Mob2p interacts with the protein kinase Orb6p to promote coordination of cell polarity with cell cycle progression

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Summary
The molecular mechanisms that temporally and spatially coordinate cell morphogenesis with the cell cycle remain poorly understood. Here we describe the characterization of fission yeast Mob2p, a novel protein required for regulating cell polarity and cell cycle control. Deletion of mob2 is lethal and causes cells to become spherical, with depolarized actin and microtubule cytoskeletons. A decrease in Mob2p protein level results in a defect in the activation of bipolar growth. This phenotype is identical to that of mutants defective in the orb6 protein kinase gene, and we find that Mob2p physically interacts with Orb6p. In addition, overexpression of Mob2p, like that of Orb6p, results in a delay in the onset of mitosis. Mob2p localizes to the cell periphery and cytoplasm throughout the cell cycle and to the division site during late anaphase and telophase. Mob2p is unable to localize to the cell middle in mutants defective in actomyosin ring and septum formation. Our results suggest that Mob2p, along with Orb6p, is required for coordinating polarized cell growth during interphase with the onset of mitosis.

Key words: Cell polarity, Cell cycle, Orb6p, Mob1p, Schizosaccharomyces pombe

Introduction
Establishment and maintenance of cell polarity is fundamental to numerous cellular functions, such as cell differentiation, intracellular transport, localized membrane growth and cell migration (Drubin and Nelson, 1996). Changes in polarity occur during the cell cycle; however, the coordination of such changes with cell cycle control is poorly understood.

The fission yeast Schizosaccharomyces pombe is an attractive model organism for studying cell polarity (Chang, 2001; Mata and Nurse, 1998; Nurse, 1994; Snell and Nurse, 1994; Verde, 1998). Fission yeast cells are cylindrical, grow by linear extension at their ends and divide by medial fission. Moreover, they undergo morphological transitions that are tightly coupled with progression through the cell cycle (Mitchison and Nurse, 1985; Nurse, 1975). During G1 and S phases, cells grow only from the old cell end (the one that was present in the previous cell cycle). Early in G2 phase, after completion of DNA replication and attainment of a critical cell length, cells switch to bipolar growth, growing from both the old end and the new end formed by the previous cell division. This transition from unipolar to bipolar growth is known as new end take off (NETO) (Mitchison and Nurse, 1985). At the onset of mitosis, polarized cell growth ceases and is only re-established after cytokinesis. The sites of cell growth and division are reflected by the distribution of the actin cytoskeleton. For example, actin patches are always observed at the growing end(s) of the cell during interphase (Marks et al., 1986; Marks and Hyams, 1985). When cell elongation ceases at mitosis, actin disappears from the cell ends and relocates to the division site.

Genetic studies show that the protein kinase Orb6p is important for maintaining cell polarity and for coordinating cell morphogenesis with cell cycle progression (Verde et al., 1998). A number of Orb6p-related kinases have been identified in other organisms, including Saccharomyces cerevisiae Cbk1p (Biddingmaier et al., 2001; Racki et al., 2000), Neurospora crassa Cot-1 (Yarden et al., 1992), Ustilago maydis Ukc1 (Durrenberger and Kronstad, 1999), Caenorhabditis elegans LET-502 and SAX-1 (Wissmann et al., 1997; Zallen et al., 2000) and Drosophila Warts/Lats and Trc (Geng et al., 2000; Justice et al., 1995; Xu et al., 1995). Functional studies of these kinases suggest that they are involved in regulation of cell polarity.

By contrast, S. pombe Sid2p, another Orb6p-related kinase, is required for cytokinesis (Sparks et al., 1999), and its S. cerevisiae homolog Dbf2p is required for the exit from mitosis and the onset of cytokinesis (Frenz et al., 2000; Toyn and Johnston, 1994). Both Sid2p and Dbf2p associate with Mob1p (Hou et al., 2000; Komarnitsky et al., 1998; Salimova et al., 2000), a member of an emerging conserved protein family (Hou et al., 2000; Justice et al., 1995; Luca and Winey, 1998; Salimova et al., 2000). Mob1p appears to be important for both the localization and the kinase activity of both Sid2p (Hou et al., 2000; Salimova et al., 2000) (M.-C.H. and D.M., unpublished) and Dbf2p (Frenz et al., 2000; Lee et al., 2001; Mah et al., 2001). Both the S. pombe and S. cerevisiae genomes contain a second
Mob1-related protein called Mob2p (Hou et al., 2000; Luca and Winey, 1998; Salimova et al., 2000). Recent studies have shown that S. cerevisiae Mob2p is not essential for growth but interacts with Cbk1p to promote polarized cell growth and to induce asymmetric cell fate by activating daughter-specific gene expression (Colman-Lerner et al., 2001; Weiss et al., 2002).

In this study, we report the characterization of fission yeast Mob2p. Mob2p is an essential protein that functions in polarized cell growth and in regulating the onset of mitosis. Mob2p associates directly with Orb6p. We propose that Mob2p and Orb6p form a kinase complex that coordinates polarized cell growth with cell cycle progression.

**Materials and Methods**

**Yeast methods and strains**

The S. pombe strains used in this study are listed in Table 1. All strains are isogenic to 972 (Leupold, 1970). Fission yeast media, growth conditions and manipulations were carried out as described previously (Moreno et al., 1991). Except where noted, cells were grown in YE medium. All experiments involving temperature-sensitive strains were done at the permissive temperature of 25°C and the restrictive temperature of 36°C, unless otherwise indicated. Standard recombinant DNA methods were used (Sambrook et al., 1989). S. pombe transformations were carried out using either a lithium acetate method (Keeney and Boeke, 1994) or electroporation (Prentice, 1992). DNA was prepared from bacteria and isolated from agarose gels using Qiagen kits (Qiagen; Valencia, CA) and from yeast cells as described previously (Hoffman and Winston, 1987). DNA sequencing was performed at the University of Massachusetts Medical School’s Nucleic Acid Facility. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA).

**Construction of the mob2-null mutant**

A strain containing the mob2-null mutation (mob2Δa) was constructed in diploid cells (YDM634) as previously described (Bähler et al., 1998) by replacing one copy of mob2 with a fragment generated by PCR using plasmid pKS-ura4 (pDM155) as a template. The two primers contained the sequences corresponding to the regions immediately upstream and downstream of the mob2 start and stop codons. The PCR fragment was gel-purified and transformed into the diploid strain using the lithium acetate method. The strain bearing the mob2 deletion (YDM 679) was identified by PCR. A mob2Δ haploid strain bearing an episomal copy of mob2 (YDM1032) was obtained by placing a spore preparation from YDM679 containing pREP3X-mob2 (see below) and selecting for Leu+ Ura+ haploid colonies.

**Tagging of Mob2p and Orb6p**

Mob2p was tagged at its C-terminus with GFP or 13 tandem copies of the Myc epitope (13 Myc) by direct chromosomal integration into a promoterless copy of the mob2 gene, tagged at the C-terminus with three copies of the HA epitope (3HA) obtained by PCR (XhoI and EcoRI sites included in the primers), was inserted into an integrative plasmid containing the sup3-5 marker. The resultant plasmid was then transformed into ade6-704 leu1-32 ura4-D18 (PN567) cells and selected for colonies capable of growing in the absence of adenine. The correct integration was confirmed by PCR. Three strains (YDM1077) were used to test the expression of Orb6p-3HA by western blotting. In all cases, a single band of the right size was recognized by the mouse anti-HA antibody.

**Construction of mob2- and orb6-containing plasmids**

To express mob2 under the control of the thiamine-repressible nmt1 promoter, the multicopy plasmid pREP3X-mob2 was constructed by cloning a 750 bp BamHI fragment containing the entire mob2 cDNA obtained by PCR (BamHI sites included in the primers) into the BamHI site of the vector pREP3X (Maundrell, 1990). pREP41MH-mob2 expresses mob2 from an attenuated nmt1 promoter with an N-terminal tag consisting of six histidine residues followed by two copies of the Myc epitope (MH); it was constructed by cloning a 750 bp Asel-BamHI fragment containing the entire mob2 cDNA obtained by PCR (Asel and BamHI sites included in the primers) into the NdeI and BamHI-cut vector pREP41MH (Craven et al., 1998). Triple HA-tagged orb6 (pREP41HA-orb6) was constructed by cloning a 1.4 kb NdeI-BamHI fragment containing the entire orb6 cDNA (NdeI and BamHI sites included in the primers) into the NdeI and BamHI-cut plasmid pREP41HA (Craven et al., 1998).

**Two-hybrid screen for proteins interacting with Orb6p**

S. cerevisiae strain Y190 (MATa gal4 gal80 his3 trpl-901 ade2-101 ura3-52 leu2-3-112, URA3::GAL→lacZ; LYS2::GAL(UAS)→HIS3 cyh2) (provided by S. Elledge, Baylor College of Medicine) was used as the host for the two-hybrid interaction experiments. Strain Y187 (MATa gal4 gal80 his3 trpl-901 ade2-101 ura3-52 leu2-3-112 met-Ura3::GAL→lacZ) was used for mating experiments (Durfee et al., 1993). orb6 was fused to the DNA-binding domain of GAL4 in a plasmid (pAS1) carrying the TRP1 marker (pAS1-ori6) and to the GAL4 activation domain in a plasmid (pACT2) carrying the LEU2 marker (pACT2-ori6). Plasmids pAS1, pACT2, pSE1111 (pACT2-SNF4), pSE1112 (pAS1-SNF1) were kindly provided by S. Elledge. Cells were cultured in YEPD or selective SC at 30°C. The test for cell growth on medium lacking histidine (and containing 100 mM 3-aminoatrazole (3-AT)), the β-galactosidase assay, and the mating test to detect interaction specificity were performed as described previously (Durfee et al., 1993).

The S. pombe library prepared in phage λ vectors (a gift from S. Elledge) was used in a two-hybrid screen to identify proteins that interact with Orb6p. After screening 2×10⁸ transformants, one of the interactions was found to be Mbp2p. The specificity of this interaction was controlled by co-transformation with pAS1-ori6 and pACT1-mob2 independently with a number of control plasmids. pAS1-ori6 was unable to activate the GAL4 promoter when co-expressed with Snf4p (pACT2-SNF4), with Pak1p (pACT2-pak1) fused to the GAL4 activation domain or with Tea1p (pACT2-tea1) fused to the GAL4 activation domain (data not shown). Similarly, pACT2-mob2 did not activate the GAL4 promoter when co-expressed with Snf1p (pAS1-SNF1), with Cdt1p (pAS1-cdt1) fused to the GAL4 DNA-binding domain or with p53 protein (pAS1-p53) fused to the GAL4 DNA-binding domain (data not shown).

**Microscopy**

Cells were prepared for immunofluorescence staining by methanol fixation as described previously (Balasubramanian et al., 1997). The actin antibody (Amersham; Arlington Heights, IL) was used at a 1:200 dilution. The tubulin antibody TAT-1 (Woods et al., 1989) was a gift from K. Gull (University of Manchester, UK) and used at a 1:30 dilution. Primary antibodies were detected with anti-mouse Alexa 488 or Alexa 594 (Molecular Probes; Eugene, OR) at a 1:200 dilution.
Calcein fluor staining was performed as described previously (Balasubramanian et al., 1997). Images were captured using a Nikon Eclipse E 600 microscope with a cooled CCD camera (Orca-ER; Hamamatsu Photonics, Japan) and IPLab Spectrum software (Scanalytics; Fairfax, VA).

Immunoprecipitation and western blotting

Cell pellets were obtained from 4-5 x 10⁸ (20-25 total OD units at 955 nm) exponentially growing cells, which were collected by centrifugation and frozen at ~80°C if not used immediately. All subsequent manipulations were carried out at 4°C or on ice. Cells were lysed in NP-40 buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 80 µg/ml benzamidine, 1 mg/ml pepstatin, 10 µg/ml leupeptin, and 120 µg/ml [4-(2-aminomethyl)benzenesulfonyl fluoride, HCI] (AEBSF, Hydrochloride) by vortexing vigorously with acid-washed glass beads (Sigma; St Louis, MO) for 1 minute. Immunoprecipitations were performed by adding either 1 µl of anti-Myc mouse monoclonal IgG (9E10; Sigma; St Louis, MO) or 1 µl of anti-HA mouse monoclonal IgG (12CA5; Amersham Pharmacia Biotech) to the NP-40 cell lysates, followed by incubation on a rocker for 1 hour at 4°C. Immune complexes were purified by adding 40 µl of a 1:1 slurry of protein G-sepharose beads (Sigma), followed by incubation on a rocker for 1 hour at 4°C and centrifugation in a microfuge for 1 minute. The beads were washed then three times with 1 ml of NP-40 buffer. For detection of Myc-tagged, HA-tagged proteins and Cdc2p, the cell lysates or the immune complex beads were subjected to SDS-PAGE (7.5%), transferred onto Immobilon P nylon (Millipore; Bedford, MA) using a tank transfer system (BioRad; Hercules, CA). The blotted membranes were probed with the anti-Myc antibody (1:2000 dilution), anti-HA antibody (1:1000 dilution) or Cdc2p mouse monoclonal antibody (anti-PSTAIRE, 1:5000 dilution; Sigma) and developed using an alkaline phosphatase chemiluminescent system (BioRad).

Table 1. S. pombe strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| PN567  | ade6-704 leu1-32 ura4-D18 h+ | P. Nurse Lab |
| YDM34  | cdc3-124 ura4-D18 h+ | P. Nurse Lab |
| YDM82  | 972 h+ | P. Nurse Lab |
| YDM83  | 975 h+ | P. Nurse Lab |
| YDM105 | ade6-M210 leu1-32 ura4-D18 h+ | K. Gould Lab |
| YDM108 | ade6-M216 leu1-32 ura4-D18 h+ | K. Gould Lab |
| YDM144 | wee1-50 leu1-32 h- | P. Nurse Lab |
| YDM151 | cdc25-22 ura4-D18 h+ | P. Nurse Lab |
| YDM308 | cdc8-110 ade6-M210 leu1-32 ura4-D18 h+ | P. Nurse Lab |
| YDM634 | ade6-M210ade6-M216 leu1-32 ura4-D18ura4-D18 h+ | P. Nurse Lab |
| YDM679 | mob2+mob2::ura4 adel6-M210adel6-M216 leu1-32leu1-32 ura4-D18ura4-D18 h+/h+ | This study |
| YDM927 | mob2-Myc::Can adel6 leu1-32 ura4-D18 h+ | This study |
| YDM928 | mob2-GFP::Can adel6 leu1-32 ura4-D18 h+ | This study |
| YDM933 | mob2-Myc::Can cdc25-22 adel6 leu1-32 ura4-D18 h+ | This study |
| YDM963 | orb6-25 adel6-M210 leu1-32 h- | F. Verde Lab |
| YDM975 | mob2-Myc::Can adel6 leu1-32 ura4-D18 h+ | This study |
| YDM1010 | mob2-Myc::Can orb6-25 adel6 leu1-32 ura4-D18 | This study |
| YDM1021 | mob2-GFP::Can adel6 leu1-32 ura4-D18 h+ | This study |
| YDM1032 | mob2::ura4 adel6 leu1-32 ura4-D18 h+ [pREP3X-mob2] | This study |
| YDM1077 | orb6HA sup3-5 adel6-704 leu1-32 ura4-D18 h+ | This study |
| YDM1151 | mob2-Myc::Can orb6HA sup3-5 adel6-704 leu1-32 ura4-D18 [pREP3X-mob2] | This study |
| YDM1023 | mob2-GFP::Can | This study |
| YDM1024 | mob2-GFP::Can orb6-25 | This study |
| YDM1025 | mob2-GFP::Can cdc3-124 | This study |
| YDM1026 | mob2-GFP::Can cdc8-110 | This study |
| YDM1252 | mob2-GFP::Can cdc7-24 | This study |
| YDM1253 | mob2-GFP::Can sid2-250 | This study |
| YDM1456 | mob2::ura4 cdc25-22 [pREP3X-mob2] adel6 leu1-32 ura4-D18 | This study |

In vitro binding assay

An MH-tagged mob2 cDNA was amplified from pREP41MH-mob2 (see above) by PCR using the 5‘ primer 5‘-CTAGTAATAGCAGT-CACTATAGGGGTCGACCCCATGGGATGCAGC-3‘, which contained the T7 promoter (underlined) and a sequence homologous to the MH tag, and the 3‘ primer 5‘-GGGGATCGATCATCAAAATGTTTCCTTGATTTTCC-3‘, which contained sequence homologous to the C-terminus of mob2. Similarly, an HA-tagged orb6 cDNA was amplified from pREP41HA-orb6 by PCR using the 5‘ primer 5‘-GCCATACCTATAGGGGCTCGCCATGGGATGCAGC-3‘ and the 3‘ primer 5‘-CAATGCATTCCTACAAATGCTCTTCTATCTTTAA-3‘, which contained the orb6 stop codon. The PCR products were gel-purified and concentrated to 30 µl with Qiagen columns, and 2 µl of DNA was then used in a 25-µl coupled in vitro transcription/translation reaction system according to the manufacturer’s instructions (TNT; Promega; Madison, WI). Each reaction contained 12.5 µl of TNT® rabbit reticulocyte lysate, 1 µl of TNT® reaction buffer, 0.5 µl of TNT® T7 RNA polymerase, 0.5 µl of 1 mM amino acid mixture (minus methionine), 1 µl of Redivue L-[35S]methionine (>1000 Ci/mmol at 10 mCi/ml; Amersham Pharmacia Biotech), 0.5 µl (2 units) of Rnasin (Promega) and 7.0 µl of nuclease-free water. Luciferase DNA (TNT®, Promega) was used as a control reaction. Reactions were incubated at 30°C for 90 minutes. To examine the amounts of each protein in each reaction, 5 µl was taken and boiled for 2 minutes in 20 µl of 2x SDS sample buffer, and the proteins were resolved by SDS-PAGE. For the in vitro binding assay, the remaining 20 µl aliquots of each specific TNT reaction were mixed and incubated at 30°C for another 30 minutes, and the mixtures were then diluted to 500 µl with NP-40 buffer. The reaction mixtures were then precleared with 40 µl of protein G-sepharose beads for 1 hour prior to immunoprecipitation using anti-Myc mouse monoclonal IgG as described above and analysis by SDS-PAGE. Gels were processed for fluorography using Amplify™ (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.
Results
Phenotypes of mob2-deficient strains

mob2 was identified in the S. pombe genome database by its homology to S. cerevisiae Mob2p, a Mob1p-related protein (Hou et al., 2000; Salimova et al., 2000) and through a two-hybrid screen using orb6 as a bait (see below). To determine whether mob2 is essential, a null mutant, mob2Δ, was created by replacing the entire mob2 coding region by ura4+ in a wild-type diploid strain (strain YDM679, Table 1; see Materials and Methods). Sporulation and dissection of tetrads revealed that for each tetrad at most two spores containing the wild-type mob2 gene were viable (n=12 tetrads); these spores were Ura−, indicating that they contained the wild-type mob2 (data not shown). mob2Δ mutant spores did germinate to form spherical cells that underwent one or two cell divisions before lysing, suggesting that mob2 is essential for growth but not for spore germination (Fig. 1A).

To further analyze Mob2p function, the phenotype of a mob2Δ strain containing a mob2 shut-off plasmid (pREP3X-mob2) was examined. In the absence of thiamine, cells were viable and showed a normal cell morphology with normal organization of the actin and microtubule cytoskeletons (Fig. 1B,C). By contrast, changes in cell morphology and reorganization of the actin cytoskeleton and microtubules were observed when mob2 expression was repressed by thiamine addition. After 15 hours, cells became shorter at cell division (Table 2) and exhibited actin patches at only one end (80% versus 28% in a wild-type strain) (Fig. 1D), but microtubule organization appeared to be normal (Fig. 1E). After 22 hours, cells became spherical or oval (Table 2), and the actin cytoskeleton was dispersed around the cell cortex (Fig. 1F) whereas microtubules became depolarized with a criss-cross pattern (Fig. 1G).

A possible explanation for the high frequency of cells with actin patches at one end after 15 hours in thiamine is that Mob2p may have a role in the activation of bipolar growth. To address this possibility, we measured new end growth by examining the distance between the new end and the most recent birth scar, the site where the previous cell division occurred. Only cells with septa were analyzed to ensure that all cells examined were at the end of their growth phase. 15 hours after thiamine addition, the new ends of ~80% of mob2 shut-off cells showed no growth (Fig. 2Ab,d), whereas the new ends of wild-type cells grew normally (Fig. 2Aa,c). Thus, a reduction in Mob2p protein level leads to a defect in activation of new end growth. However, our results also showed that a decrease in Mob2p level resulted in entry into mitosis at a reduced size (Table 2). It is possible that cells go through mitosis before bipolar growth is initiated, thereby leading to accumulation of monopolar cells. If this is the case, then monopolar cells would not be observed if they had sufficient G2 phase to grow. To test this possibility, mob2 shut-off cells carrying the cdc25-22 mutation (YDM1456) were grown in the presence of thiamine at permissive temperature (25°C) for 12 hours then arrested at G2 phase by shifting to 36°C for an additional 4 hours. These cells displayed ~30% of total cell population with monopolar growth (Fig. 2Ba,d). By contrast, both strain YDM1456 without thiamine addition and a cdc25-22 single mutant strain YDM151 showed that ~10% of cells are monopolar and ~90% are bipolar (Fig. 2Ba-c). This result showed that a decrease in Mob2p level results in a bipolar growth defect even after a prolonged delay in G2. Interestingly, the cdc25-22 mob2 shut-off double mutant after elimination of mob2 expression did not elongate to the same extent as the double mutant before elimination of mob2 expression or the cdc25-22 single mutant, suggesting that Mob2p is important for general polarized growth (Fig. 2Bb,c,d).

Mob2p functions in regulation of the onset of mitosis
As noted above, reduced expression of mob2 caused cells to
Table 2. The effect of reduced expression of mob2 on cell length and width

| Time after thiamine addition (hour) | 0   | 1   | 11  | 12  | 13  | 14  | 15  | 16  | 22  |
|-------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Length (µm)                         | 14.6±1.2 | 14.2±0.9 | 12.8±0.7 | 12.6±0.8 | 12.2±0.9 | 11.4±0.8 | 10.6±0.8 | 8.8±0.6 |
| Width (µm)                          | 3.9±0.3 | 4.0±0.2 | 4.0±0.2 | 4.0±0.2 | 4.2±0.2 | 4.2±0.3 | 4.7±0.4 | 6.2±0.5 |

YDM1032 strain was grown in the absence of thiamine at 30°C overnight and then grown in the presence of thiamine. Samples were taken at the indicated times. Cell length and width of thirty cells at division are measured for each sample. The mean values±s.d. (µm) are shown.

Fig. 2. A decrease in Mob2p level leads to a defect in activation of bipolar growth.
(A) Wild-type (YDM105) (a) and mob2 shut-off cells (YDM1032) (b) were grown to exponential phase at 30°C, then grown in the presence of 5 µg/ml thiamine for 15 hours and stained with Calcofluor. Areas of previous cell division (birth scars) were marked as dark bands (a,b; arrowheads). Note that in b, these cells did not grow at their new ends causing the new ends (arrowheads) not to be visible. New end growth was measured in YDM105 (c) and YDM1032 (d) strains with septa as the distance between the most recent birth scar and the cell end (see brackets). Fifty cells were analyzed in each strain.

(B) cdc25-22 (YDM151) and cdc25 mob2::ura4+[pREP3X-mob2] (YDM1456) (16 hours +thiamine) were grown to exponential phase at 25°C, then grown for 12 hours in the presence of thiamine prior to shift to 36°C for 4 hours. In addition, mob2::ura4+[pREP3X-mob2] (YDM1456) cells were grown in the absence of thiamine at 25°C, then shifted to 36°C for 4 hours (0 hour +thiamine). Cells were fixed with 4% paraformaldehyde, stained with Calcofluor and scored with monopolar or bipolar growth. The result shown in a was obtained from two independent experiments. The corresponding Calcofluor-stained images are shown in (b) YDM151, (c) YDM1456 (0 hour + thiamine) and (d) YDM1456 (16 hours + thiamine). Arrowheads in d indicate the cells with monopolar growth.
become shorter at division, although the cell width was not significantly changed (Table 2; 12-15 hours). By contrast, mob2 overexpression in wild-type cells resulted in an increase in cell length at division owing to a delay in G2 phase (Table 3; data not shown). These data suggested that Mob2p plays a role in regulating the onset of mitosis. Control of entry into mitosis is regulated temporally by the timing of activation of Cdc2p. The protein kinase Wee1p inhibits the activation of Cdc2p and is a key regulator of cell size at mitosis. Thus, if Wee1p is required for the delay of the onset of mitosis caused by mob2 overexpression, then a wee1 mutation should compromise this effect. To test this possibility, mob2 was overexpressed in the wee1-50 temperature-sensitive strain at the restrictive temperature. In this case, mob2 overexpression failed to increase cell length at division, and the cells were the same size as wee1-50 cells with a control plasmid (Table 3). The same result was also obtained in a wee1 deletion mutant overexpressing mob2 (M.-C.H. and D.M., unpublished). Thus, the regulation of the onset of mitosis by Mob2p may require Wee1p activity. Interestingly, the same phenotype was observed upon overexpression of Orb6p (Verde et al., 1998).

Localization of Mob2p to the cell periphery/cytoplasm and the division site

To investigate the localization of Mob2p, we constructed a strain (YDM1203) expressing a C-terminally GFP-tagged Mob2p fusion protein from its chromosomal locus under the
control of its own promoter (see Materials and Methods). YDM1203 cells showed normal morphology and had no significant defect in growth rate. Mob2p-GFP localization was observed in living cells, since in fixed cells the GFP fluorescence of Mob2p-GFP was much fainter and the localization not perfectly maintained (M.-C.H. and D.M., unpublished). During interphase, Mob2p-GFP localized faintly at the cell periphery and the cytoplasm (Fig. 3A, cells 1 and 2; also see Fig. 4 and Fig. 6, wild-type cells). The signal at the cell periphery was slightly enriched at the cell tips but seemed to be present all around the cell cortex. Some faint punctate signals were observed in the cytoplasm as well as around the nucleus, particularly at high temperatures, that seem to be caused by autofluorescence as it was observed in cells not expressing Mob2p-GFP (M.-C.H. and D.M., unpublished). During septum formation, Mob2p-GFP was clearly localized to the division site, where it remained during septation and cytokinesis (Fig. 3A, cells 3-5). Following cell separation, Mob2p-GFP relocalized evenly at the cell periphery and the cytoplasm (Fig. 3A, cell 6).

Because the Mob2p-GFP signal was more intense at the division site during cytokinesis than at the cell periphery and the cytoplasm during interphase, we examined whether Mob2p levels varied during the cell cycle. A strain (YDM933) that expressed a C-terminally 13 Myc-tagged Mob2p fusion protein from its chromosomal locus under the control of its own promoter (see Materials and Methods) was synchronized by cdc25-22 block and release, and Mob2p-13Myc levels were determined at 15 minute intervals. It appeared that Mob2p-13Myc levels did not change significantly as cells progressed through the cell cycle (Fig. 3B).

To further investigate whether the localization of Mob2p at the division site requires the actomyosin ring, Mob2p-GFP localization was examined in living temperature-sensitive mutant cells of cdc3 (encoding profilin) (Balasubramanian et al., 1994) and cdc8 (encoding tropomyosin) (Balasubramanian et al., 1992). In these mutants, actomyosin rings are not formed and the septal material is disorganized at the restrictive temperature. At the permissive temperature, Mob2p-GFP localization in cdc3-124 (YDM1205) and cdc8-110 (YDM1206) cells appeared normal (Fig. 4A). However, after incubation at the restrictive temperature for 2.5 hours, when the majority of cells of both mutant strains had two separate nuclei but no actomyosin ring or septa (Balasubramanian et al., 1992; Balasubramanian et al., 1994), Mob2p-GFP did not
localize to the cell middle in either mutant but localized normally in wild-type cells (Fig. 4A, bottom panels), suggesting that Mob2p requires actomyosin ring assembly to localize to the division site. However, it is likely that septum formation affects Mob2p-GFP localization to the actomyosin ring. Mob2p-GFP localization to the actomyosin ring was examined in living cdc7-24 and sid2-250 temperature-sensitive mutant cells. Both Cdc7p and Sid2p are serine/threonine protein kinases and components of seption initiation network (SIN) (Fankhauser and Simanis, 1994; Sparks et al., 1999). In these mutants, no septum formation occurs, and thus cells become multinuclear at the restrictive temperature. At the permissive temperature, Mob2p-GFP localization in cdc7-24 (YDM1252) and sid2-250 (YDM1253) cells appeared normal (Fig. 4B). However, after incubation at the restrictive temperature for 2.5 hours, when the great majority of cells of both mutant strains had two separate nuclei but no septa, Mob2p-GFP did not localize to the cell middle in either mutant (Fig. 4B) but localized normally in wild-type cells (Fig. 4A).

Mob2p interacts with Orb6p kinase directly

Given that Orb6p is a Sid2p-related kinase and orb6 mutants have the same phenotype as the mob2Δ mutant (Verde et al., 1998), it seemed likely that Mob2p may interact with Orb6p. In fact, Mob2p was identified independently in a two-hybrid screen for Orb6p-binding partners (see Materials and Methods). Mob2p and Orb6p interacted specifically with each other but not with negative controls (Fig. 5A). To further confirm the interaction between Mob2p and Orb6p in vivo, co-immunoprecipitation was performed. Using a strain (YDM1151) expressing chromosomal Mob2p-13Myc and Orb6p-3HA, we found that the two proteins could be co-immunoprecipitated (Fig. 5B). Mob2p and Orb6p were also found to interact by a binding assay using in vitro translated proteins (Fig. 5C), suggesting that their interaction is direct. Because the Mob2p-related protein Mob1p interacts with the Orb6p-related kinase Sid2p, we also tested for interaction between Mob1p and Orb6p or between Mob2p and Sid2p by co-immunoprecipitation. No interactions were detected between them (M.-C.H. and D.M., unpublished). These results indicate that Mob2p interacts with Orb6p specifically in vivo.

Relationship between Mob2p and Orb6p localization

Since Mob2p associates with Orb6p, we tested whether Orb6p was important for Mob2p localization. In an orb6-25 temperature-sensitive mutant strain (YDM1204) at the permissive temperature, Mob2p-GFP was localized the same as in wild-type cells (Fig. 6A). By contrast, at the restrictive temperature, Mob2p-GFP was no longer observed at the cell cortex or the division site (Fig. 6A), even though the expression level of Mob2p remained unchanged (Fig. 6B), indicating that the absence of normal Mob2p-GFP localization is not due to degradation. Thus, Orb6p appears to be required for proper Mob2p localization.

We then examined the localization Orb6p-3HA localization in a mob2 shut-off strain (YDM1080). In the absence of thiamine, Orb6p-3HA was localized to the cell tips and the medial region, but under repressing conditions, no signal was observed (data not shown). To explore why no Orb6p signal...
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was observed, we examined the Orb6-3HA protein levels in the mob2 shut-off strain and found that a decrease in Mob2p protein levels resulted in a reduction in Orb6p-3HA protein levels (Fig. 6C). Thus, we could not determine if Mob2p is required for Orb6p localization, but it does seem that Mob2p is required for the stability of Orb6p-3HA.

Discussion

Role of Mob2p in cell polarization

Our studies show that Mob2p plays an important role in polarized cell growth. Deletion of mob2 results in disorganization of the actin and microtubule cytoskeletons and an inability of cells to grow in a polarized manner. In addition, a decrease in Mob2p level leads to a defect in activation of bipolar growth. It is possible that the remaining concentration of Mob2p is only sufficient for its ability to grow at the old end but is not enough to initiate bipolar growth at the new end. We also observed an intense cortical signal for Mob2p-GFP at the division site, which will become the new ends, consistent with a role for Mob2p in marking the new end as a site for growth. Since Mob2p did not localize to the division site until septum formation and cytokinesis are initiated, and deletion of mob2 did not result in detectable defects in septum formation and cytokinesis, it seems unlikely that Mob2p plays a direct role in cytokinesis. Instead, Mob2p may localize to the cell division site to mark the new end after cell division.

Mob2p localization to the division site appears to depend on actomyosin ring assembly. The question of how the actomyosin ring assembly promotes Mob2p localization to the division site remains to be answered. One possibility is that Mob2p is directly recruited by components of the actomyosin ring to the division site. However, Mob2p localization corresponds more to the septum and not the actomyosin ring; thus, the failure of Mob2p to localize in the actomyosin ring mutants may be due to an indirect effect of actomyosin ring failure on septum...
formation. Indeed, Mob2p is no longer localized to the medial region in SIN mutants, which make actomyosin rings, but fail to make septa.

Functional relationship of Mob2p and Orb6p
Our results show that Mob2p and Orb6p form a complex to regulate cell polarity and cell cycle progression. Orb6p has been suggested to function downstream of the Cdc42-activated Pak1p kinase (Verde et al., 1998). Overexpression of Orb6p partially suppressed the phenotype of pak1 temperature-sensitive mutants, and Pak1p-dependent establishment of cell polarity is required for Orb6p to localize correctly. However, overexpression of Mob2p cannot complement the phenotypes of pak1 and orb6 temperature-sensitive mutants (M.-C.H. and D.M., unpublished). One plausible explanation is that Orb6p kinase activity is important for Mob2p function, and overexpression of Mob2p is unable to bypass the requirement for Orb6p kinase. Elucidation of how Orb6p kinase is regulated will be helpful to resolve this question.

At present, nothing is known about whether Mob2p is important for Orb6p kinase activity. Previous studies have shown that Mob1p associates with Dbp2p and Sid2p in S. cerevisiae and S. pombe, respectively (Hou et al., 2000; Komarnitsky et al., 1998; Salimova et al., 2000). In both yeasts, Mob1p plays an essential role in kinase activity. In S. cerevisiae, Dfb2p kinase activity is eliminated in mob1 temperature-sensitive mutants (Lee et al., 2001; Mah et al., 2001), and in S. pombe, Sid2p kinase activity is abolished in mob1 temperature-sensitive mutants (M.-C.H. and D.M., unpublished). Thus, it is possible that Mob2p is essential for Orb6p kinase activity. Unfortunately, we have been unable to detect strong Orb6p kinase activity in vitro using artificial substrates, and thus were unable to assess whether Mob2p is required for the Orb6p kinase activity. Resolution of this issue may have to wait until in vivo substrates of Orb6p are identified.

Genetic studies suggest that Mob2p and Orb6p play a role in regulation of the onset of mitosis, which may require the activity of Wee1p kinase, an inhibitor of p34cdc2 mitotic kinase activity. Overexpression of either Mob2p or Orb6p resulted in a delay in entry into mitosis, whereas a decrease in their levels caused cells to divide at a reduced size (Verde et al., 1998). In addition, overexpression of Mob2p or Orb6p in wee1 mutants showed no delay in the onset of mitosis (Verde et al., 1998). Thus, it is possible that Mob2p-Orb6p kinase complex functions directly in activating Wee1p kinase or inactivating its inhibitor(s), thereby activating Wee1p kinase. However, the onset of mitosis is triggered by simultaneous activation of Cdc25 phosphatase and inactivation of Wee1 kinase (Ohi and Gould, 1999). Overexpression of Mob2p or Orb6p in cdc2 mutants that are able to bypass the requirement for Cdc25 phosphatase exhibited a delay in the onset in mitosis, but the effect was not as dramatic as that in wild-type cells (M.-C.H., F.V. and D.M., unpublished). Thus, at this moment we cannot exclusively rule out the possibility that Mob2p-Orb6p regulates the onset of mitosis through inhibition of Cdc25 phosphatase activity. Interestingly, it was recently found that the human tumor suppressor LAT51, which shares sequence similarity with Orb6p, complexes with and inhibits Cdc2p (Tao et al., 1999) and causes G2/M arrest when overexpressed (Xia et al., 2002; Yang et al., 2001). This raises an alternative possibility that Mob2p-Orb6p complexes with and inactivates Cdc2p kinase directly. Further studies will be needed to distinguish between these models.

Finally, we propose that the interaction between the Mob1p/Mob2p protein family and Sid2p/Orb6p protein kinase family are likely to be evolutionarily conserved. Along with the known Orb6p-related kinases (see Introduction), there are at least four Mob1p/Mob2p-related proteins in animal cells (M.-C.H. and D.M., unpublished), suggesting that the specific interaction between these two protein families may be evolutionarily conserved in higher eukaryotes. Thus, it will be quite interesting in future studies to determine if all Mob1p/Mob2p-related proteins function together with Sid2p/Orb6p family protein kinases.

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