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Fission yeast Mor2/Cps12, a protein similar to *Drosophila* Furry, is essential for cell morphogenesis and its mutation induces Wee1-dependent G_2 delay

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Fission yeast cells identify growing regions at the opposite ends of the cell, producing the rod-like shape. The positioning of the growth zone(s) and the polarized growth require CLIP170-like protein Tip1 and the Ndr kinase Orb6, respectively. Here, we show that the *mor2/cps12* mutation disrupts the localization of F-actin at the cell ends, producing spherical cells and concomitantly inducing a G_2 delay at 36°C. Mor2 is important for the localization of F-actin at the cell end(s) but not at the medial region, and is essential for the restriction of the growth zone(s) where Tip1 targets. Mor2 is homologous to the *Drosophila* Furry protein, which is required to maintain the integrity of cellular extensions, and is localized at both cell ends and the medial region of the cell in an actin-dependent fashion. Cellular localization of Mor2 and Orb6 was interdependent. The tyrosine kinase Wee1 is necessary for the G_2 delay and maintenance of viability of the *mor2* mutant. These results indicate that Mor2 plays an essential role in cell morphogenesis in concert with Orb6, and the mutation activates the mechanism coordinating morphogenesis with cell cycle progression.

Keywords: actin/checkpoint/CLIP170/Furry/growth polarity

Introduction

Cell morphogenesis and the cell cycle are coordinately regulated. The fission yeast *Schizosaccharomyces pombe* is an ideal system to study cell morphogenesis (Snell and Nurse, 1993; Verde *et al*., 1995; Hirata *et al*., 1998b; Mata and Nurse, 1998; Brunner and Nurse, 2000). The fission yeast cell has a cylindrical rod-like shape (8–14 μm in length and 3 μm in diameter), and cell growth occurs only at the tips (Mitchison, 1970). The growth polarity dynamically changes during three stages of the cell cycle: (i) the initiation of growth upon cell division; (ii) new end take off (NETO; Mitchison and Nurse, 1985); and (iii) septum formation after anaphase. After cell division, cortical F-actin moves from the new end that is newly produced by division to the old end that existed in the previous cell cycle. The cell growth is initiated from only the old cell end. Upon NETO, which occurs at 0.34 of the way through the cell cycle (Mitchison and Nurse, 1985), F-actin localization dynamically shifts from the old cell end to both cell ends. After NETO, the growth polarity changes from monopolar to bipolar. At the onset of mitosis, cell growth ceases, and the cortical F-actin patches at both ends then disappear. Prior to cytokinesis, actin reassembles into an F-actin ring in the middle region of the cell where septation will occur (Marks and Hyams, 1985; Marks *et al*., 1986).

In general, microtubules (MTs) mediate long-range transport of membranous organelles to the cell periphery, whereas actin mediates short-range transport and anchorage (Goode *et al*., 2000). Rod-shaped fission yeast cells grow in a polarized manner, and unlike in the case of the budding yeast, the correct positioning of the growth sites at cell ends requires interphase MTs. The Tea1 protein and interphase MTs are important for cell polarity in fission yeast (Mata and Nurse, 1997). Tea1 is localized on the plus ends of interphase MTs. Tea1 localization requires MTs, a kinesin-like protein Tea2, and the CLIP170-like protein Tip1 (Browning *et al*., 2000; Brunner and Nurse, 2000). The protein Tip1, which becomes localized at the distal tips of cytoplasmic MTs, enables the MTs to discriminate between the cell end(s) and other cortical regions, and regulates their dynamics accordingly (Brunner and Nurse, 2000). It has been suggested that there is a pre-existing marker at the cell ends recognized by Tip1 that promotes catastrophe by causing the dissociation of Tip1 from the MT tip (Brunner and Nurse, 2000). The mechanism targeting Tip1 to the cell ends remains to be clarified.

A series of checkpoints ensures that events proceed normally during the cell cycle. Several checkpoints, which monitor DNA replication, DNA damage, spindle assembly to the kinetochore and spindle orientation, have been identified. Cell morphogenesis and cell cycle progression are coordinately regulated, indicating that cells have a checkpoint(s) that monitors the cell morphogenesis at a specific stage(s) of the cell cycle. Indeed, the checkpoints that monitor the bud formation in budding yeast (Lew, 2000) and the completion of cytokinesis in fission yeast have been identified (Goff *et al*., 1999; Liu *et al*., 2000). In the 'morphogenesis checkpoint' monitoring the bud formation in budding yeast, the Cdc28 inhibitory kinase Swe1 is important for the G_2 delay. In response to actin perturbations, the Swe1 protein is continuously accumulated during G_2 delay by both increases in *SWE1* transcription and inhibition of Swe1 degradation (Lew, 2000). The activation of the cytokinesis checkpoint in

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fission yeast requires the septation initiation network (SIN) pathway and the Cdc2 inhibitory kinase Wee1 (Goff *et al*., 1999; Liu *et al*., 2000).

The *Drosophila* protein Furry is important for maintaining the integrity of cellular extensions during morphogenesis (Cong *et al*., 2001). The *furry* mutation causes the formation of branched arista laterals, branched bristles and strong multiple hair cells. The Furry-like proteins have been evolutionarily conserved from yeast to humans. Genetic analysis has suggested that the *furry* gene functions in the same pathway as the *tricornered* gene that encodes Ndr (nuclear DBF2-related) kinase (Geng *et al*., 2000; Cong *et al*., 2001). The Ndr family is related to *S.pombe* Orb6 (Verde *et al*., 1998), *Saccharomyces cerevisiae* Cbk1 (Racki *et al*., 2000; Bidlingmaier *et al*., 2001; Colman-Lerner *et al*., 2001), *Neurospora* Cot1 (Yarden *et al*., 1992), *Drosophila* Warts/Lats (Justice *et al*., 1995; Xu *et al*., 1995), *Caenorhabditis* let-502 and Sax1 (Wissmann *et al*., 1997; Zallen *et al*., 2000), mammalian Rho-associated kinase (Leung *et al*., 1995; Ishizaki *et al*., 1996; Matsui *et al*., 1996) and human myotonic dystrophy kinase (DMPK) (Brook *et al*., 1992), and is required for the regulation of cell morphology and division. The Orb6 kinase of fission yeast is localized at the polarized growing sites, and its mutation results in a round morphology (Verde *et al*., 1995, 1998). Recently, it was shown that a budding yeast Furry-like protein, Pag1, is required for cell morphogenesis and forms a complex with the Ndr kinase Cbk1 (Du and Novick, 2002).

Here we show that the fission yeast Furry-like protein, Mor2, is required for the establishment of growth polarity and was localized to the cell ends and septation site in an actin-dependent fashion. The *mor2* mutation disrupts the localization of F-actin, inducing a Wee1-dependent G_2 delay. Furthermore, we demonstrate that the localization of Mor2 and Orb6 is interdependent for their function.

Results

Isolation of temperature-sensitive round mutants

To identify a mechanism coordinating morphogenesis with cell cycle progression in fission yeast, we visually screened for mutants that showed a round morphology and arrest of cell growth at 36°C. To distinguish the cell wall-defective mutants, we examined the sensitivity of the cells to the protein kinase inhibitor staurosporine (STS), because the PKC-related kinase Pck2 is important for the maintenance of the cell wall integrity and its mutation causes super-sensitivity to STS (Toda *et al*., 1993; Katayama *et al*., 1999). By this screening, four STS-insensitive mutants were isolated and were designated *mor* (morphological round). Complementation analyses between *mor* and other round mutants showed that the *mor* mutants could be classified into three loci, and that *mor1* and *mor2* were allelic to *orb1* (Snell and Nurse, 1994) and *cps12* (Ishiguro and Uhara, 1992), respectively. The *orb1*

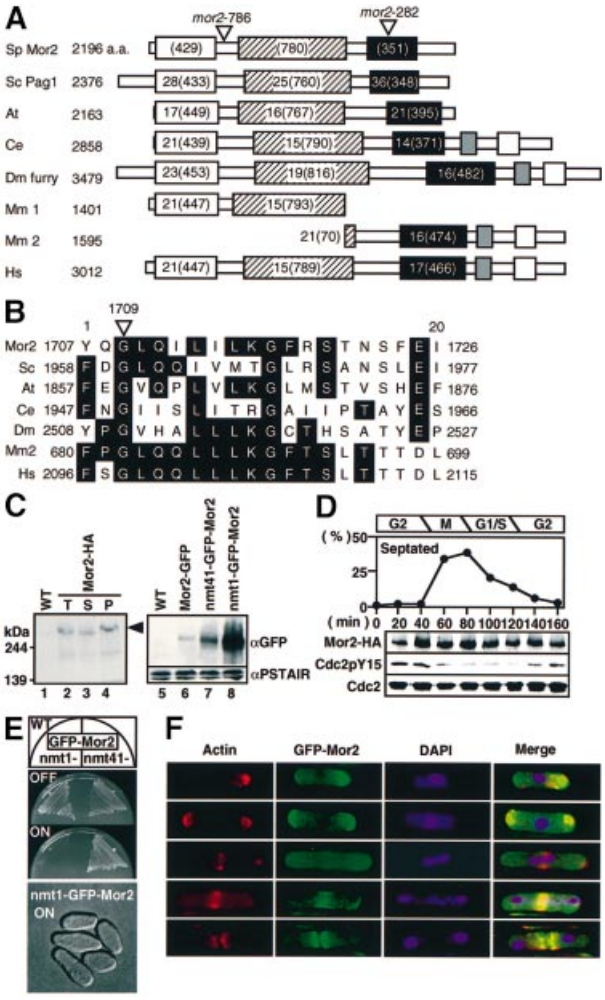

Fig. 1. Identification of Mor2. (**A**) A schematic presentation of the homology between Mor2 and Furry homologous proteins. *Schizosaccharomyces pombe* Mor2 is aligned with *S.cerevisiae* Pag1/Tao3 (Sc), *Arabidopsis thaliana* T51397 (At), *C.elegans* AAF99910 (Ce), *D.melanogaster* Furry (Dm), *Mus musculus* CAC42175.1 (Mm1), CAC42196.1 (Mm2) and *Homo sapiens* CAB42442 (Hs). Identities (%) and the number of amino acids are indicated. (**B**) The mutation site of the *mor2-282* allele. (**C**) Identification of Mor2 protein. Lanes 1–4: total cell extract prepared from wild type (WT, 972), and the cells having *mor2*$^+$*:3HA:kan*^{r} (Mor2-HA, YS78-1) grown in YPD medium were electrophoresed on SDS–PAGE gels and immunoblotted with anti-HA (lanes 1 and 2). Total cell extracts (T, lane 2) prepared from the Mor2-HA strain were fractionated into soluble (S, lane 3) and insoluble fractions (P, lane 4) by centrifugation at 14 000 *g*. Lanes 5–8: wild type, and cells with *mor2*$^+$*:GFP:kan*^{r} (Mor2–GFP, YS77), *kan*^{r}*:nmt41-GFP:mor2*$^+$ (nmt41–GFP–Mor2, YS84-1) or *kan*^{r}*:nmt1-GFP:mor2*$^+$ (nmt1-GFP–Mor2, YS85) grown in Edinburgh minimal medium (EMM) containing 4 μM thiamine were transferred into EMM. After cultivation for 14 h, the cells were collected. Total cell extracts prepared from the cells were immunoblotted with anti-GFP or anti-PSTAIR antibodies. (**D**) Mor2 protein during the cell cycle. Early G_2 cells of Mor2-HA strain were collected by centrifugal elutriation and cultured in YPD medium at 28°C. Samples of the culture were taken at the indicated times for calculating the percentage of septated cells. Total cell extracts prepared from the samples of the same cultures were immunoblotted with anti-HA (for Mor2-HA), anti-PSTAIR (for Cdc2) or anti-Cdc2 phosphorylated on tyrosine-15 (for Cdc2-P) antibodies. (**E**) Growth (upper) and morphology (lower) of the Mor2-over-expressing cells. Upper panel: the nmt1-GFP–Mor2 and nmt41–GFP–Mor2 strains were cultured on EMM (ON) or EMM plates containing 4 mM thiamine (OFF) at 25°C for 3 days. Lower panel: the nmt1-GFP–Mor2 strain grown in EMM containing 4 μM thiamine was transferred into EMM medium and cultured for 18 h at 25°C. (**F**) Localization of Mor2 and F-actin. The nmt41–GFP–Mor2 strain cells grown in EMM containing 4 μM thiamine were transferred into EMM medium. After cultivation for 14 h, cells were fixed and stained with rhodamine–phalloidin. Merged image (Merge): F-actin (red), GFP–Mor2 (green) and DAPI (blue).

mutant was isolated as one of the 12 *orb* loci that showed a round shape at 36°C, and the *cps12* mutant as one of the 14 *cps* loci that showed super-sensitivity to the spindle poison chlorpropham (isopropyl *N*-3-chlorophenyl carbamate). In this paper, we focused on the novel mutant *mor2/cps12*. Hereafter, the originally isolated *mor2* and *cps12* mutants are referred to as *mor2-786* and *mor2-282*, respectively.

Mor2 is a Drosophila Furry-like protein

The *mor2*$^+$ gene was cloned by complementation of the phenotype of the mutant (temperature sensitivity and round morphology). Nucleotide sequencing of the cloned DNA fragment identified a novel gene that encoded 2196 amino acids (molecular mass 250 kDa) matching the genomic sequence of ORF SPBP19A11.04C in the *S.pombe* genome. Blast analysis indicated Mor2 to be a *Drosophila* Furry-like protein. The Furry-like proteins have been evolutionarily conserved from yeast to humans. The proteins exist in *S.cerevisiae* (Pag1/Tao3), *Drosophila melanogaster* (Furry), *Caenorhabditis elegans* (AAF-99910), *Arabidopsis* (T51397) and humans (HS85D21.1) (Figure 1A). The *D.melanogaster*, *C.elegans* and human proteins contain five similarity regions, but the *S.pombe, S.cerevisiae* and *Arabidopsis* proteins contain only the first three regions of similarity. Among these genes, the *Drosophila* essential gene *furry* was first to be identified and found to be important for maintaining the integrity of cellular extensions during morphogenesis (Cong *et al*., 2001). The *S.cerevisiae tao3/pag1* mutant was originally identified as a mutant that caused altered transcription of the *OCH1* gene (*Saccharomyces* Genome Database); however, recently it was shown that Pag1/Tao3 is important for cell morphogenesis (Du and Novick, 2002).

The mutation sites of the two *mor2* alleles were determined. The *mor2-282* and *mor2-786* mutants showed the substitution of an evolutionarily conserved glycine 1709 with aspartic acid [G<u>G</u>T to G<u>A</u>T (the mutated nucleotide is underlined)] and the substitution of tyrosine 558 (T<u>A</u>T) with cysteine (T<u>G</u>T), respectively (Figure 1A and B).

The *mor2*$^+$ gene was disrupted by the one-step gene disruption method using the *ura4*$^+$ gene as marker. Tetrad analysis indicated that two viable and two non-viable spores were obtained, and the viable spores produced Ura$^-$ colonies with an intact *mor2*$^+$ gene. Microscopic observation of the non-viable spores showed that the putative Δ*mor2* spores failed to germinate or ceased growing immediately after germination. These results indicate that the *mor2*$^+$ gene was essential for cell growth.

Mor2 is localized at cell ends and the septation site

To investigate the cellular localization of the Mor2 protein, we first introduced an epitope tag (3HA or 13Myc) or a green fluorescent protein (GFP) gene at the end of the chromosomal *mor2*$^+$ gene in wild-type cells by using the polymerase chain reaction (PCR) tagging method (Bahler *et al*., 1998). The C-terminal tagging did not interfere with the function of the Mor2 protein, as the tagged strains grew as well as wild-type cells. We detected the proteins by western blotting using these tagged strains (Figure 1C), but we could not determine the specific cellular localization of Mor2. The Mor2-HA was found in both the soluble and insoluble fractions, suggesting that the protein was present in both cytoplasm and membrane regions (Figure 1C, lanes 2–4). The Mor2-HA protein level did not change drastically during the cell cycle (Figure 1D). To investigate the localization of the Mor2 protein, we next attempted to increase the expression level of the *mor2*$^+$ gene by introducing the thiamine-repressible *nmt41* or *nmt1* promoter-driven *GFP* gene in front of the chromosomal *mor2*$^+$ gene. The level of GFP–Mor2 protein expressed by the *nmt41-GFP-mor2*$^+$ or *nmt1-GFP-mor2*$^+$ strain grown under the inducible condition increased by 5- or 50-fold, respectively, compared with that expressed from the *mor2*$^+$ promoter (Figure 1C, lanes 6–8). The excessive expression of GFP–Mor2 from the *nmt1* promoter caused the defects in growth and morphology (Figure 1E), but the induced GFP–Mor2 protein from the *nmt41* promoter did not affect growth and morphology. Therefore, we used an nmt41–GFP–Mor2 strain for the observation of the localization of Mor2 protein. In the monopolar growing cells in which the majority of F-actin localized to the old ends, GFP–Mor2 was localized predominantly at the growing end and faintly at the non-growing end. In the bipolar growing cells, GFP–Mor2 was localized at both ends of the cell. During mitosis, GFP–Mor2 was concentrated at the medial region where the septum formation would later occur (Figure 1F).

To investigate further the precise order of the localization of Mor2 and actin, we used synchronous cultures of *cdc25-22* mutant cells (Figure 2A). The cells were arrested in late G_2 phase at 36°C and were released at the permissive temperature of 25°C. After the release, we compared the appearance of the actin ring and the accumulation of GFP–Mor2 at the medial region in the time-course. In the G_2-arrested cells, F-actin and GFP–Mor2 were localized at both ends of the cells (Figure 2A, a). At 40 min after the release, 60% of the cells formed an actin ring (Figure 2A, f). At this time, an actin ring formed in 40% of the cells, but the localization of GFP–Mor2 at the medial region was not observed (Figure 2A, b, c and f). The localization of GFP–Mor2 at the medial region followed the actin localization during cell division (Figure 2A, f). We next examined the appearance of actin dots and the accumulation of GFP–Mor2 at the old end of the cells. At the growth initiation after the cell division, the appearance of actin dots and the accumulation of GFP–Mor2 at the old end were observed simultaneously (Figure 2A, e and g). In the monopolar growing cells in which F-actin was predominantly localized at the old end, a small number of actin dots and the localization of GFP–Mor2 at the new end were observed. These results suggested that the appearance of actin dots but not the formation of an actin ring depends on Mor2.

The actin cytoskeleton is important for the cellular localization of Mor2

To investigate whether the actin or MT cytoskeleton is important for the cellular localization of GFP–Mor2, we examined the localization of GFP–Mor2 in the cells treated with the F-actin inhibitor latrunculin-B (Lat-B; Spector *et al*., 1983) or the tubulin-binding drug thiabendazole (TBZ; Umesono *et al*., 1983). The normal localization of GFP–Mor2 was disrupted by Lat-B but not

by TBZ (Figure 2B and C). Consistent with this result, the disruption of the actin cytoskeleton by the profilin mutation *cdc3* (Balasubramanian *et al*., 1994), but not the disruption of MTs by the β-tubulin mutation *nda3* (Hiraoka *et al*., 1984), prevented the normal localization of GFP–Mor2 (Figure 2D and E). Further, the deletion of the *tip1*$^+$ gene, essential for the guidance of MT ends to their target region (Brunner and Nurse, 2000), did not affect the normal cellular localization of GFP–Mor2 (Figure 2F). These results thus indicated that the actin cytoskeleton, but not the MTs, was required to locate Mor2 at the cell ends and the septation site, and that the localization of Mor2 and F-actin at the cell end was interdependent.

Morphology of the mor2 mutant is reversed by temperature shift-down

To investigate the morphological change in the *mor2* mutant cells in detail, the mutant cells grown at the permissive temperature of 25°C were shifted up to the restrictive temperature of 36°C and kept there for 6 h, and then shifted down to 25°C and incubated for 6 h. The polarized growth ceased immediately after the shift-up, and the morphology completely changed to the round type after 6 h of the incubation at 36°C (Figure 3A). The cells finally showed two phenotypes: unseptated round with one nucleus (70%) and septated round with two nuclei (30%), indicating that Mor2 is important for the polarized growth during interphase and after mitosis. Fluorescence-activated cell sorting (FACS) analysis and the hemispherical nuclear structure indicated that the *mor2* mutation induced a G_2 delay at 36°C (Figure 3B). After the shift-down of the culture, the cell poles were re-established, and the morphology returned to a rod-shape through round with pole(s) (Figure 3A). The temperature-dependent reversible phenotype indicated that viability of the mutant was maintained at 36°C. To confirm G_2 delay of the *mor2* mutant, we performed FACS analysis of the unseptated round cells, selected by cell elutriation of the mutant culture at 36°C. The result indicated that the mutant was delayed in G_2 (Figure 3C). Further, the double mutant cells (either *mor2cdc10* or *mor2cdc25*) showed abnormal spherical morphology (data not shown), indicating that Mor2 is required for the maintenance of growth polarity in both G_1 and G_2 phases of the cell cycle.

The *mor2* mutant cells could mate normally. To investigate whether Mor2 is important for the mechanism of cell polarization during conjugation, we examined the shmoo morphology of the mutant. As shown in Figure 3D, the shmoo morphology of the mutant was indistinguishable from that of wild-type cells.

We evaluated the cell wall integrity of the mutant by examining the kinetics of cell lysis upon β-glucanase treatment (Toda *et al*., 1996). The sensitivity of the *mor2* mutant to the treatment was indistinguishable from that of wild-type cells (data not shown). We further examined whether an osmotic stabilizer, sorbitol, would rescue the *mor2* mutant, because the phenotype of the cell wall-

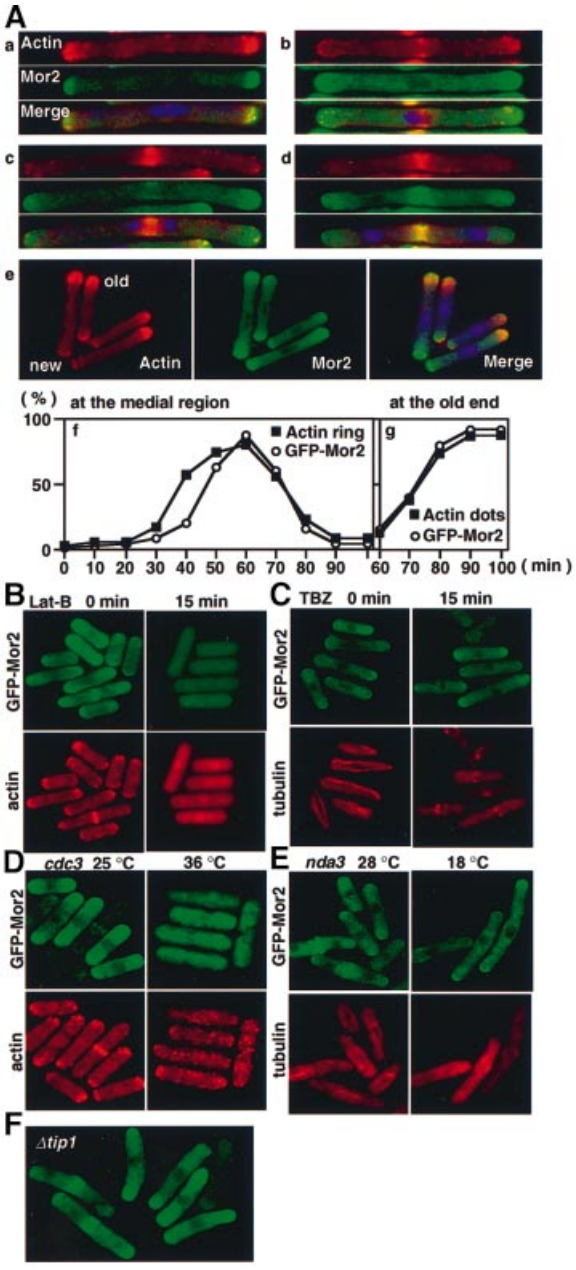

Fig. 2. Role of cytoskeletons in Mor2 localization. (**A**) Mor2 localization in the cells released from G_2 block. The *cdc25-22* mutant cells having *kan*^{r}*:nmt41-GFP:mor2*$^+$ (DH294-4A) grown in EMM containing 8 μM thiamine were transferred into EMM medium. After cultivation for 11–12 h at 25°C, the cells were transferred to 36°C and kept there for 4 h. The medium was then was returned to 25°C, and the cells were fixed and stained with rhodamine–phalloidin (a–e). The appearance of the actin ring at the medial region (f) and actin dots at the old end (g) was examined with respect to Mor2 accumulation at their sites in the time course. (**B** and **C**) Effect of Lat-B and TBZ on Mor2 localization. The nmt41–GFP–Mor2 strain (YS84-1) cells grown in EMM containing 8 μM thiamine were transferred into EMM medium. After cultivation for 14–15 h at 25°C, the cells were incubated with 100 μM Lat-B (B) or 100 μM TBZ (C). Cells taken at the indicated times were fixed and stained with rhodamine–phalloidin (B) or anti-tubulin antibody (C). (**D**) Mor2 localization in the *cdc3* mutant. The *cdc3-6* (profilin) mutant cells with *kan*^{r}*:nmt41-GFP:mor2*$^+$ (DH299-2) grown in EMM containing 8 μM thiamine were transferred into EMM medium. After cultivation for 12 h at 25°C, the cells were transferred to 36°C for 3–4 h, fixed, and stained with rhodamine–phalloidin. (**E**) Mor2 localization in the *nda3* mutant. The *nda3*-KM311 (β-tubulin) mutant cells with *kan*^{r}*:nmt41-GFP:mor2*$^+$ (DH297-1C) grown in EMM containing 8 μM thiamine were transferred into EMM medium. After cultivation for 11–12 h at 28°C, the cells were transferred to 18°C for 6 h, fixed, and immunostained with anti-tubulin antibodies. (**F**) Mor2 localization in *tip1*-deleted cells. The Δ*tip1* mutant cells with *kan*^{r}*:nmt41-GFP:mor2*$^+$ (DH421-2B) grown in EMM containing 8 μM thiamine were transferred into EMM medium and cultivated for 13 h at 25°C.

defective mutant is often suppressed by sorbitol (Ribas *et al*., 1991; Shiozaki and Russell, 1995). Neither the ts-growth nor round morphology of the mutant was restored by 1 M sorbitol (data not shown). These results indicated that the cell wall integrity of the *mor2* mutant was not compromised.

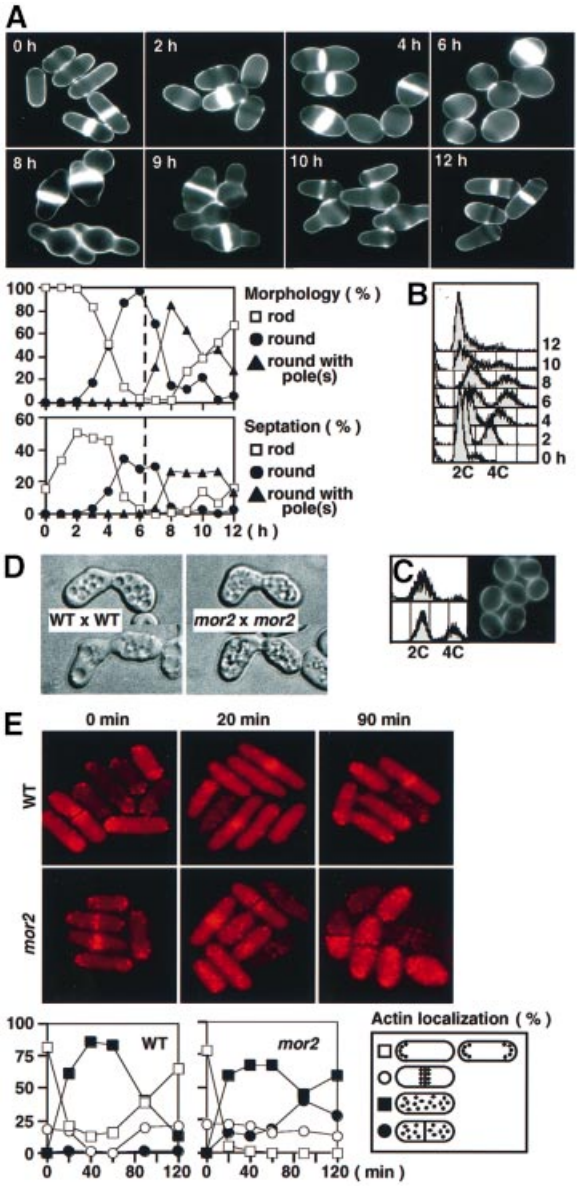

Fig. 3. Phenotypes of the *mor2* mutant. (**A** and **B**) Reversible phenotype of the *mor2* mutant. *mor2* mutant cells (DH107-4C) grown in YPD medium at 25°C were transferred to 36°C (time 0 h). After incubation for 6 h, the cells were shifted down to 28°C and incubated for 6 h (total 12 h). The cells at the indicated times were taken for observation of cell morphology after having been stained with Calcofluor (A) and for FACS analysis (B). (**C**) *mor2* mutant cells (DH107-4C) grown in YPD medium at 25°C were transferred to 36°C and incubated for 4 h. The unseptated round cells in the culture were collected by centrifugal elutriation for FACS analysis (upper panels, the collected round cells; lower panels, the culture) and for observation of morphology (isolated round cells). (**D**) Shmoo morphology of the *mor2* mutant. Wild-type (h^-) or *mor2* mutant cells (h^- *mor2-786*) were crossed with wild-type (h^+) or *mor2* mutant cells (h^+ *mor2-786*), respectively. After incubation for 24 h on malt extract (MEA) plate, the shmoo morphology of the cells was observed. (**E**) F-actin localization in the *mor2* mutant. The wild-type (972) and *mor2* mutant cells (DH192-1A) grown in YPD medium at 25°C were transferred to 36°C (time 0 h). The cells were taken at the indicated times for observation of F-actin localization after being stained with rhodamine–phalloidin.

Mor2 is required for re-localization of F-actin to the cell end(s)

To investigate the role of Mor2 in locating F-actin at the polarized growing sites, we examined the distribution of F-actin in the *mor2* mutant at 36°C. Cortical F-actin in the wild-type cells is localized as patches at the growing end(s) of the cell and the septation site at 25°C (Marks and Hyams, 1985; Marks *et al*., 1986) (Figure 3E). In wild-type cells, F-actin at the cell end(s) but not at the septation site was dispersed throughout the cells within 20 min after the shift, presumably due to heat shock stress. By 90 min after the shift, however, F-actin had re-localized at the cell end(s). In the *mor2* mutant cells, on the other hand, F-actin at the cell end(s) dispersed as in wild-type cells, but the re-localization of F-actin at the cell ends was not observed (Figure 3E). These results indicated that Mor2 was required for the re-localization of F-actin at the cell end(s).

Mor2 is important for the restriction of the growth zone(s)

To investigate the role of Mor2 in the organization of the MT cytoskeleton, we examined the MT cytoskeleton and the MT end factor Tip1, which is required for the guidance of the MT ends to their target region at the cell end(s), in the *mor2* mutant cells (Figure 4A). In the mutant cells at 25°C, cytoplasmic MTs and mitotic spindles were observed (Marks *et al*., 1986; Hagan and Hyams, 1988) (Figure 4A), and Tip1 was localized to both MT end(s) and cell tips, as in wild-type cells (Brunner and Nurse, 2000) (Figure 4A). At 36°C, with up to 3 h incubation, the MT-targeting region and normal cellular localization of Tip1 was maintained, but after 6 h the MT-targeting regions became dispersed, which resulted in Tip1 localizing throughout the cell. Despite this, Tip1 was still associated with MT end(s) (Figure 4A), suggesting that the Tip1 dispersion results from deposition by MT at random locations in the cell, but not from random dissociation from its localization sites. These results indicated that Mor2 is important for the restriction of the growth zone(s) to which Tip1 specifically targets.

To investigate further whether Mor2 is involved in polymerization of MTs, we examined the nucleation of MTs disrupted by cold shock treatment. MTs were depolymerized by cold shock treatment, and then the cells were transferred to warm medium (Mata and Nurse, 1997). We performed two experiments. In the first experiment, the *mor2* mutant cells grown at 25°C were incubated on ice for 30 min and then returned to 25°C (Figure 4B, upper panels). In the second experiment, the cells grown at 25°C were shifted up to 36°C for 6 h, incubated on ice for 30 min, and then returned to 36°C (Figure 4B, lower panels). Upon cold shock, no MTs were observed (Figure 4B, 0 min). At 25°C, the disrupted MTs were nucleated within 5 min after the return to 25°C (Figure 4B, upper panels). At 36°C, nucleation of the MTs was observed as it was at 25°C (Figure 4B, lower panels). These results indicated that Mor2 is not important for the nucleation of MTs.

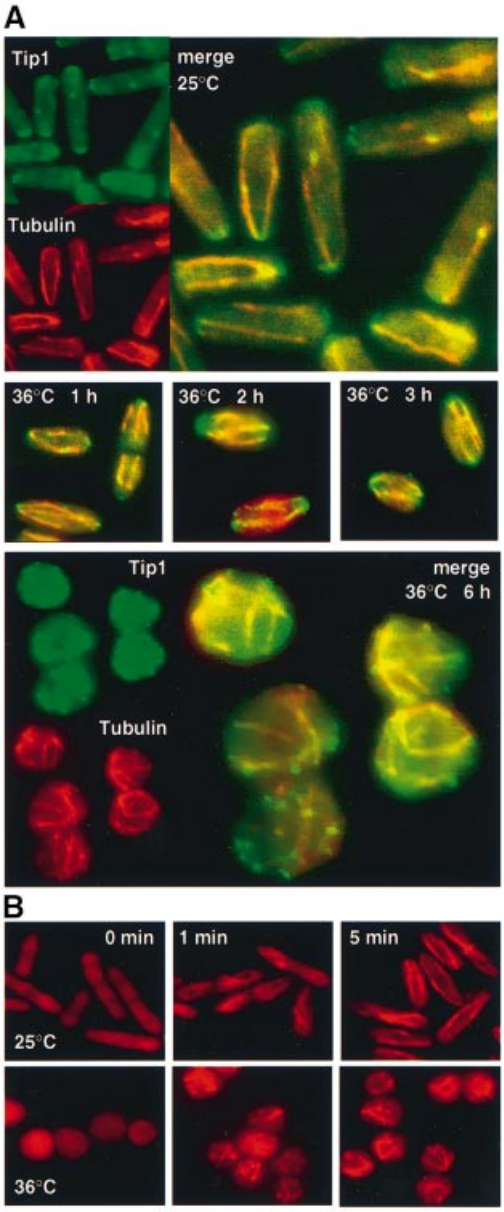

Fig. 4. Role of Mor2 in the organization of the MT cytoskeleton. (**A**) MT structure and Tip1 localization in the *mor2* mutant. The *mor2* mutant cells (DH107-4C) grown in YPD medium at 25°C were transferred to 36°C. The cells were fixed and immunostained with anti-tubulin and anti-Tip1 antibodies at the times indicated. (**B**) MT nucleation in the *mor2* mutant. Upper panels: the *mor2* mutant cells having *tip1*$^{+}$*:GFP:kan*^{r} (DH360-2B) grown in YPD medium at 25°C were incubated on ice for 30 min, re-warmed to 25°C, and collected at the indicated times for immunostaining with anti-tubulin antibodies. Lower panels: the *mor2* mutant cells grown in YPD medium at 25°C were transferred to 36°C and kept there for 6 h, incubated on ice for 30 min, and then re-warmed to 36°C and collected at the indicated times.

The mor2 mutant protein is localized as dots at 36°C

To compare the localization of the wild-type and mutant Mor2 proteins, we introduced an *nmt41* promoter-driven *GFP* gene in front of the chromosomal *mor2-282* mutant gene by the PCR-tagging method. The molecular weight of the Mor2 mutant protein was the same as that of the wild-type GFP–Mor2 protein, as judged by immunoblot analysis (Figure 5A). Using the nmt41–GFP–Mor2-282

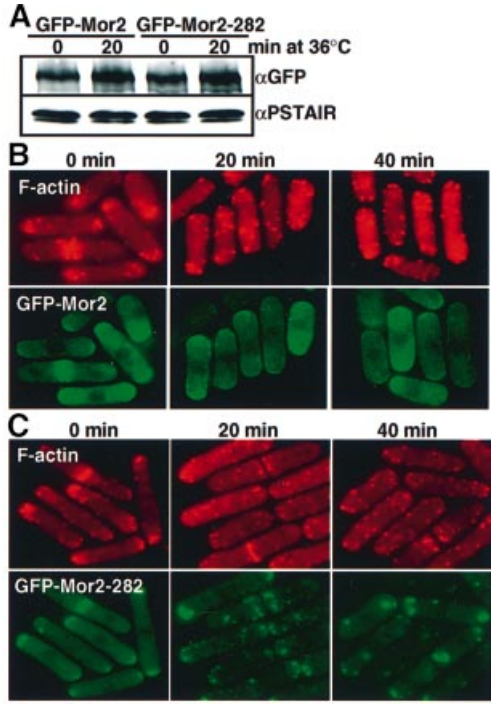

Fig. 5. Localization of the Mor2 mutant protein. (**A–C**) The nmt41–GFP–Mor2 [YS84-1 (B)] and nmt41–GFP–Mor2-282 [YY5-5 (C)] strains grown in EMM containing 8 μM thiamine were transferred into EMM medium. After cultivation for 14 h at 25°C, the cells were transferred to YPD medium at 36°C, fixed at the indicated times, and stained with rhodamine–phalloidin. The cells of the 36°C culture were taken at the indicated times for examination of the GFP–Mor2 and GFP–Mor2-282 proteins by immunoblotting (A).

cells, we examined the localization of the GFP–Mor2-282 mutant protein. At 25°C, the GFP–Mor2-282 mutant protein was localized at both ends and the septation site of the cell, as in the case of the wild-type protein (Figure 5B and C). After the shift to 36°C, a portion of the wild-type GFP–Mor2 protein was localized at the cell ends (Figure 5B), whereas the majority of GFP–Mor2-282 mutant protein was found as discrete punctate dots close to cell ends and the septation site (Figure 5C). After 6 h incubation, the GFP–Mor2-282 mutant protein was localized in cytoplasm as single large dot (data not shown). The expression levels of the wild-type and mutant Mor2 proteins were similar and no obvious change in the levels of these proteins was observed at 36°C (Figure 5A). This result indicates that the Mor2-282 protein is defective in proper cellular localization, namely at cell end(s) and the medial region of the cell, and supports the notion that Mor2 plays an essential role in the growth polarity control at the cell ends.

Survival of the mor2 mutant at 36°C requires Wee1

At present, five mitotic checkpoints, i.e. DNA structure (damage and replication), spindle assembly, spindle orientation and cytokinesis, have been identified in fission yeast. To investigate whether these checkpoint pathways are required for the survival and G_2 delay of the *mor2* mutant at the restrictive temperature, we examined the viability of the double mutants between Mor2 and Rad3 (essential for DNA structure checkpoint; Bentley *et al.*,

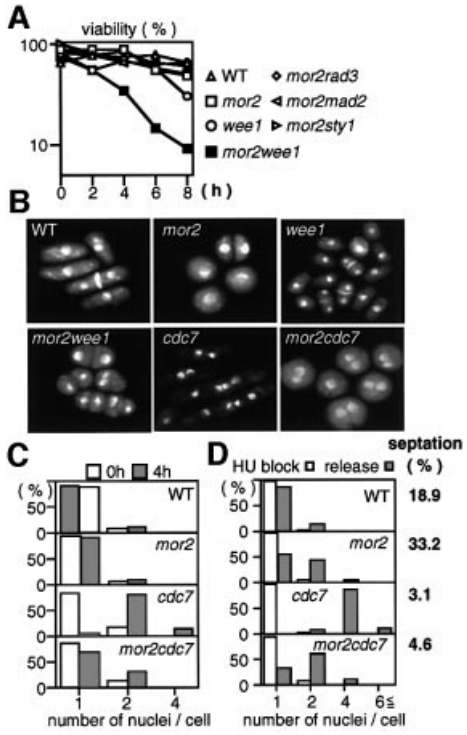

Fig. 6. Wee1-dependent G_2 delay in the *mor2* mutant. (**A**–**C**) Viability and nucleus number of the mutant. Wild-type (22), *mor2* (DH107-4C), Δ*wee1* (IY1078), *mor2*Δ*wee1* (DH203-6A), *mor2*Δ*rad3* (DH205-5D), *mor2*Δ*mad2* (DH207-5A), *mor2*Δ*sty1* (DH214-3B), *cdc7* and *mor2cdc7* (DH295-2B) grown in YPD medium at 25°C were transferred to 36°C and collected at the indicated times. The cells were spread on YPD plates, incubated for 3–4 days at 28°C, and assessed for cell viability (A). The nuclear structures of the cells incubated at 36°C for 4 h were observed by DAPI staining (B), and the number of nuclei in the cells at the indicated times was examined (C). (**D**) Wild-type, *mor2*, *cdc7* and *mor2cdc7* mutant cells grown in YPD medium at 25°C were treated with 12 mM hydroxyurea (HU) for 3.5 h and shifted to 36°C for 1 h. HU-treated cells were washed with pre-warmed YPD medium, inoculated into fresh YPD medium, incubated at 36°C for a further 4 h, fixed, and stained with DAPI. HU-treated cells prior to release from HU are labelled 'HU block', and cells released from the HU block are labelled 'release'. The septation index in 'release' is indicated.

1996), Mad2 (for spindlle assembly; He *et al*., 1997; Kim *et al*., 1998), Sty1 MAPK (for spindle orientation; Gachet *et al*., 2001) or Wee1 (inhibitory tyrosine kinase of Cdc2; Nurse, 1975; Lundgren *et al*., 1991). Among these mutants, only *mor2wee1* substantially lost viability at 36°C (Figure 6A). Microscopic observation showed accumulation of the abnormally septated or anucleate cells (64% at 4 h; Figure 6B). We further examined the appearance of the abnormal cells in the *mor2wee1* mutant by using the early G_2 cells selected by centrifugal elutriation. After 3 h of the incubation at 36°C, 48% of the mutant cells had abnormal septa that were caused by undergoing a second mitosis before the completion of cytokinesis. These results indicated that the survival and G_2 delay of the *mor2* mutant required the function of Wee1, but not that of Rad3, Mad2 or Sty1.

As a portion of the *mor2* mutant cells at 36°C (30% for 6 h) showed the septated round with two nuclei, we examined whether the G_2 delay of the *mor2* mutant was dependent on the cytokinesis checkpoint mechanism, which induces a Wee1-dependent G_2 delay until the previous cytokinesis has been completed. The 1,3-β-glucan synthase-defective mutant *cps1* arrests G_2 at the restrictive temperature by this checkpoint (Goff *et al*., 1999; Liu *et al*., 2000). The double mutants between *cps1* and the septation initiation mutant are unable to arrest the cell cycle and produce multi-nuclear cells at 36°C, indicating that the G_2 delay induced by this checkpoint requires the SIN pathway (Goff *et al*., 1999; Liu *et al*., 2000). If the G_2 delay of the *mor2* mutant was caused by this checkpoint, the G_2 delay would also require the SIN pathway, and the double mutant between *mor2* and the SIN mutant *cdc7* (Nurse *et al*., 1976) should have had multi-nuclei as in the case of *cps1*. However, the *mor2cdc7* double mutant cells at 36°C had one or two nuclei, as in the *mor2* mutant (Figure 6B and C), and the actin ring was not observed. These results indicated that the major mechanism inducing the G_2 delay of the *mor2* mutant was distinct from the cytokinesis checkpoint mechanism.

Fission yeast have a cell size control, such that the attainment of a critical cell size is required for the onset of mitosis (Nurse, 1975). Previously, Liu *et al*. (2000) showed that the *cps1* mutant arrests at a two-nuclei stage without undergoing cytokinesis, even in the hydroxyurea (HU)-treated cells that are long enough to divide. The authors then concluded that the delay of the mutant is not cell size-dependent, instead being dependent upon cytokinesis *per se* (Liu *et al*., 2000). To investigate whether the G_2 delay in the *mor2* mutant is cell size- or cytokinesis-dependent, we performed the HU experiment, and asked whether nuclear division occurred in the *mor2* mutant cells (at 36°C) that had been released from previous S-phase block at 25°C. If the G_2 delay of the *mor2* mutant was dependent on cell size control, the elongated mutant cells released from S-phase block should have accumulated more than two nuclei. This, however, was not the case. The HU-treated *mor2* mutant cells elongated and arrested with one or two nuclei (Figure 6D). In contrast, in *cdc7* mutant cells, after the release from S-phase block, 84% of cells accumulated more than four nuclei. Note that 33.2% of *mor2* mutant cells had a septum, raising the possibility that in this case, G_2 delay in *mor2* mutants is induced by the cytokinesis checkpoint (Figure 6D). Therefore, to confirm further that the G_2 delay of the *mor2* mutant is not dependent on either cell size or cytokinesis, we performed the same experiment using the *mor2cdc7* double mutant. After the release from S-phase block, 90.4% of *mor2cdc7* double mutant cells arrested at the one- or two-nucleus stage (Figure 6D). These results indicate that the major mechanism inducing the G_2 delay of the *mor2* mutant is distinct from cell size control. However, it remains possible that cell size control partially contributes to the G_2 delay in the mutant.

Wee1 protein levels are not reduced in the mor2 mutant

The Wee1 protein is known to oscillate during the cell cycle, its level decreasing in M and G_1 phases (Aligue *et al*., 1997). To investigate whether the Wee1 protein and the inhibitory tyrosine phosphorylation of Cdc2 on tyrosine-15 (Nurse, 1990) were maintained in the *mor2* mutant, we examined the amount of the tyrosine-phosphorylated form of Cdc2 in the mutant. The early G_2 cells grown at 25°C were selected by elutriation and incubated

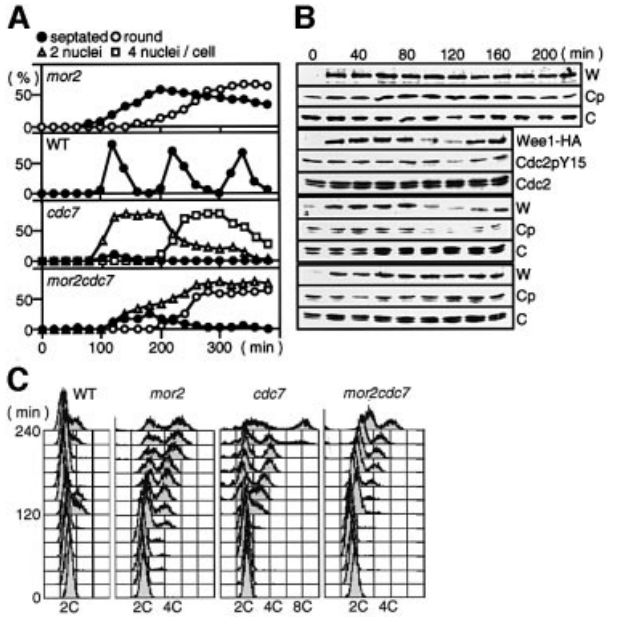

Fig. 7. Maintenance of Wee1 protein levels in the *mor2* mutant. (A–C) Early G_2 cells of wild type (RA1224), *mor2* (DH274-10B), *cdc7* (DH306-8A) and *mor2cdc7* (DH306-12B) were collected by centrifugal elutriation. The cells were released into YPD medium at 36°C, and taken at the indicated times for observation of cell morphology (**A**), immunoblotting (**B**) and FACS analysis (**C**). The total proteins (100 μg) were electrophoresed on SDS–PAGE gels. Wee1-HA protein, tyrosine phosphorylation of Cdc2, and Cdc2 protein in the samples were analysed by immunoblotting with antibodies specific for the HA epitope (Wee1-HA, W), Cdc2 phosphorylated on tyrosine-15 (Cdc2pY15, Cp) and an anti-PSTAIR antibody (Cdc2, C), respectively.

at 36°C (Figure 7). In wild-type cells, the level of the phosphorylated Cdc2 was decreased by 120 min upon mitotic entry. The Wee1 protein level in wild-type cells was decreased by 100 min, just before the decrease in the phosphorylated Cdc2. In contrast, in the *mor2* mutant, the Wee1 protein and the phosphorylated Cdc2 were maintained at high levels at 36°C until at least 4 h, when the septation index had already been decreased (Figure 7A and B).

To confirm that the G_2 delay of the *mor2* mutant was independent of the cytokinesis checkpoint, we examined the Wee1 protein and the phosphorylated Cdc2 in the *mor2cdc7* double mutant. In the *cdc7* mutant, the Wee1 protein and the phosphorylated Cdc2 oscillated as in the wild-type cells. In the *mor2cdc7* double mutant, however, both the Wee1 protein and the phosphorylated Cdc2 were maintained, as in the case of the *mor2* mutant (Figure 7A and B). The FACS profile of the double mutant was also the same as that of the *mor2* mutant (Figure 7C). Furthermore, the double mutant did not form a septum and arrested at the two-nuclei stage (Figure 7A). These results establish that the major mechanism inducing G_2 delay of the *mor2* mutant is independent of the cytokinesis checkpoint.

Functional relationship of Mor2 and Orb6

It has been suggested that a Furry-like protein functions in the same pathway as the Ndr kinase in flies and budding yeast (Cong *et al*., 2001; Du and Novick, 2002). The budding yeast Furry-like protein Pag1 forms a complex with the Ndr kinase Cbk1 (Du and Novick, 2002). To investigate the functional interaction of Mor2 with Orb6, we first examined by means of an immunoprecipitation experiment whether Mor2 could form a complex with Orb6. However, no interaction of Mor2 with Orb6 under our experimental conditions was found (data not shown). We next examined whether Mor2 and Orb6 could affect the cellular localization of each other. In the *orb6* mutant cells at 36°C, the normal cellular localization of GFP–Mor2 was disrupted (Figure 8A). Similarly, in the *mor2* mutant cells at 36°C, the Orb6–GFP protein was dispersed throughout the cell (Figure 8B). These results indicate that the localization of Mor2 and Orb6 is interdependent.

As the overexpression of the $orb6^+$ gene induces a Wee1-dependent G_2 delay (Verde *et al*., 1998), we examined whether the maintenance of the Wee1 protein in the *mor2* mutant required the function of Orb6. This was not the case. In the *mor2orb6* double mutant, the G_2 delay and the maintenance of Wee1 protein were observed as in the *mor2* mutant (Figure 8C and D). In the *orb6* mutant, Wee1 protein was maintained as in the *mor2* mutant (Figure 8C and D). Further, the phenotype of the *mor2orb6* double mutant was similar to that of the *mor2* single mutant. These results suggested that Mor2 and Orb6 function in the same pathway for cell morphogenesis.

To investigate the functional relationship of Mor2 and Orb6 further, we examined the effect of $mor2^+$ over-expression in the *orb6* mutant. In the wild-type background, the over-expression of the $mor2^+$ gene caused defects in growth (at 25 and 36°C) and morphology (round with poles) (Figure 8E and F). The over-expression of the $mor2^+$ gene in the *orb6* mutant background caused more severe defects in growth (no growth even at 30°C) and morphology (round) than it did in the wild-type background (Figure 8E and F). This result suggested that $mor2^+$ over-expression prevented the polarity control in which Orb6 functions.

Discussion

Mor2 is essential for the establishment and maintenance of growth polarity

We have demonstrated that the essential protein Mor2 is required for the establishment and maintenance of growth polarity. In the absence of Mor2 function, cells lose polarity completely, which results in the failure of F-actin to localize at the cell ends. The Mor2-mediated polarity control is important for the restriction of the growth zone(s), where the MT end factor Tip1 targets. Mor2 is localized at both ends of the cell and the septation site. The maintenance of Mor2 localization is dependent on the actin cytoskeleton. Actin localization is also dependent on Mor2, except during cell division, when Mor2 follows actin localization. This result suggests that the actin ring, which forms first, does not depend on Mor2, but that the appearance of actin dots at the cell end(s) does. The localization of Mor2 and F-actin at the cell ends is interdependent. It is possible that Mor2 has a general role in organizing the growth machinery and the associated actin. The identification of the protein(s) interacting with Mor2 is important for the understanding of the role of Mor2 in cell morphogenesis.

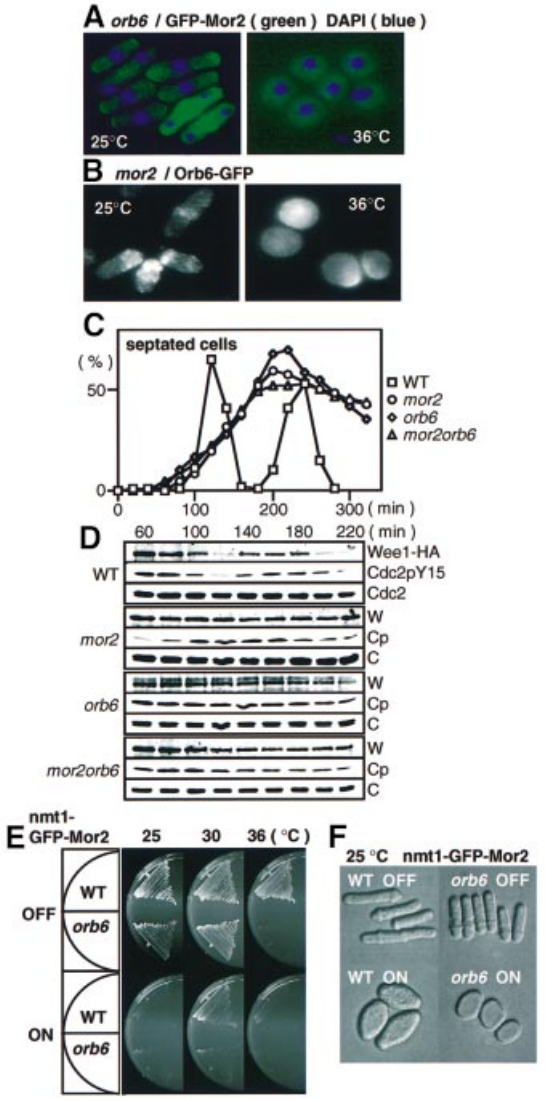

Fig. 8. Functional interaction between Mor2 and Orb6. (**A**) Localization of Mor2 in the *orb6* mutant. The *orb6* mutant cells with *kan*^{r}*:nmt41-GFP:mor2*$^+$ (DH451-3D) grown in EMM containing 8 μM thiamine were transferred into EMM medium. After cultivation for 12 h at 25°C, the cells were transferred to 36°C and kept there for 6 h. (**B**) Localization of Orb6 in the *mor2* mutant. The *mor2* mutant cells with *orb6*$^+$*:GFP:kan*^{r} (DH454–2B) and grown in YES5 medium at 25°C were transferred to 36°C and kept there for 6 h; they were then fixed and immunostained with anti-GFP antibodies. (**C** and **D**) Early G_2 cells of wild type (RA1224), *mor2* (DH274-10B), *orb6* (DH463-2C) and *mor2orb6* (DH436-12B) were collected by centrifugal elutriation. The cells were released into YPD medium at 36°C, and taken at the indicated times to determine frequency of the septated cells (C) and for immunoblotting (D). (**E**) Overexpression of Mor2 in the *orb6* mutant. Wild-type and *orb6* mutant cells with *kan*^{r}*:nmt1-GFP:mor2*$^+$ (WT, YS85; *orb6*, DH475-1C) were cultured on EMM (ON) or EMM plates containing 4 μM thiamine (OFF) at the indicated temperatures for 3 days. (**F**) The strains grown in EMM containing 4 μM thiamine were transferred into EMM medium and cultured for 18 h at 25°C.

The actin perturbation caused by Lat-B treatment induces the activation of the Sty1-Atf1-dependent mitotic checkpoint that ensures proper spindle orientation in fission yeast. It has been proposed that the formation of the cortical actin ring that encircles the early mitotic nucleus is monitored by this checkpoint (Gachet *et al*., 2001). The G_2 delay in the *mor2* mutant does not require the function of Sty1, suggesting that Mor2 is not important for the actin ring formation. Indeed, Mor2 follows actin localization during cell division. A mechanism that determines the growth polarity in septum formation would be different from that in interphase.

In fission yeast, Ras1, Cdc42 and Pak1/Shk1 participate in a pathway that regulates morphogenesis and mating (Chang *et al*., 1994; Miller and Johnson, 1994; Marcus *et al*., 1995; Ottilie *et al*., 1995). The mating and the shmoo morphology of the *mor2* mutant seem to be normal, indicating that Mor2 is not important for the mating process. However, it remains a possibility that Mor2 participates in the Ras1–Cdc42–Pak1/Shk1 branch that is important for morphogenesis during vegetative growth. It is interesting to examine whether Mor2 interacts with this pathway.

Functional conservation and divergence of Furry-like proteins in yeasts

The Furry-like proteins in yeasts are involved in cell morphogenesis. However, we have found functional divergence between the fission yeast Mor2 and the budding yeast Pag1 proteins. Mor2 was important for the localization of actin at the cell ends during interphase, but not for the formation of shmoo during mating. In contrast, Pag1 was important for the organization of actin cytoskeleton during mating but not during vegetative growth (Du and Novick, 2002). These results indicate that the Furry-like proteins of the fission and budding yeasts are implicated, at least in part, in the different cellular processes, although both proteins are essential for cell morphogenesis. Furthermore, as the *tao3*/*pag1* mutant was originally isolated as a mutant that caused the altered transcription of the *OCH1* gene (J.Horecka and Y.Jigami, the *Saccharomyces* Genome Database), we examined the transcription level of the *och1*$^+$ gene in the *mor2* mutant. However, the *mor2* mutation did not affect its transcription (N.Kishimoto and D.Hirata, unpublished results). This result also suggests that different proteins are functionally related to the Furry-like proteins in the budding and fission yeast.

Functional interaction between Furry-like Mor2 and Ndr kinase Orb6

It has been suggested that Furry-like proteins function in the same pathway as the Ndr kinase in *D.melanogaster* and budding yeast (Cong *et al*., 2001; Du and Novick, 2002). Although we have not found a physical interaction between Mor2 with Orb6, it is possible that these two proteins function in the same pathway. Indeed, the localization of Mor2 and Orb6 is interdependent, and phenotypes of the *mor2orb6* double mutant were similar, if not identical, to those of single mutants. It remains possible that Mor2 interacts with Orb6 in the specific stage of the cell cycle. However, we also found a difference in the phenotype between the *mor2* and *orb6* mutants. The phenotype of the *orb6* mutant but not that of the *mor2* mutant was rescued by sorbitol (Y.Sogabe and D.Hirata, unpublished results), suggesting that Orb6 but not Mor2 is important for the maintenance of cell-wall integrity. Mor2 and Orb6 would therefore have somewhat different functions in cell morphogenesis.

The mor2 mutation induces a Wee1-dependent G_2 delay

The *mor2* mutation causes the loss of growth polarity, inducing G_2 delay, suggesting that the defect in the establishment of growth polarity activates the mechanism coordinating morphogenesis with the cell cycle. A target of the mechanism is the tyrosine kinase Wee1. The mechanism inducing the G_2 delay of the *mor2* mutant might be similar to the morphogenesis checkpoint monitoring the establishment of polarity in bud formation in budding yeast (reviewed in Lew, 2000).

In the morphogenesis checkpoint, the Cdc28 inhibitory kinase Swe1 is continuously accumulated during the G_2 delay by increases in both *SWE1* transcription and inhibition of Swe1 degradation. Swe1 is targeted for rapid degradation in G_2, but its degradation is inhibited by the checkpoint. Hsl1 (budding yeast homologue of the fission yeast Nim1 kinase) and Hsl7 play a direct role in targeting Swe1 for degradation. The downregulation of the Hsl1–Hsl7 pathway plays a role in the morphogenesis checkpoint response (McMillan *et al*., 1999; Shulewitz *et al*., 1999). In fission yeast, it is not known whether the Nim1 kinase targets Wee1 for degradation. The overexpression of the *nim1*$^+$ gene did not affect the abundance of Wee1 (M.Suda and D.Hirata, unpublished results). Nim1 directly phosphorylates and inhibits Wee1 in fission yeast, but it is not known whether Hsl1 inhibits Swe1 activity in budding yeast. Further, Skb1 (fission yeast homologue of the budding yeast Hsl7) has a role as a mitotic inhibitor (Gilbreth *et al*., 1998). The putative role for Skb1 is opposite to that of Hsl7, as a negative regulator of Swe1. These results suggest that the regulatory mechanisms of the Cdc2/Cdc28 inhibitory kinase Wee1/Swe1 in the checkpoint response in both yeasts are different from each other.

Previously, Verde *et al*. (1998) showed that the overexpression of Orb6 induces Wee1-dependent G_2 delay, and thereby concluded that the control of cell size required for division is linked via Orb6 to the growth machinery. We therefore examined whether Orb6 is important for the maintenance of Wee1 protein in the *mor2* mutant; however, this was not the case. Furthermore, in the *orb6* single mutant, the Wee1 protein was maintained as in the *mor2* mutant. This result indicates that both loss and gain of Orb6 functions induce a Wee1-dependent G_2 delay. It would be of interest to examine the Orb6 kinase activity in the *mor2* mutant.

What defect(s) in the *mor2* mutant produces a signal activating this mechanism and how Wee1 is upregulated by the signal are the next subjects to be addressed. To understand better the mechanism monitoring growth polarity in fission yeast, a G_2-delay-defective mutant(s) in the *mor2* mutant should be isolated.

Materials and methods

Media and general methods

All media and standard methods were followed as described previously (Moreno *et al*., 1991).

Isolation of conditional lethal mutants with polarity defects

Wild-type cells (HM123, h$^-$*leu1-32*) were mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as described previously (Uemura and Yanagida, 1984). In total, 200 960 colonies were screened for temperature sensitivity, and 2822 ts mutants were isolated. Mutants that showed altered cell shape were then selected visually by fluorescence microscopy after staining with Calcofluor White (Hirata *et al*., 1998a; Radcliffe *et al*., 1998). The isolated round mutants were examined further for sensitivity to the protein kinase inhibitor staurosporine (STS; 1.5 μg/ml).

Cloning and disruption of the mor2$^+$ *gene*

An *S.pombe* genomic library constructed in the vector pAL-KS (a gift from Dr Nakamura) was used for the isolation of plasmids that complemented the ts *mor2* mutant (DH107-4C). One out of 9600 colonies was capable of growing at 36°C and showed normal rod shape. The segregation analysis showed that the Ts$^+$ phenotype was plasmid dependent. One plasmid (pMR301) containing 9.3 kb insert was isolated. The result of the transposon insertion indicated that an internal region of ~6.5 kb was essential for the complementing activity of pMR301. The resulting plasmid (pMR310-9) lost the complementing activity, but Ts$^+$ colonies appeared from transformants (DH107-4C) containing pMR310-9. We found that pMR310-9 containing the *LEU2* marker was integrated into the genome via homologous recombination in Ts$^+$ transformants. Tetrad analysis between the Ts$^+$ integrant and a wild-type strain yielded 4 Ts$^+$ spores in each tetrad, and further tetrad analysis between the integrant and the *mor2* mutant yielded four viable spores, in which Ts$^+$ Leu$^+$ and Ts$^-$ Leu$^-$ segregated 2:2 in each tetrad. This demonstrated that pMR310-9 contained the *mor2*$^+$ gene itself. For disruption of the *mor2*$^+$ gene, a 5.1 kb *Bam*HI–*Sac*I fragment of pMR301 was subcloned into pUC119, and a 3.0 kb *Hin*dIII–*Hin*dIII fragment of the insert was replaced by a *ura4*$^+$ fragment, yielding pMR309 (pΔmor2::ura4$^+$). A 3.9 kb *Bam*HI–*Sac*I fragment containing the deleted *mor2* gene (Δmor2::ura4$^+$) was used to disrupt the *mor2*$^+$ gene. The disruption was verified by Southern hybridization.

Epitope and GFP tagging

C- or N-terminal tagging of proteins with 3HA or GFP was performed using PCR-generated fragments (Bahler *et al*., 1998).

Immunological assay

Total cell extracts were prepared as described in Matsusaka *et al*. (1995). Protein samples were electrophoresed on SDS–PAGE gels and electrophoretically transferred onto nitrocellulose filters. The primary antibodies, anti-GFP (8362-1; Clontech), anti-HA antibodies (HA.11; BabCO), anti-PSTAIRE (sc-53; Santa Cruz Biotechnology) and anti-phospho-Cdc2 (9111S; Cell Signaling Technology), were used for detection of GFP–Mor2, Mor2-HA, Cdc2 and Cdc2 phosphorylated on Tyr15, respectively. Horseradish peroxidase-conjugated sheep anti-rabbit IgG or anti-mouse IgG (Amersham) was used as the second antibody. Enhanced chemiluminescence (ECL; Amersham) was used to detect bound antibody.

Cytological techniques

Cytological techniques were performed according to Alfa *et al*. (1993) and Matsusaka *et al*. (1995). For F-actin staining, rhodamine–phalloidin was used. For tubulin staining, a monoclonal anti-tubulin antibody (TAT-1; a gift from Dr Keith Gull) was used as the primary antibody, and Cy3-conjugated sheep anti-mouse IgG (Sigma) as the second antibody. Calcofluor white (Sigma) was used to monitor cell wall growth, and 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was used for observing chromatin regions.

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