understanding the role of this dual specificity protein kinase in SPB duplication and in cell cycle control.

Materials and methods

Strains, cell culture and genetic techniques

The yeast strains used in this study are listed in Table I. Yeast media and genetic and molecular techniques were as described by Ausubel *et al.* (1994). *Escherichia coli* DH5α (Sikorski and Hieter, 1989) was cultured and transformed as described by Ausubel *et al.* (1994). Yeast 5-FOA plates were prepared and used as described by Boeke *et al.* (1987).

For *in vivo* labeling, yeast strains containing the *GST::MPS1Δ2* fusion construct (described below) and *GST* alone were grown to 0.4 OD_{600}/ml in uracil-minus, 2% raffinose medium at 30°C. Transcription was induced by addition of galactose to 4% final concentration (Miller *et al.*, 1993). Cells were induced for 5 h at 30°C. Cells were then starved for phosphate in uracil-minus, 4% galactose, phosphate-depleted medium (Warner, 1991) for 1 h. 2.5 mCi of [^{32}P]orthophosphate were then added to the cells and allowed to incorporate for 2 h. The cells were collected and resuspended in buffer B (described below) plus phosphatase inhibitors (10 mM NaF and 10 mM sodium orthovanadate). Following glass-bead lysis with 2 vol of acid-washed glass beads (0.45–0.52 mm, Thomas Scientific), the extracts were incubated with glutathione agarose (100 ml agarose suspended at 50% v/v in buffer B; Sigma) for 1 h at 4°C. The agarose was washed extensively in buffer B plus phosphatase inhibitors. Thrombin cleavage was carried out as described by Miller *et al.* (1993). Post-cleavage supernatants were pooled and acetone precipitated (Ausubel *et al.*, 1994), and subjected to SDS−PAGE analysis and autoradiography.

Nucleic acid techniques

DNA was manipulated by standard techniques as described in Ausubel *et al.* (1994). DNA was prepared from *E.coli* using Wizard kits (Promega), and from yeast by the method of Hoffman and Winston (1987). Sequence analysis was accomplished by using fragments of the *MPS1* gene introduced in M13 mp18 or mp19 (Messing, 1983) or by using custom oligonucleotides as primers (DNA Express, Fort Collins CO). DNA sequencing was carried out using the Sequenase 2.0 kit designed for this purpose (United States Biochemical) following the instructions provided by the supplier. Analysis of the DNA sequence of *MPS1* and its derived amino acid sequence was carried out using programs in the GCG software package from the Genetics Computer Group, Madison, WI.

DNA hybridization analysis utilized a labeled *Eco*RV fragment internal to the ORF of the *MPS1* gene. DNA was labeled by random primed incorporation of digoxigenin (DIG)-labeled deoxyuridine triphosphate, using the Genius Nonradioactive Nucleic Acid Detection System (Boehringer Mannheim Biochemicals) and following the labeling protocol provided by the supplier. The DIG-labeled probe was hybridized to the Olson collection of overlapping genomic clones (Riles *et al.*, 1993; American Type Culture Collection, Rockville Pike, MD) in 5× SSC, 50% deionized formamide, 5% (w/v) Genius blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine (sodium salt), 0.02% SDS at 42°C for 16 h. An anti-DIG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) was bound to hybridized DIG-labeled DNA, and the chemiluminescent alkaline phosphatase substrate Lumiphos (Boehringer Mannheim) was applied to the filters at alkaline pH. Light emission was then detected by exposure of filters to X-ray film.

A PCR protocol was used to confirm proper integration of the *MPS1* null allele (*mps1Δ::HIS3*, see Figure 1) into the genome of a diploid yeast recipient strain (D8bx5cA, Table I). Reactions were carried out under standard conditions using *Taq* polymerase (Promega) in a Perkin-Elmer 480 Thermal cycler with priming oligos synthesized by DNA-Express (Fort Collins, CO). Three primers were used together in the analysis. One (BKS1, 5′-GAATCTTTTCATTATTG-3′) binds 3′ of the site of integration and should prime both chromosome IV homologs in the transformants. A second oligonucleotide (BSO2, 5′-CGATGAC-TTTGACAAA-3′), specific for the region of *MPS1* removed in *mps1Δ::HIS3*, was used to detect the non-recombinant chromosome, yielding a 1542 nucleotide fragment with the above common primer (BKS1). The *mps1Δ::HIS3* allele was detected by an oligonucleotide specific for the *HIS3* sequence (HIS3A, 5′-GATTAGCGACCAGCCGG-3′) and, in correct integrants, oligos HIS3A and the common oligonucleotide (BKS1) yield a band of 1401 nucleotides. PCR was performed on four independent His$^+$ transformants with the *mps1Δ::HIS3* allele, and all four yielded the two expected bands. Tetrad analysis of all four diploids showed that a recessive lethal mutation was segregating and was linked to *HIS3*. Cells from the viable spore clones were analyzed by PCR and found to contain only the 1542 nucleotide band indicative of the normal *MPS1* gene.

PCR was also used to create a point mutation in the kinase domain of the *MPS1* gene. PCR was carried out as described above, but plasmid DNA containing the *MPS1* gene was used as the template for mutagenesis. A 42 residue oligonucleotide (MPSC, 5′-CGGTACCGCGTTTGC-TATACCAAAAGCAATGATTTTTAA-3′, the bold nucleotide being the mutation) was synthesized that incorporated the *Kpn*I site in the kinase domain and the point mutation (nucleotide $A_{1739} \rightarrow C_{1739}$ changing a D_{580} to an A_{580}, numbering after Poch *et al.*, 1994) in this primer. A second primer (MPSG, 5′-CATTGGATGATGCAATAA-3′) was used so the PCR product could be cleaved with *Xba*I and *Kpn*I for re-introduction into the *MPS1* gene (see Figure 1). Once cloned, the DNA sequence of the entire *Xba*I−*Kpn*I fragment in *GST::mps1Δ2D/A* was determined to confirm that only the intended mutation had been introduced into the gene.

Plasmid constructs

The original isolate of *MPS1* in YCp50 is shown in Figure 1. An integrative plasmid containing the *MPS1* region was constructed by inserting the *Eco*RI−*Sal*I fragment of *MPS1* into pRS306 (Sikorski and Hieter, 1989). The integration of this plasmid into yeast chromosomal DNA was promoted by linearizing the plasmid in the *MPS1* gene. The null allele, *mps1Δ::HIS3*, was constructed as an insertion/deletion mutation. The *Cla*I−*Bam*HI fragment of *MPS1* (internal to the ORF,

Figure 1) was replaced with the 1692 bp *Cla*I–*Bam*HI fragment containing the *HIS3*$^+$ genes (Struhl, 1985). This allele was excised from the plasmid in which it was constructed as an *Eco*RI–*Kpn*I fragment for transformation in a one-step gene transplacement (Rothstein, 1991). Gene transplacements were confirmed by the PCR protocol described above.

Two plasmids were constructed for affinity purification of the *MPS1* gene product from yeast. A fusion of *MPS1* to the *LexA* gene of *E.coli* was accomplished by inserting the *Cla*I–*Sal*I *MPS1* fragment, after converting the *Cla*I site to an *Eco*RI by the addition of linkers (New England Biolabs), into the *Eco*RI and *Sal*I sites of the plasmid pEG202 (Golemis and Brent, 1992). This chimeric gene (*LexA*::*MPS1Δ63*) is under the control of the constitutive *ADH1* promoter, and deletes the first 63 amino acids of the *MPS1* ORF. A second fusion of the *MPS1* gene was made to the *Schistosoma japonicum GST* gene under the control of the inducible *GAL1* promoter in the pEG(KT) vector described by Miller *et al.* (1993). In this construct (*GST*::*MPS1Δ2*), an ~3 kb *Hin*cII fragment containing *MPS1* was inserted into the *Pvu*II site of pEG(KT), resulting in removal of only the first two amino acids of the *MPS1* ORF.

Protein techniques and kinase assays

Immunoblot analysis of protein samples separated by 12% SDS–PAGE (Ausubel *et al.*, 1994) involved transferring the proteins to Immobilon PVDF membrane (Millipore) for 2 h at 0.75 A in transfer buffer (20% MeOH, 0.1% SDS, 5 mM Tris, 0.65 M glycine) at 4°C using a Hoefer apparatus. Membranes were incubated in blocking buffer (100 mM Tris–HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20, 2% BSA) for 30 min at room temperature, or overnight at 4°C. Membranes were then washed extensively in TBST (100 mM Tris–HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20) and subsequently incubated in the primary antibody for 1 h at room temperature. For detection of GST–Mps1Δ2p, the primary antibody was used at 1:500 (Pharmacia goat anti-GST). Rabbit polyclonal anti-LexA antibody (a gift from Dr Roger Brent) was used at a 1:1000 dilution. After washing in TBST, the membranes were then incubated in secondary antibody for 1 h at room temperature. Secondary antibodies were used at a 1:20 000 dilution for detection using the ECL chemiluminescence system (Amersham).

Yeast strain EGY48 (Table I) transformed with the *LexA*::*MPS1Δ63* plasmid was grown in histidine-minus selective medium to 0.2–0.5 OD_{600}/ml. Cells were collected by centrifugation, rinsed twice in ice-cold sterile distilled water, and disrupted by vortex mixing 10 times (30 s vortex, 30 s on ice) in 0.5 ml buffer B (50 mM Tris–HCl, pH 7.5; 50 mM NaCl; 0.2% Triton X-100; leupeptin, 5 mg/ml; soybean trypsin inhibitor, 5 mg/ml; aprotinin, 5 mg/ml; PMSF, 1 mM) with 2 vol of 0.45–0.52 mm glass beads (Thomas Scientific). The supernatant from the lysed cells was incubated with anti-LexA antibody prebound to protein A–Sepharose (Pharmacia) for 2 h at 4°C. The Sepharose was then washed in buffer B three times, buffer B1 [50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% Triton X-100] three times, buffer A [50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.02% Triton X-100] three times, and non-detergent buffer [50 mM Tris–HCl (pH 7.5), 50 mM NaCl] once, and used in kinase assays (described below) or for immunoblot analysis.

The yeast strain BJ2168 (Table I) containing the *GST*::*MPS1Δ2* or *GST*::*mps1Δ2D/A* construct was grown to 0.4 OD_{600}/ml in selective medium (uracil-minus, 2% raffinose) at 30°C. The cells were then induced to express the chimeric genes by the addition of galactose (Sigma) to 4% final concentration. Following 6 h of induction, the cells were collected by centrifugation, rinsed twice in ice-cold sterile distilled water and glass-bead beaten as described above. The supernatant from the lysis was incubated with glutathione agarose (200 ml agarose suspended at 50% v/v in buffer B, Sigma) for 1 h at 4°C. For use in kinase assays or for imumunoblot analysis, the beads were washed three times each in buffer B, buffer B1 and buffer A sequentially, and once in non-detergent buffer.

The affinity-enriched proteins were incubated in 25 μl kinase assay buffer minus ATP [KAB–ATP: 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM PMSF] for 5 min at 30°C. The buffer was removed, and 25 μl of kinase assay buffer was added. The assay buffers were: KAB–autophosphorylation (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.5 mM DTT, 1 mM PMSF, 10 μM ATP, 10 μCi [γ-^{32}P]ATP) and KAB–substrate phosphorylation [50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 10 μM ATP, 10 μCi [γ-^{32}P]ATP, 4 μg MBP (Sigma)]. Reactions were incubated at 30°C for 30 min and then terminated by the addition of 6.5 μl of 5× Laemmli sample buffer (Ausubel *et al.*, 1994) and boiled for 5 min.

Phosphoamino acid analysis was performed as described in Kamps and Sefton (1989) and Cooper *et al.* (1983) on material from *in vitro* kinase assays. The kinase assay reactions were run on 12% SDS–PAGE and transferred to Immobilon PVDF, as described above. Following exposure of the membrane to film, the bands of interest (autophosphorylated Mps1p fusion proteins and phosphorylated MBP) were excised and eluted from the membrane (Cooper *et al.*, 1983; Kamps and Sefton, 1989).

Affinity-purified GST–Mps1Δ2 and GST–Mps1Δ2D/A protein was phosphatase treated using protein phosphatase 2A (a gift from Dr Natalie Ahn) while still conjugated to the glutathione agarose (in non-detergent buffer, see above). PP2A was added to the proteins at a final concentration of 500 U/ml and incubated at 30°C for 1 h. The phosphatase inhibitor microcystin was added to identical phosphatase reactions at a final concentration of 2 μM. Following the incubation, the glutathione agarose was needle-aspirated dry, resuspended in 20 μl of 2× Laemmli buffer, boiled for 5 min and separated by SDS–PAGE.

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