Ste20/GCK kinase Nak1/Orb3 polarizes the actin cytoskeleton in fission yeast during the cell cycle

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This article is dedicated to our colleague and friend Heidi Browning, who died last year

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Summary

Polar growth is a crucial process during cell morphogenesis. The microtubule and actin cytoskeletons, and vesicular transport are tightly regulated to direct cellular growth and to generate specific cell forms. We demonstrate here that the Ste20-related protein kinase Nak1/Orb3 is required in fission yeast to polarize the actin cytoskeleton at the tips of the cells and for cell separation, and so is involved in controlling both cell shape and late stages of cytokinesis. The localization of the Nak1/Orb3 kinase to the cell tips, a medial ring and the spindle-pole bodies changes during the cell cycle, and the accumulation of F-actin at the cell tips is dependent on Nak1/Orb3 kinase. The phosphorylation of Nak1/Orb3 is periodic during the cell cycle and could be part of a mechanism that relocalizes a constitutively active kinase from the cell tips to the middle of the cell, thereby coordinating reorganization of the actin cytoskeleton and regulation of cell separation with cell-cycle progression.

Key words: Nak1 kinase, Orb3, Morphogenesis, Actin cytoskeleton, Polar growth, Cell separation

Introduction

The ability of tissues and organs to develop form depends on single cells having defined shapes. Polar growth contributes to shape generation in many cell types including neurons and epithelial cells, and the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (Chang and Peter, 2003; Drubin and Nelson, 1996; Fukata et al., 2002; Hayles and Nurse, 2001). The cylindrical fission-yeast cells grow from the two tips at the cell ends, which are positioned in opposition to each other (Snell and Nurse, 1993). The growth zones are marked by cortical F-actin patches associated with cell-wall deposition at the growing ends of interphase cells (Marks et al., 1986). Upon the onset of mitosis, growth ceases and F-actin patches disappear from the ends and are re-established in the middle of the cell, where the actomyosin ring forms. After cytokinesis, the growth zones become repositioned to the ends of the cells (Marks et al., 1986; Mitchison and Nurse, 1985). These rearrangements of actin-based growth zones are linked to progression through the cell cycle.

Genetic screens have identified genes involved in generating cell form in fission yeast (Brunner and Nurse, 2000; Hirata et al., 1998; Radcliffe et al., 1998; Snell and Nurse, 1994; Verde et al., 1995), showing that both the actin and the microtubule cytoskeletons are important to establishing and maintaining cell shape. A group of T-shaped mutants define a microtubule-based system that is involved in the exact positioning of the growth zones at opposite cell ends, thereby maintaining long-distance order over the length of the cell. The local organization of the growth zones themselves is not affected in these mutants. Rather, normal growth zones are formed but are not placed correctly at the ends of the cell, leading to branched cells growing at an angle off the longitudinal axis. Tip1, a CLIP170-like protein acts as a microtubule guidance system to orient the microtubules along the longitudinal axis of the cell during interphase, so that three or four parallel bundles of microtubules extend from the vicinity of the nucleus to the cell tips (Brunner and Nurse, 2000). Tea1 protein is transported along the microtubules to the ends of the cell and is deposited there (Mata and Nurse, 1997). It serves as a marker protein for the cell ends and probably directs components of the growth machinery to their correct position. Finally, the kinesin-like motor protein Tea2 is needed to localize Tea1 to the poles and to maintain normal interphase microtubules, which terminate at the ends of the cell (Browning et al., 2000). Tea2 itself travels on the tips of polymerizing microtubules to the ends of the cell, where it needs Tea1 to become anchored (Browning et al., 2003).

A second group of mutants identifies genes involved in the local organization of polar F-actin at growth zones (Hirata et al., 1998; Snell and Nurse, 1994; Verde et al., 1995). Mutants with defects in actin polarization often display round phenotypes, indicating a loss of the ability to generate localized growth zones. Several small GTPases and associated proteins such as Ras1, Cdc42 and Rho1 have been implicated in polar growth (Miller and Johnson, 1994; Nakano et al., 1997; Pichova and Streiblova, 1992), and the protein kinases Pak1/Shk1 and Orb6, and the membrane protein Mor2 are involved in polarization of the actin cytoskeleton in fission-yeast cells (Hirata et al., 2002; Marcus et al., 1995; Verde et al., 1998).

To gain further insight into the mechanisms by which the

fission-yeast cell generates and maintains growth zones, and coordinates them with cell-cycle progression, we have analysed the orb-shaped mutant *orb3* (Snell and Nurse, 1993; Verde et al., 1995). We show here that *orb3* codes for the essential Ste20-related kinase Nak1 (Huang et al., 2003). The Nak1/Orb3 kinase domain shows the highest homology to the eukaryotic germinal centre kinase (GCK) III subfamily of Ste20 kinases, including SOK1, Mst3 and Mst4 (Dan et al., 2001). We demonstrate that the Nak1/Orb3 kinase is essential for polarizing the actin cytoskeleton in fission-yeast cells and for cell separation. It is needed for F-actin to become concentrated at the cell poles during interphase and to regulate late stages of cytokinesis and cell separation. Nak1/Orb3 protein kinase shows constitutive kinase activity but undergoes periodic phosphorylation, and its localization changes during the cell cycle.

Materials and Methods

Fission-yeast methods and strains

Unless otherwise stated, cells were grown in rich medium containing yeast extract plus supplements (YES), and genetic manipulations and lithium-acetate transformations were performed as described previously (Moreno et al., 1991). Strains used in this study are listed in Table 1.

Cloning and disruption of *orb3/nak1*

Orb3-167 cells were transformed with a cDNA library that is based on the vector pREP4X and expressed using the *nmt1* promoter (Forsburg, 1993; Maundrell, 1990). Cells were replica plated to yeast-extract plates containing 2.5 μg ml^{-1} phloxine B and incubated at 36°C for 2 days. 150,000 colonies were visually screened and five colonies showing wild-type morphology and plasmid instability were identified. They all contained the full open reading frame SPBC17F3.02. Diploid cells were obtained by crossing of haploid strains PN67 and PN558, and selection on Edinburgh minimal medium (EMM) plates (Moreno et al., 1991) lacking adenine. Disruption of the full open reading frame was performed in diploid cells by homologous recombination with a disruption fragment that was generated by PCR using the pFA6-kanMX6 plasmid (Bähler et al., 1998).

Myc and GFP tagging

Tagging of Nak1/Orb3 and of temperature-sensitive Nak1/Orb3-167 was performed by PCR-based gene targeting, as described previously (Bähler et al., 1998). The Myc tag was added directly to the C-terminus of Nak1/Orb3 using the pFA6a-13Myc-kanMX6 plasmid. Between the protein and the C-terminal green fluorescent protein (GFP) tag, a pentaglycine stretch was inserted that was encoded by the 5′ primer for the PCR reaction with the pFA6a-GFP(S65T)-kanMX6 plasmid. Both constructs are expressed from the endogenous promoter and integrated at the genomic locus.

Actin staining and live-cell microscopy

Exponentially growing cells (~1×10^8) were fixed by adding formaldehyde (TAAB Laboratories) up to a final concentration of 2.7%. Cells were incubated for 35 minutes at 25°C or for 10-20 minutes at 35°C. Samples were washed three times in PEM buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM Mg_2SO_4), once in 1% Triton X-100 in PEM and a further three times in PEM. The cells were stained with rhodamine-phalloidin (Cambridge Bioscience) at 4°C overnight (Alfa et al., 1993). Cells were visualized at room temperature using a Zeiss Axioplan2 microscope (Plan Neofluar 40×/0.75 and Plan Apochromat 100×/1.3) equipped with a chilled Hamamatsu CCD camera (C4742-95) and Kinetic Imaging AQM software or with a Coolsnap HQ camera and Metamorph software. For live-cell imaging, cells were grown in filtered EMM medium to mid-log phase. To increase the signal strength of polarity markers at cell ends (Browning et al., 2000) for quantitative analysis, cultures were grown at 25°C for 2 days into starvation and diluted 1:20 in fresh filtered EMM medium. Cells were imaged 3 hours after dilution.

Immunofluorescence microscopy

Cells (~5×10^8) grown in minimal medium were collected by filtration and fixed in –70°C methanol for 8 minutes. They were washed three times in PEM buffer, resuspended in PEM and treated with 100 μg ml^{-1} Novozyme and 50 μg ml^{-1} Zymolyase for 15 minutes at 32°C. After washing the samples three times with PEM containing 1.2 M sorbitol, the cells were permeabilized with 1% Triton X-100 in PEM and again washed three times with PEM buffer. Cells were blocked in PEMBAL [PEM + 100 mM lysine hydrochloride, 0.1% NaN_3, 1% bovine serum albumin (BSA)] for 30 minutes. For tubulin staining, TAT1 monoclonal antibody (a gift from K. Gull, University of Manchester, UK) (Woods et al., 1989) was used at 1:50 dilution. For actin and Nak1/Orb3-GFP co-staining, monoclonal actin N350 (Amersham) and a polyclonal antibody against GFP (Sawin et al., 1999) were used at 1:250 and 1:200, respectively, together with Alexa-488-conjugated goat anti-rabbit and Alexa-546-conjugated goat anti-mouse antibodies as secondary antibodies (Molecular Probes) at 1:500 dilution. Images were acquired with a laser scanning confocal microscope LSM510 (Zeiss).

Table 1. Fission-yeast strains

Strain	Genotype	Reference
PN1004	*orb3-167 ade6-M210 leu-32 h⁻*	Snell and Nurse, 1994
PN4279	*orb3-167 ade6-M216 leu1-32 ura4-D18 h⁻*	This study
PN67	*ade6-M216 h⁻*	P. Nurse collection
PN71	*ade6-M210 leu1-32 h⁻*	P. Nurse collection
PN557	*ade6-M210 ura4-D18 leu1-32 h⁻*	P. Nurse collection
PN4371	*orb3::KAN ade6-M210 leu1-32 ura4-D18 h⁻ [pREP4X-nmt1-orb3]*	This study
PN4694	*orb3-GFP KAN ade6-M216 leu1-32 ura4-D18 h⁻*	This study
PN10142	*orb3-GFP KAN leu1-32 ura4-D18 h⁻*	This study
PN10141	*orb3-GFP KAN SAD1-DsRed KAN leu1-32 ura4-D18 h⁻*	This study
PN4705	*orb3-167 cdc2-M26 ade6-M210 leu1-32 ura4-D18 h⁻*	This study
PN4524	*orb3-167 cdc25-22 leu1-32 h⁻*	This study
PN4703	*orb3-MYC KAN ade6-M210 leu1-32 ura4-D18 h⁻*	This study
PN1420	*cdc25-22 leu1-32 h⁺*	P. Nurse collection
PN4707	*orb3-MYC KAN cdc25-22 leu1-32 h⁺*	This study
PN4715	*orb3-167-MYC KAN ade6-M210 leu1-32 h⁻*	This study
PN4710	*orb3-167-GFP KAN ade6-M219 leu1-32 h⁻*	This study

Protein extracts

Cells (2×10^8) were washed in ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 1 mM NaN_3, 10 mM EDTA, pH 8.0), resuspended in HB buffer [25 mM MOPS, pH 7.2, 15 mM $MgCl_2$, 15 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and frozen in liquid nitrogen. They were thawed again and extracted by vortexing with glass beads. Protein concentrations were determined by the bicinchoninic-acid assay (Sigma), and for each sample equal amounts of protein were incubated at 95°C in 2× sample butter for 3 minutes and separated by SDS-PAGE (Laemmli, 1970). Proteins were transferred onto nitrocellulose membranes and detected with monoclonal antibodies against Myc (monoclonal 9E10, 1:1000).

Phosphatase treatment

λ-Phosphatase (New England Biolabs), λ-phosphatase buffer and 2 mM $MnCl_2$ were added to protein extracts. The samples were incubated at 30°C for 20 minutes and the reaction was stopped with 2× SDS sample buffer (Laemmli, 1970) and by incubating at 95°C for 3 minutes.

In vitro kinase assay

For each time point, 2×10^8 cells were harvested and frozen in liquid nitrogen. Cells were thawed on ice, resuspended in 1 ml ice-cold stop buffer and transferred in 1.5 ml reaction tubes. After a centrifugation for 30 seconds at 1000 ***g*** and 4°C, the pellet was resuspended in 100 µl lysis buffer [25 mM MOPS, pH 7.2, 15 mM $MgCl_2$, 15 mM EGTA, 0.2% Triton X-100, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 2.4 mg ml^{-1} complete EDTA-free protease inhibitor cocktail (Roche)]. Then, 1.5 ml glass beads (425-600 µm) were added and cells were broken in a FastPrep cell breaker (Thermo) for 4 seconds at full speed. Another 100 µl lysis buffer were added and the lysate was cleared by centrifugation at 21,000 ***g*** for 8 minutes at 4°C. The cleared lysate was diluted with 320 µl lysis buffer, 15 µl prewashed protein-G/Sepharose (Amersham) and 6 µg anti-Myc antibody 9E10 were added, and the immunoprecipitation was performed at 4°C for 35 minutes. The precipitates were washed once with 1 ml wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA) including 0.2% Triton X-100 and all inhibitors used in the lysis buffer, once with wash buffer containing 0.2% Triton X-100 without inhibitors, once with wash buffer containing 0.1% Triton X-100 without inhibitors and twice with kinase buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$). Samples were split in half with the last wash. One half was denatured with SDS sample buffer, proteins were subjected to SDS-PAGE, transferred to PVDF membranes and detected with anti-Myc antibodies 9E10 as a control for equal amounts of precipitated Nak1/Orb3-Myc protein. The other half was used for the kinase reaction. The precipitates were resuspended in 15 µl kinase buffer supplemented with 1 mM dithiothreitol, 0.33 µg $µl^{-1}$ casein, 1 µM ATP and 5 µCi [^{32}P]γATP (Amersham) per reaction. The samples were incubated at 32°C for 30 minutes. The reaction was stopped with 15 µl 2× SDS sample buffer and subjected to SDS-PAGE. The gels were dried and quantified using a phosphor imager.

Results

The *orb3* gene encodes the essential Ste20-related kinase Nak1

We cloned the *orb3* gene by complementation of *orb3-167* mutant cells using visual screening. The *orb3* mutants were transformed with a cDNA library and five independent clones were identified that rescued the morphology defect. The plasmids isolated from these clones all contained a complete open reading frame coding for an N-terminal Ser/Thr kinase that has been described as the essential Nak1 kinase (Huang et al., 2003). Based on the homology of the kinase domains, Nak1/Orb3 is most closely related to the germinal centre kinase III subfamily of eukaryotic Ste20 kinases (Dan et al., 2001). The known human members of this subfamily (SOK1, MST3 and MST4) have been implicated in the regulation of stress response, apoptosis and proliferation but the molecular mechanisms involved and their cellular targets are largely unknown (Huang et al., 2002; Pombo et al., 1996; Qian et al., 2001; Sung et al., 2003).

Deletion of the fission yeast *nak1/orb3* gene resulted in

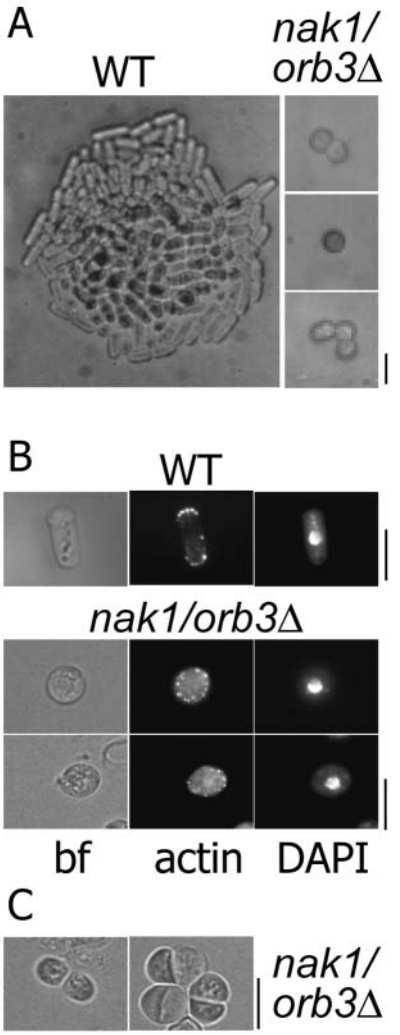

Fig. 1. Effect of the *nak1/orb3* deletion on morphology, actin cytoskeleton and cytokinesis of fission-yeast cells. (A) The *nak1/orb3* deletion phenotype. Diploid cells lacking one copy of *nak1/orb3* were sporulated; the spores were dissected and incubated at 25°C, and microphotographs were taken 48 hours later. One example of wild-type (WT) and three of *nak1/orb3*Δ microcolonies are shown. (B,C) Phenotype of *nak1/orb3*Δ cells after loss of a complementing *nak1/orb3* plasmid. A *nak1/orb3*Δ strain complemented with the plasmid pREP4X-*nmt1-nak1/orb3* and a wild-type strain were grown without selection. Fixed cells were stained with rhodamine-phalloidin for actin and with DAPI to show the nuclei. Bars, 10 µm.

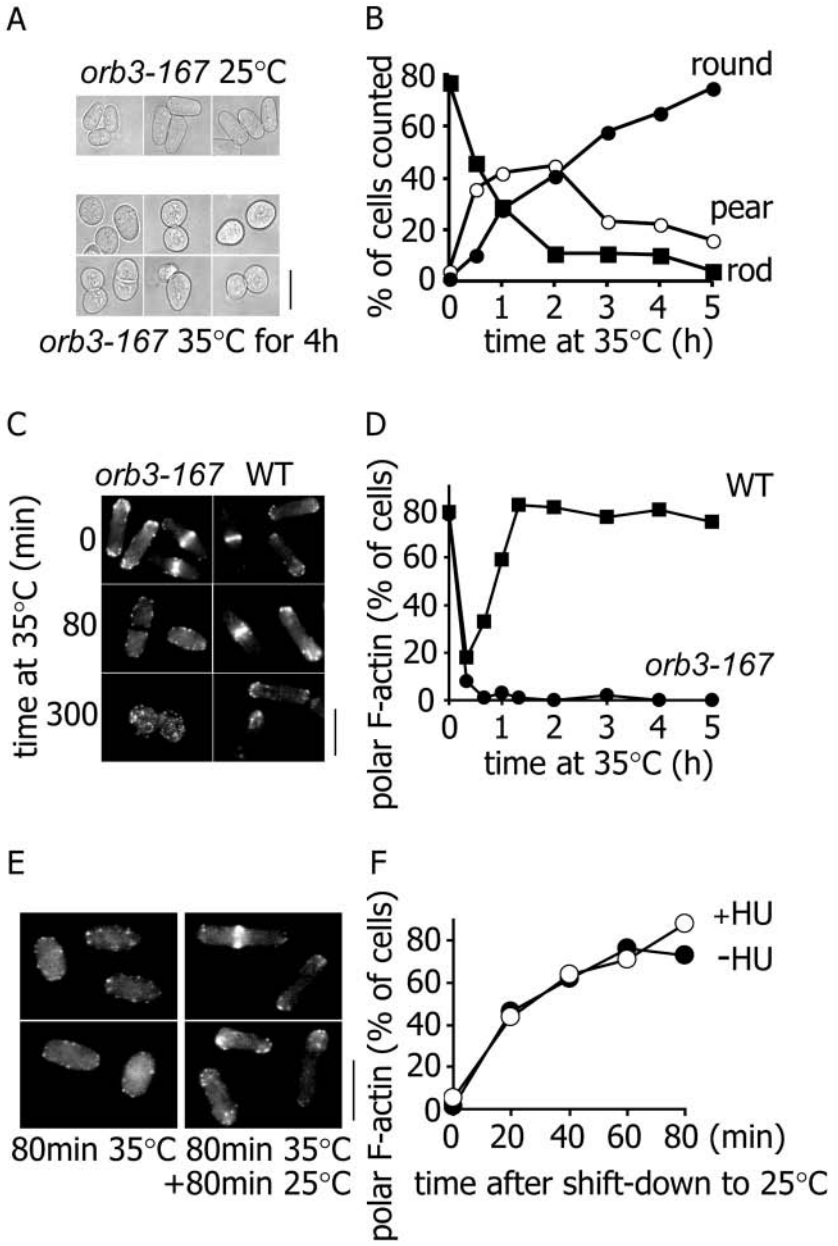

Fig. 2. Changes in shape and F-actin distribution in *nak1/orb3-167* cells. (A) Morphology of *nak1/orb3-167* cells at 25°C (top) and 4 hours after the shift from 25°C to 35°C (bottom). (B) Morphological changes of *nak1/orb3-167* cells up to 5 hours after temperature shift to 35°C. Exponentially growing cells were transferred from 25°C to 35°C and the proportions of rod-shaped, pear-shaped and round cells were determined for each time point. (C) F-Actin delocalization in *nak1/orb3-167* cells. Wild-type (WT) and *nak1/orb3-167* cells were grown at 25°C and samples taken before (0) and 80 minutes (80) and 300 minutes (300) after transfer to 35°C. The cells were fixed and stained with rhodamine-phalloidin for actin. (D) Changes of polar F-actin patches in wild-type and *nak1/orb3-167* cells after a temperature shift from 25°C to 35°C. Cells were grown at 25°C and samples were taken before (time point 0) and after transfer to 35°C at time points indicated. The proportions of fixed and rhodamine/phalloidin-stained cells with polar F-actin patches was determined. (E,F) Repolarization of the actin cytoskeleton after a shift of *nak1/orb3-167* cells from 35°C to 25°C. The *nak1/orb3-167* cells were grown at 25°C, transferred to 35°C for 80 minutes (E, left) and shifted down to 25°C (E, right). Cells were fixed in formaldehyde and stained with rhodamine-phalloidin. (F) Samples were taken of *nak1/orb3-167* cells up to 80 minutes after transfer from 35°C to 25°C (–HU, closed circles). The *nak1/orb3-167* cells were blocked in 15 mM hydroxyurea for 3 hours at 25°C, then shifted to 35°C for 80 minutes and shifted back to 25°C to follow actin relocalization in blocked cells (+HU, open circles). At the time points indicated, the cells were fixed and stained with rhodamine-phalloidin. The proportions of cells with polarized F-actin patches at the cell tips. 200 cells were counted for each time point and experiments were performed three times. Bars, 10 μm.

round cells that lost their normal rod-shaped morphology completely and stopped proliferating after no more than four rounds of division (Huang et al., 2003) (Fig. 1A). To determine the reasons for this change in cell shape, we examined the cytoskeletons of cells lacking *nak1/orb3*. A deletion strain complemented with a plasmid expressing *nak1/orb3* was grown without selection for the *nak1/orb3* plasmid and cells were fixed in formaldehyde and stained for F-actin with rhodamine-phalloidin. The staining of spherical cells, which had lost the complementing plasmid, was examined. The polar organization of the actin cytoskeleton seen in wild-type cells was fully disrupted in *nak1/orb3*Δ cells and F-actin dots were scattered all around the cortex (Fig. 1B). DAPI staining showed single nuclei in non-septated cells (Fig. 1B). Additionally, about 50% of the round cells accumulated with a septum, suggesting some defect in late stages of cytokinesis (Fig. 1C).

nak1/orb3 is required for polar actin localization to the cell tips and cell separation

To investigate further the function of the essential Nak1/Orb3 kinase in cell polarity and morphogenesis, we studied the temperature-sensitive mutant *orb3-167*, which enabled us to analyse the effects directly following Nak1/Orb3 inactivation. *Orb3-167* mutant cells gradually lost polarity when transferred from the permissive temperature of 25°C to the restrictive temperature of 35°C. The number of pear-shaped cells increased within the first 2 hours at 35°C and the cells became increasingly rounded until they were completely spherical after 5 hours (Fig. 2A,B). Analysis of the actin cytoskeleton showed that the F-actin patches at the tips of growing cells become delocalized after the shift to a high temperature before the appearance of major changes in cell shape. At 80 minutes, F-actin dots could be seen to be evenly distributed under the cortex, even though cells were still rod shaped (Fig. 2C).

Temporary delocalization of actin patches from the tips shortly after a temperature shift to 35°C was also observed in wild-type cells but, by 30 minutes at 35°C, this was reversed and, by 80 minutes, F-actin had completely returned to the poles of wild-type cells (Hirata et al., 2002) (Fig. 2D). By contrast, *orb3* mutant cells did not repolarize actin at the restrictive temperature (Fig. 2D). Actin localization to the tips of the cell and polar growth in general therefore requires a functional *nak1/orb3* gene. Inactivation of *nak1/orb3* quickly leads to a complete and permanent loss of polar F-actin distribution.

The *nak1/orb3* mutant cells shifted from 35°C back to 25°C could reverse the observed depolarization of the actin cytoskeleton. After incubation at 35°C for 80 minutes, the cells had delocalized F-actin but were still rod shaped (Fig. 2E, left, 2F); 20 minutes after shifting back to 25°C, these cells began to accumulate actin at their tips and, within 80 minutes, they had repolarized the actin cytoskeleton completely (Fig. 2E, right, 2F, –HU). Spherical *orb3-167* cells that had been incubated for several hours at 35°C were also able to repolarize after shift-down (data not shown), which demonstrates that the temperature-sensitive effect of the *orb3-167* mutation on the actin cytoskeleton is reversible and allows the function of Nak1/Orb3 kinase to be switched on and off. To test whether *nak1/orb3* mutant cells have to go through mitosis to restore polar F-actin, we blocked the cells in S-phase with hydroxyurea (HU) at the permissive temperature. The blocked cells were shifted to 36°C for 80 minutes to delocalize F-actin and back to 25°C to assess its relocalization. In the following 80 minutes, blocked cells in HU repolarized with similar kinetics to dividing cells without HU, showing that *nak1/orb3-167* mutant cells do not have to go through mitosis to relocalize F-actin to the cell tips (Fig. 2F, +HU).

At the restrictive temperature, not only the polar shape of the cells but also cytokinesis and cell separation were affected by the *nak1/orb3* mutation. Cultures of *nak1/orb3-167* cells accumulated up to 50% of unseparated cells at restrictive temperature (Fig. 1C). To analyse the stage at which cytokinesis is affected by the *nak1/orb3* mutation, we followed the formation of actomyosin rings in mitotic cells (Fig. 3A, red arrow, Fig. 3B). At restrictive temperature *nak1/orb3-167* cells can produce actin rings that constrict forming a septum (Fig. 3A, bottom, Fig. 3B). However, separation of the cells was strongly impaired, leading to 30% of cells having a CalcoFluor-stainable septum (Fig. 3B). Also, the number of cells with a constricting actomyosin ring and a partially closed septum nearly doubles (Fig. 3A, bottom, Fig. 3B), which suggests a delay in ring constriction in *nak1/orb3* mutant cells. These results indicate that, in addition to its involvement in polar growth, *orb3* has a role in late stages of cytokinesis, when it is required for a fully functional actomyosin ring and for cell separation.

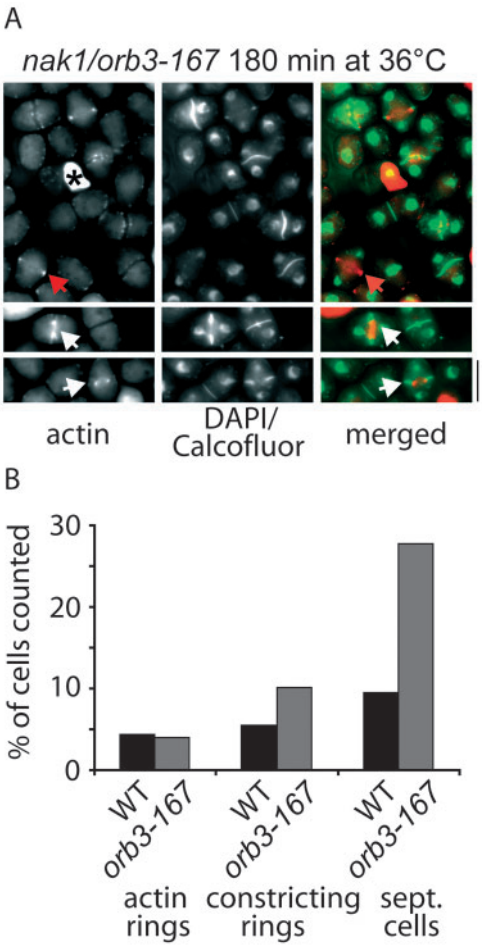

Fig. 3. Effect of *nak1/orb3-167* during cytokinesis. Wild-type and *nak1/orb3-167* cells were shifted to 36°C for 3 hours. (A) Cells were fixed and stained with rhodamine-phalloidin for F-actin, DAPI and CalcoFluor. Mitotic cell (red arrow), constricting actomyosin rings (white arrows), lysed cell (star); bar, 10 µm. (B) The proportions of cells displaying F-actin rings, constricting rings and incomplete septa, and complete septa were determined for wild-type and *nak1/orb3-167* cells. 200 cells have been counted for wild-type and for *nak1/orb3-167*; the experiments were repeated three times.

Microtubules are not immediately affected by Nak1/Orb3 inactivation

The terminal phenotype of the *orb3-167* mutant includes disorganized microtubules that span the round cells in all directions, mainly running under the cell surface (Verde et al., 1995). Analysis of fixed cells by immunofluorescence 30 minutes and 60 minutes after the transfer from 25°C to 35°C, however, did not show major changes in the organization of interphase microtubules of *nak1/orb3* mutant cells. As in wild-type cells, microtubules did span the whole length of the cell in three or four parallel bundles (Fig. 4). This indicates that the microtubule system does not immediately change after Nak1/Orb3 inactivation. After longer incubations of 4-6 hours at 35°C, the microtubule cytoskeleton was disorganized in *nak1/orb3* mutant cells, confirming the earlier described terminal phenotype (Verde et al., 1995). The timing and extent of the observed effects on the cytoskeleton suggest that the defect of the *nak1/orb3* mutation primarily affects the actin cytoskeleton.

Nak1/Orb3 function is required throughout the cell cycle

Because F-actin patches in fission-yeast cells delocalize at the end of G2 phase and relocalize to the cell ends after mitosis, it is possible that the actin localization defect in *nak1/orb3* mutant cells will only be seen if cells proceed through the cell

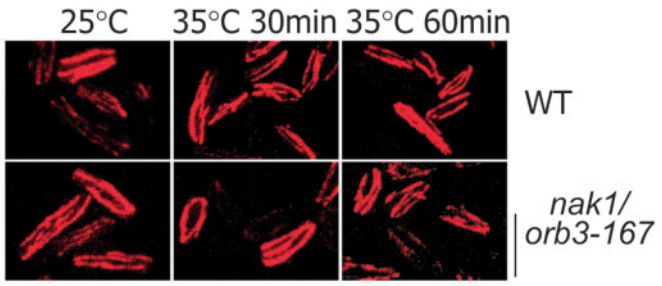

Fig. 4. Effect of *nak1/orb3-167* on the microtubule cytoskeleton. Exponentially growing wild-type (WT) and temperature-sensitive *nak1/orb3-167* cells were transferred from 25°C to 35°C. Samples were taken before and 30 minutes and 60 minutes after a shift from 25°C to 35°C, fixed and stained with an antibody against tubulin. Bars, 10 μm.

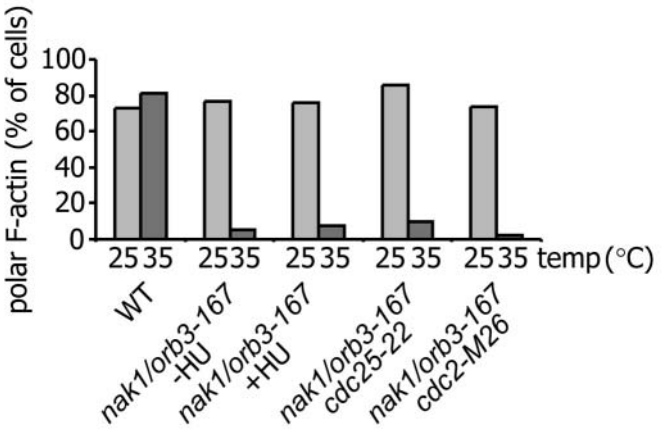

Fig. 5. F-Actin delocalization in *nak1/orb3-167* cells blocked at different stages of the cell cycle. Wild-type (WT), *nak1/orb3-167*, *nak1/orb3-167 cdc2-M26* and *nak1/orb3-167 cdc25-22* cells were grown at 25°C and shifted to 35°C for 2 hours. The *nak1/orb3-167* culture was split in half before the temperature shift. One half was directly shifted to 35°C (*orb3-167* –HU) and the other half was incubated with 15 mM hydroxyurea for 4 hours at 25°C before the shift up (*orb3-167* +HU). Cells were fixed and stained with rhodamine-phalloidin, and the proportion of cells with polar F-actin patches at the tips was determined. 200 cells were counted for each mutant; experiments were performed at least twice.

cycle. To block cell-cycle progression, *orb3-167* was crossed to both a *cdc2-M26* and a *cdc25-22* mutant, temperature-sensitive cell-division-cycle mutants that arrest mainly in G2 phase. Analysis of the F-actin distribution 2 hours after temperature shift to 35°C showed that actin was completely delocalized in arrested cells (Fig. 5). Similarly, F-actin dots became distributed over the entire cortex if *nak1/orb3* mutant cells were blocked in S-phase by the addition of HU (Fig. 5). Arrested *nak1/orb3* mutant cells changed their shape into spheres with roughly the same kinetics as non-arrested ones (data not shown). Therefore, in contrast to our earlier conclusion (Verde et al., 1995), *nak1/orb3* function is needed throughout the cell cycle for polar F-actin and polar growth; re-examination of the *orb3-167 cdc2-33* mutant strain used for this earlier study showed that it inadvertently carried a wild-type as well as a mutant copy of the *nak1/orb3* locus.

Nak1/Orb3-GFP localizes to the cell tips, medial ring and spindle-pole body

A localized signal of GFP directly fused to Nak1/Orb3 kinase could not be detected (Cope et al., 1999; Huang et al., 2003), so we inserted a pentaglycine linker between Nak1/Orb3 and GFP, and expressed the construct that is integrated at its genomic locus using the endogenous promoter. A similar linker has been shown to improve the GFP signal of a Tea1-GFP fusion protein, probably because it supports efficient folding of the GFP tag (Behrens and Nurse, 2002). With this construct, which rescues the *nak1/orb3* mutant phenotypes, we could detect a Nak1/Orb3-GFP signal at the ends of growing cells as well as more diffuse staining throughout the cell indicating a cytoplasmic or a vesicular localization in addition to the accumulation of the protein at the ends (Fig. 6A). As cells went into mitosis, Nak1/Orb3-GFP disappeared from the ends and was found as a ring in the middle of the cells. This ring constricted and split into two when cells separated. In addition, Nak1/Orb3-GFP signal could be detected as one or two dots in the vicinity of the nucleus (Fig. 6A). Colocalization with Ds-Red-tagged spindle-pole protein Sad1 showed that Nak1/Orb3-GFP is at the spindle pole body (SPB) throughout mitosis and can be found at both SPBs (Fig. 6B).

We also analysed the distribution of both the kinase and actin, because Nak1/Orb3 plays an essential role in organizing F-actin. Exponentially growing cells expressing Nak1/Orb3-GFP were fixed and stained with antibodies against actin and GFP. The signals for actin and tagged Nak1/Orb3 kinase generally colocalized in growing cells (Fig. 6C, top and middle). In dividing cells both proteins were found as a ring in the middle of the cell (Fig. 6C, bottom). Localization to the ends of the cells and as a ring in the middle was confirmed by staining fixed Nak1/Orb3-Myc cells with anti-Myc antibodies (data not shown). This localization is in agreement with a role of Nak1/Orb3 in organizing F-actin at the ends of growing cells and in regulating cytokinesis.

Nak1/Orb3 kinase is periodically phosphorylated and shows constitutive kinase activity during the cell cycle

Given that F-actin distribution is strictly coupled to the progression through cell cycle and that Nak1/Orb3 kinase is involved in F-actin organization, we asked whether changes in the expression levels of Nak1/Orb3 might contribute to the coordination of actin localization and cell cycle. A strain expressing a Myc-tagged Nak1/Orb3 kinase under the endogenous promoter (Nak1/Orb3-Myc) was crossed to the temperature-sensitive cell-division-cycle mutant *cdc25-22*. The resulting Nak1/Orb3-Myc *cdc25-22* cells were blocked in G2 phase and released into mitosis, while we monitored the expression of Nak1/Orb3-Myc. No major changes in Nak1/Orb3 protein levels were detected throughout two complete cell cycles (Fig. 7A,B). However, after separating the protein extracts by SDS-PAGE at low bisacrylamide concentration, a more-slowly migrating form of the Nak1/Orb3-Myc was detected when cells entered into mitosis that disappeared at the end of mitosis and reappeared in the next cycle (Fig. 7C). λ-Phosphatase treatment of the protein

extracts abolished the observed higher-molecular-weight form of Nak1/Orb3, indicating that the kinase is periodically phosphorylated during the cell cycle (Fig. 7C). This phosphorylation coincides with the relocalization of the protein from the cell poles to the middle of the cell at the end of G2 phase.

In order to test whether the differences in phosphorylation state correlate with changes in the activity of Nak1/Orb3 kinase, we performed in vitro kinase assays. We followed Nak1/Orb3 protein kinase activity in synchronous cultures obtained from block-and-release of Nak1/Orb3-Myc *cdc25-22* cells and of *cdc25-22* cells with untagged Nak1/Orb3 as a control (Fig. 8A,B). Extracts were prepared from samples taken every 20 minutes, the tagged kinase was immunoprecipitated and in-vitro kinase assays were performed using casein as a substrate. A clear Nak1/Orb3-kinase-dependent signal was obtained with precipitated Nak1/Orb3-Myc protein compared with background phosphorylation in control samples, which were made from synchronized cells with untagged Nak1/Orb3 protein (Fig. 8A). Analysis throughout the cell cycle did not show major changes in Nak1/Orb3 protein-dependent-kinase activity (Fig. 8A,B). Protein-kinase assays were also performed with the tagged temperature-sensitive mutant *orb3-167*, sequencing of which revealed a single-nucleotide change, C581 to T, leading to the exchange of serine194 for lysine in a highly conserved area of the Nak1/Orb3 kinase domain (data not shown).The kinase activity of the mutant protein was already reduced somewhat at the permissive temperature of 25°C, even though the expression levels of the mutant protein were very similar to those detected in Nak1/Orb3

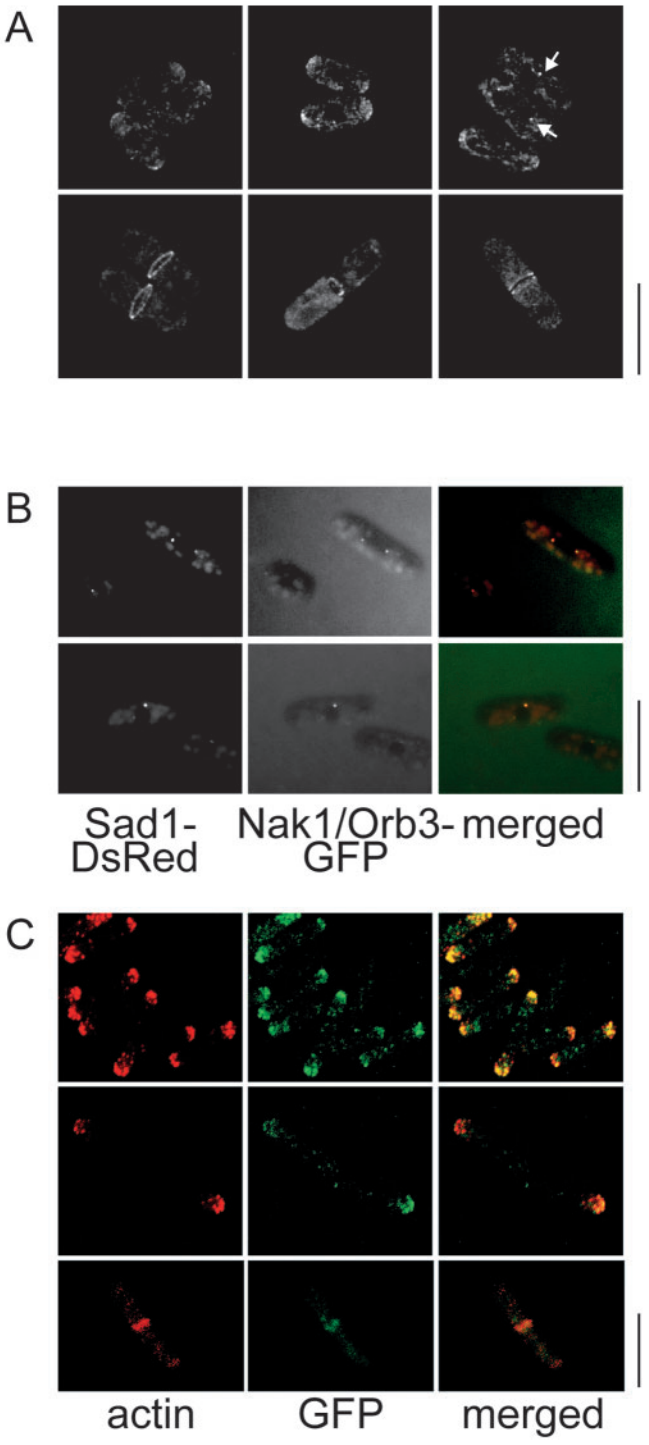

Fig. 6. Localization of Nak1/Orb3. (A) Localization of Nak1/Orb3-GFP in vivo. Living cells expressing Nak1/Orb3 tagged with GFP under the endogenous promoter. Arrows point at dots at the nuclei that might correspond to the spindle pole body. (B) Colocalization of Nak1/Orb3-GFP and Sad1-DsRed in live cells. (C) Colocalization of Nak1/Orb3-GFP and actin. Cells expressing GFP-tagged Nak1/Orb3 were fixed and double stained with antibodies against actin (red) and GFP (green). Overlay of both channels is shown in yellow. Bars, 10 μm.

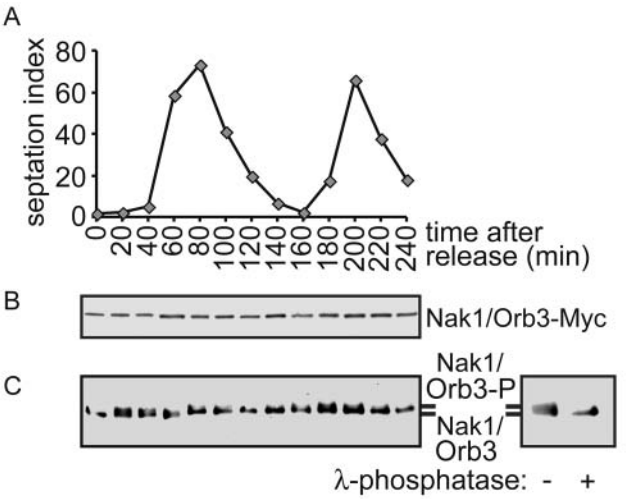

Fig. 7. Nak1/Orb3 protein levels in synchronized Nak1/Orb3-Myc *cdc25-22* cells. The cells were blocked in G2 phase at 36.5°C for 3.5 hours and synchronously released into mitosis by cooling the culture to 25°C. Samples were taken every 20 minutes. (A) Septation index. The proportion of cells displaying a septum was determined for each time point. (B,C) Nak1/Orb3-Myc protein levels. Denatured protein extracts were prepared and equal amounts for each time point were separated by SDS-PAGE. The proteins were detected with monoclonal antibody 9E10 against the Myc tag. (B) Separation on a 4-12% acrylamide gradient gel. (C) Separation on a 8% acrylamide/0.1% bisacrylamide gel (left). Phosphorylated (Nak1/Orb3-P) and unphosphorylated (Nak1/Orb3) forms of Nak1/Orb3 are visible. The extract of the 40 minutes time point was either incubated with λ-phosphatase (+) or buffer only (–) (right).

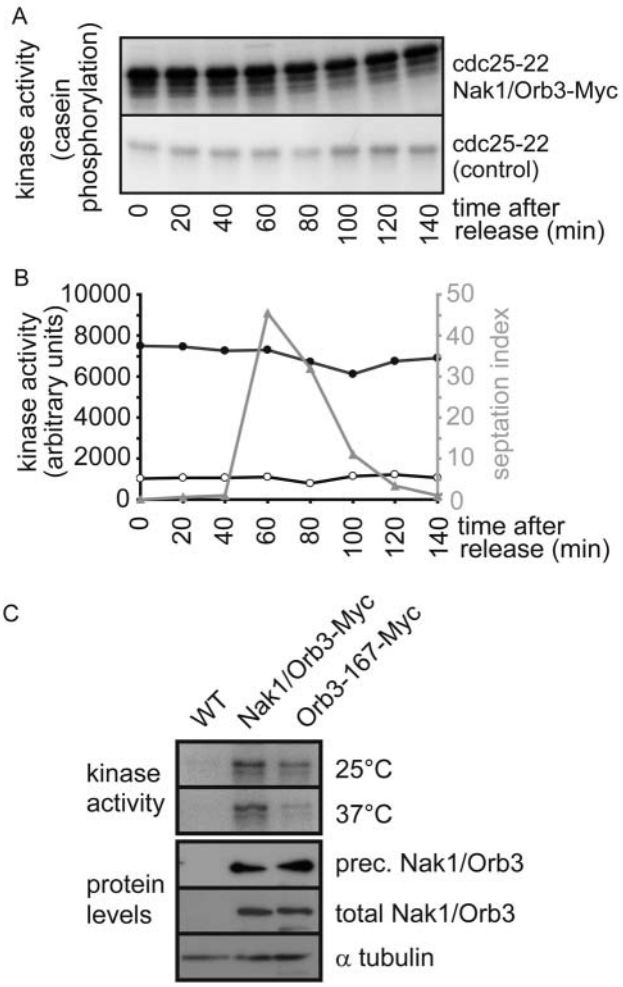

Fig. 8. Protein-kinase activity of Nak1/Orb3 in vitro. (A) Nak1/Orb3-dependent kinase activity throughout the cell cycle. Samples were taken from cultures synchronized by *cdc25-22* block-and-release at the time points indicated and kinase assays were performed with casein as a substrate. Synchronous *cdc25-22* cells expressing untagged Nak1/Orb3 were used as a control. Protein levels of precipitated Nak1/Orb3-Myc were checked by western blotting and immunodetection with anti-Myc antibodies (data not shown). (B) Kinase activity of Nak1/Orb3-Myc samples (closed circles) and of untagged control samples (open circles) was quantified using a phosphor imager. Septation index (closed triangles). (C) Temperature-sensitive kinase activity of Nak1/Orb3-167/Myc in vitro. Kinase assays were performed at 25°C and 37°C, with samples taken from asynchronous cultures of wild-type cells (WT) expressing untagged Nak1/Orb3, tagged wild-type Nak1/Orb3 or tagged mutant Nak1/Orb3-167. Protein levels of precipitated (prec.) Nak1/Orb3 and of total Nak1/Orb3 and α-tubulin in the extracts were checked by western blotting and immunodetection.

wild-type cells (Fig. 8C). The slightly reduced activity might explain small morphological defects seen in *orb3-167* cells at 25°C (Fig. 2A). Furthermore, kinase activity of Orb3-167/Myc protein was strongly reduced at 37°C (Fig. 8C) and the specific kinase activity per unit of mutant protein at 37°C was reduced to 27% compared with wild-type protein, showing that the activity of orb3-167/Myc is temperature sensitive in vitro (data not shown). This indicates that the measured kinase activities are dependent on the Nak1/Orb3 protein itself, not on co-precipitating kinases.

Thus, the changes in actin-cytoskeleton organization during the cell cycle are not regulated by changes in Nak1/Orb3 protein-kinase activity but by relocalization of the constantly active kinase between the tips and the middle of the cell. The observed periodic phosphorylation of the protein might contribute to the regulation of its cell-cycle-dependent relocalization.

F-Actin and Nak1/Orb3 kinase activity but not microtubules are required for Nak1/Orb3 localization

To investigate the mechanism that determines the subcellular distribution of Nak1/Orb3 kinase, we examined the role of F-actin. The localization of Nak1/Orb3-GFP was analysed in cells treated with latrunculin A (Cambridge Bioscience), a drug that promotes F-actin depolymerization by binding to actin monomers (Spector et al., 1983). The Nak1/Orb3-GFP signal at the poles was completely lost after drug treatment (Fig. 9A,B, arrows). Similarly, the ring formed by Nak1/Orb3-GFP in dividing cells faded after the addition of latrunculin A (Fig. 9A,B). We next examined the role of microtubules in the intracellular distribution of Nak1/Orb3. Microtubules of cells expressing Nak1/Orb3-GFP were depolymerised by the addition of the drug carbendazim (MBC, Sigma). However, the Nak1/Orb3-GFP signal at the tips of the cells and at the medial ring did not change compared with the control (Fig. 9A,C). We conclude that the localization of Nak1/Orb3 kinase is dependent on F-actin but does not require functional microtubules.

To test whether Nak1/Orb3 kinase activity is needed for localization of the protein to the cell tips and to the medial ring, we stained fixed Nak1/Orb3-167/GFP cells grown at 25°C and at 36°C with anti-GFP antibodies. We could detect an accumulation of GFP signal at the cell ends and in the middle of dividing cells at the permissive but not at the restrictive temperature (Fig. 9D). This suggests that kinase activity of Nak1/Orb3 protein is necessary for its own localization, because Nak1/Orb3 protein levels of mutant *nak1/orb3-167* cells is similar to the levels found in wild-type cells but kinase activity is significantly reduced in the mutant at 36°C (Fig. 8C).

Discussion

Inactivation of the Nak1/Orb3 kinase causes complete delocalization of F-actin away from the tips of fission-yeast cells, leading to changes in shape from cylindrical to spherical cells. F-Actin dots become randomly spread over the cortex of the cell and are not organized into patches at the ends of the cells. Thus, functional Nak1/Orb3 kinase is needed for the organization of polar F-actin. Because F-actin polarization can also be disturbed in cells lacking non-essential genes such as *ras1* (Pichova and Streiblova, 1992), the delocalization of actin from the cell tips does not necessarily explain why an active Nak1/Orb3 kinase is essential for cell survival. The organization of Nak1/Orb3 kinase as a ring in the middle of dividing cells and the strong impact of the *orb3-167* mutation on cell separation suggest an essential function for the protein is during late stages of cytokinesis. The fact that cells lacking *nak1/orb3* often arrest as two unseparated, round cells is in

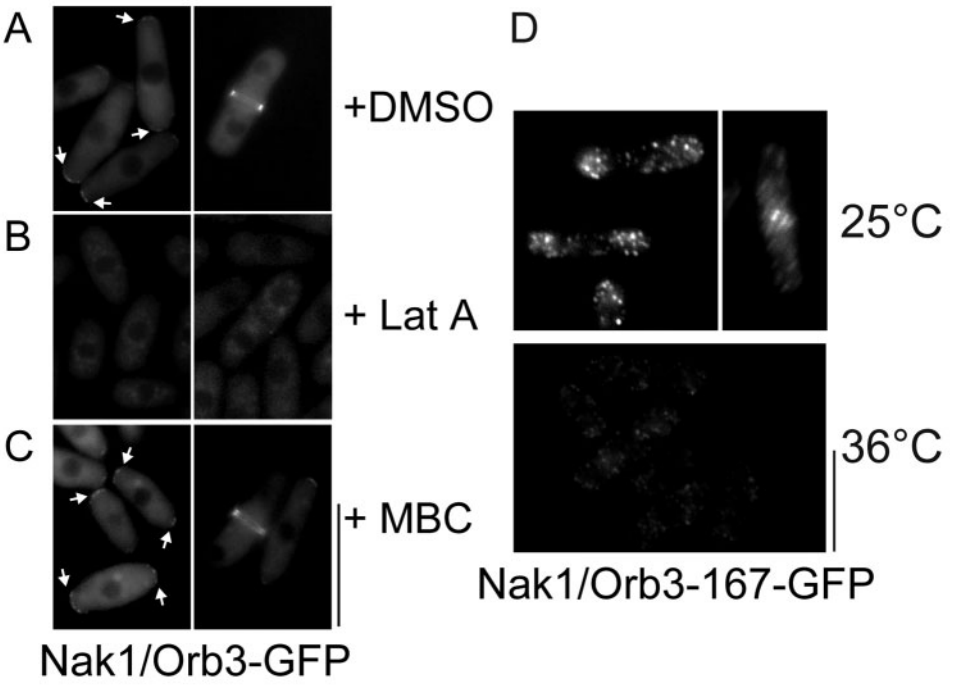

Fig. 9. The roles of the actin and the microtubule cytoskeletons, and Nak1/Orb3 kinase activity in localizing the protein. (A-C) The effects of F-actin and microtubule depolymerization on the localization of Nak1/Orb3-GFP. Cells were incubated with 10 μM latrunculin A for 20 minutes (+ Lat A), with 25 μg ml^{-1} of the microtubule-depolymerizing agent carbendazim (+ MBC) or with their solvent dimethylsulfoxide as a control (+ DMSO). Arrows show patches of Nak1/Orb3-GFP. Samples with and without MBC were fixed in methanol and stained with anti-tubulin antibodies to control for microtubule polymerization (data not shown). (D) Effects of *nak1/orb3-167* mutation on Nak1/Orb3-GFP localization. Nak1/Orb3-GFP cells grown at 25°C and cells shifted to 36°C for 60 minutes were fixed in formaldehyde and stained with anti-GFP antibodies. Bars, 10 μm.

agreement with this proposed role for the Nak1/Orb3 kinase in cell separation (Fig. 1C). A genetic interaction between *nak1/orb3-167* and cytokinesis mutant *cps1-191* might give an additional hint to a function of Nak1/Orb3 in cell separation. Mutant *cps1-191* cells arrest as unseptated cells with a stable actin ring (Liu et al., 1999). The double mutant *cps1-191 nak1/orb3-167*, however, arrests later with a CalcoFluor stainable septum without an actomyosin ring, which might suggest a functional link between *nak1/orb3* and *cps1* (data not shown).

Cells expressing mutant Nak1/Orb3 without kinase activity display round cells with delocalised actin and unseparated cells similar to cells deleted for *nak1/orb3* (Huang et al., 2003) (data not shown). We conclude that the organization of F-actin at the tips of cells and regulation of late stages of cytokinesis and cell separation depend on the kinase activity of Nak1/Orb3.

In contrast to F-actin, interphase microtubules do not show rapid changes after inactivation of Nak1/Orb3 function. After shifting *orb3-167* cells to the restrictive temperature, microtubule bundles can still be found, running from the vicinity of the nucleus to the cell ends. When cell shape starts changing after incubation at the restrictive temperature, microtubules become disorganized until they are strongly bent and distorted, mostly running under the cortex of spherical cells (Huang et al., 2003; Verde et al., 1995). From the temporal sequence of events, we conclude that the delayed disorganization of the microtubule cytoskeleton is likely to be a secondary effect of Nak1/Orb3 inactivation caused by the complete loss of polarity of the actin cytoskeleton, cell shape and potentially associated markers. Microtubules constantly explore the shape of the cell to find the ends (Brunner and Nurse, 2000). As soon as the loss of polarity of the actin cytoskeleton leads to spherical cells, microtubules might continue to grow underneath the cell surface because they do not reach an area specified as cell ends. This could lead to the microtubule disorganization seen in round *orb3-167* cells after prolonged incubation at restrictive temperature (Verde et al., 1995).

Nak1/Orb3 is a Ste20/GCK kinase, a group that is characterized by a conserved kinase domain and a highly diverse noncatalytic domain. These kinases can be divided into the p21-activated kinase (PAK) family, with a C-terminal kinase domain, and the germinal-centre kinase (GCK) family, with its catalytic domain at the N-terminus and no defined p21-binding site in the noncatalytic domain (Dan et al., 2001). Members of both families have been identified in all eukaryotes from yeast to human, and are involved in the regulation of stress response, apoptosis, cytoskeletal rearrangements, changes of cell shape and cell motility. Most Ste20-related kinases seem to be upstream activators of mitogen-activated protein kinase (MAPK) cascades (Bagrodia and Cerione, 1999; Kyriakis, 1999). Based on phylogenetic classification, PAKs and GCKs have been grouped into several subfamilies (Dan et al., 2001). Sequence homology of the kinase domains shows that Nak1/Orb3 is most closely related to the GCKIII subfamily, which includes the human proteins SOK1, MST3 and MST4/MASK. None of these apparently activate any of the well-characterized MAPK-kinase pathways like JNK or p38 MAPK, although there are contradictory results about the activation of ERK for MST4/MASK (Lin et al., 2001; Pombo et al., 1996; Qian et al., 2001; Schinkmann and Blenis, 1997). SOK1 is specifically stimulated by oxidative stress and both Mst3 and Mst4/MASK can be activated by caspase-3 cleavage of the C-terminus during apoptosis (Dan et al., 2002; Huang et al., 2002; Pombo et al., 1996). MST4/MASK overexpression leads to increased proliferation and transformation rates, and to increased tumorigenicity (Lin et al., 2001; Sung et al., 2003). Additionally, members of the closely related GCKII kinase subfamily, human Mst2 and *Drosophila hippo*, which also has a cell morphology phenotype, have a strong influence on cell

proliferation, whereas the other more distant GCK subfamilies have not been implicated in cell proliferation (Harvey et al., 2003; Wu et al., 2003). In the case of Nak1/Orb3, interconnection with the cell cycle is indicated by periodic phosphorylation of the kinase and its changing localization during the cell cycle.

Interestingly, all human GCKIII kinases seem to be negatively regulated by the C-terminal domain, and SOK1 and Mst3 were shown to be activated by phosphorylation (Dan et al., 2002; Huang et al., 2002; Pombo et al., 1996; Schinkmann and Blenis, 1997). By contrast, the observed phosphorylation of Nak1/Orb3 at the beginning of mitosis did not have any influence on the protein kinase activity of Nak1/Orb3. Kinase activity against casein in vitro did not undergo significant changes as synchronous cells proceeded through the cell cycle. Therefore, we conclude that Nak1/Orb3 exhibits a constitutive level of kinase activity during the vegetative cell cycle. Because a kinase-dead mutant of Nak1/Orb3 does not complement the phenotype (Huang et al., 2003) (data not shown) and the temperature-sensitive mutant *orb3-167* shows normal protein levels but strongly reduced activity (Fig. 8C), Nak1/Orb3 protein-kinase activity is essential for its function in polarizing the actin cytoskeleton and regulating cell separation. Relocalization of F-actin between the tips of the cell and the actin ring correlates tightly with the fission-yeast cell cycle (Marks et al., 1986) but it is not clear how cell-cycle control and regulation of actin polarity are linked. Nak1/Orb3 kinase might provide such a connection between the cell cycle and organization of the actin cytoskeleton. Because Nak1/Orb3 protein levels and kinase activity seem to be largely unchanged during progression through the cell cycle, periodic changes in phosphorylation of the kinase peaking at mitosis and cytokinesis might be involved in Nak1/Orb3 localization. Phosphorylation of Nak1/Orb3 at the onset of mitosis might be responsible for its relocalization from the poles to the medial actomyosin ring. It is not known which kinases are responsible for the periodic phosphorylation of Nak1/Orb3. However, a strong synthetic phenotype of *orb3-167* with *cdc11-119*, a mutant anchoring components of the SIN pathway to the SPB, already at 25°C with a high proportion of pear-shaped and round cells (data not shown), and the localization of Nak1/Orb3 to the SPB might suggest that the relocalization of Nak1/Orb3 is regulated at the SPB (Krapp et al., 2001).

The closest homologue of Nak1/Orb3 in budding yeast is Kic1p, with a homology of nearly 50% between the kinase domains. Kic1p was identified as kinase interacting with the SPB component Cdc31p, a homologue of mammalian centrin (Sullivan et al., 1998). The *kic1* gene is essential and its mutants show morphological defects and cell-wall alterations. The defects include thicker cell walls, cell lysis, F-actin changes at the bud neck and growth in unseparated chains or clusters, which indicate a separation defect (Sullivan et al., 1998; Vink et al., 2002). Several other genes have been identified that might contribute to the same regulatory pathway as Nak1/Orb3 because their deletion phenotypes are very similar to that of *nak1/orb3*. Deletions of *orb6*, *mob2* and the *furry* homologue *mor2* are lethal and result in completely round cells that arrest after two to four rounds of division and have unpolarized F-actin (Hirata et al., 2002; Hou et al., 2003; Verde et al., 1998). Furthermore, the encoded proteins localize to the tips of growing and to the middle of dividing cells, mirroring F-actin distribution. Orb6 physically interacts with Mob2 and belongs to the Orb6/COT-1/Warts kinase family, members of which are implicated in cell-shape regulation (Verde et al., 1998; Zallen et al., 2000). The homologues of these proteins in *S. cerevisiae* (Cbk1p, Mob2p and Tao3p), together with the Nak1/Orb3 homologue Kic1p, form a recently described signalling network called RAM that regulates Ace2p activity and morphogenesis (Nelson et al., 2003). It seems likely that Nak1/Orb3 kinase is part of a similar signalling pathway in *S. pombe*, including Orb6, Mob2 and Mor2 regulating F-actin polarization and cell separation, which might be regulated at the SPB in coordination with the SIN pathway (Sohrmann et al., 1998).

We have shown that the Nak1/Orb3 kinase localizes with F-actin and that its localization also depends on the integrity of the actin cytoskeleton. Depolymerization of F-actin by latrunculin A leads to the loss of Nak1/Orb3 from both the tips and the ring. Therefore, the Nak1/Orb3 kinase has an essential function in localizing F-actin to the poles, and polarized F-actin is required in turn to localize the kinase. These results indicate that Nak1/Orb3 is recruited to the sites where F-actin polymerization occurs first. There, the kinase could increase F-actin polymerization, thereby functioning as a positive-feedback loop that concentrates F-actin at the tips or in the middle of the cell. The generation of cellular polarity through a positive-feedback loop involving the small GTPase Cdc42 has been suggested for bakers' yeast cells (Wedlich-Soldner et al., 2003). In fission yeast, the ability of the cell to polymerize F-actin itself does not seem to be abolished by Nak1/Orb3 inactivation because, in *nak1/orb3-167* mutant or *nak1/orb3*-deficient cells, F-actin dots are still visible (Fig. 1B, Fig. 2C). In fact, the organization of F-actin in concentrated patches seems to be dependent on Nak1/Orb3 function. In growing cells, a Nak1/Orb3-dependent feedback mechanism might be recruited to sites at the cell tips that are determined by a microtubule-based system involving Tea1, Tea2 and Tip1 (Browning et al., 2000; Brunner and Nurse, 2000; Mata and Nurse, 1997), leading to a stable accumulation of F-actin at these sites. Furthermore, Nak1/Orb3 function seems to be essential for the de novo establishment of cell polarity during spore maturation, because *nak1/orb3*Δ spores never establish a cellular axis (Fig. 1A). For the de novo formation of polarity, Nak1/Orb3 could be recruited to sites already specified during spore formation or to a random site at the cortex of the spores in order to establish a new zone of polar growth. We conclude that the Nak1/Orb3 kinase plays an essential role in regulating F-actin polarity and late stages of cytokinesis.

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