The small GTPase Rho3 and the diaphanous/formin For3 function in polarized cell growth in fission yeast

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Summary

We identified a novel Rho gene *rho3*$^+$ and studied its interaction with diaphanous/formin *for3*$^+$ in the fission yeast *Schizosaccharomyces pombe*. Both *rho3* null cells and *for3* null cells showed defects in organization of not only actin cytoskeleton but also cytoplasmic microtubules (MTs). *rho3 for3* double null cells had defects that were more severe than each single null cell: polarized growth was deficient in the double null cells. Function of For3 needed the highly conserved FH1 and FH2 domains, an N-terminal region containing a Rho-binding domain, and the C-terminal region. For3 bound to active forms of both Rho3 and Cdc42 but not to that of Rho1. For3 was localized as dots to the ends of interphase cells and to the mid-region in dividing cells. This localization was probably dependent on its interaction with Rho proteins. Overexpression of For3 produced huge swollen cells containing depolarized F-actin patches and thick cytoplasmic MT bundles. In addition, overexpression of a constitutively active Rho3Q71L induced a strong defect in cytokinesis. In conclusion, we propose that the Rho3-For3 signaling system functions in the polarized cell growth of fission yeast by controlling both actin cytoskeleton and MTs.

Key words: Actin cytoskeleton, Cell polarity, Cytokinesis, Microtubule, Rho

Introduction

Establishment of cell polarity and polarized growth are essential for various cellular events such as cell morphogenesis, cell movement, intracellular transportation, and cell differentiation (Drubin, 2000). In these processes, microtubules (MTs) and actin filaments (F-actin) are considered to be involved. It has also been demonstrated that the Rho family small GTPases play an important role in the polarization of eukaryotic cells (Narumiya, 1996; Aelst and Schorey, 1997; Hall, 1998).

Molecular genetics is powerful for analyses of cell polarity. The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are generally used as excellent systems for this purpose (Bähler and Peter, 2000). Budding yeast cells grow asymmetrically by localized vesicle fusion and cell wall synthesis to form a bud (Pruyne and Bretscher, 2000a; Pruyne and Bretscher, 2000b). The bud site assembling-proteins function in assembling components required for bud growth. During bud formation, patches of F-actin are localized at the cell surface of the bud, and cables of F-actin are oriented along the axis of bud formation. In contrast to F-actin, MTs are not required for the bud emergence and bud growth. Cells of fission yeast are cylindrical in shape and grow at the ends (Nasim et al., 1989). They divide at the mid-region of the cell by contraction of the contractile ring like many animal cells. In this organism, both F-actin and MTs contribute to the polarized cell growth again like many animal cells. Actin is organized as longitudinal F-actin cables and cortical F-actin patches at the growing ends of the interphase cell where the cell wall is newly synthesized (Marks and Hyams, 1985). MTs extend along the long axis of the cell during interphase (Hagan and Hyams, 1988) and are essential for maintaining the linear growth of the cell (Hiraoka et al., 1984; Mata and Nurse, 1997). In addition, various morphological mutants have been isolated in fission yeast, which show a round, swollen, bent, or branched shape (Snell and Nurse, 1993; Verde et al., 1995; Mata and Nurse, 1998). Abnormal organization of F-actin and/or MTs has frequently been observed in these mutants.

Both the yeasts have two conserved essential Rho family small GTPases, Rho1 and Cdc42, which are counterparts of human RhoA and Cdc42Hs, respectively. Cdc42 plays an essential role in the establishment of cell polarity in both yeast cells (Johnson and Pringle, 1990; Miller and Johnson, 1994), while Rho1 promotes cell wall synthesis for polarized cell growth (Drgonova et al., 1996; Qadota et al., 1996; Arellano et al., 1996; Nakano et al., 1997; Nakano et al., 2001a). In addition, budding yeast has another Rho family protein, Rho3p, which is involved in maintenance of cell polarity for bud growth (Matsui and Toh-e, 1992a). It has been suggested that budding yeast Rho3p plays this important role by properly organizing actin cytoskeleton during bud formation (Imai et al., 1996). Thus, it has been desirable to identify and characterize the counterpart of Rho3p in fission yeast in order to clarify the mechanism of polarized growth of the cell in general.

Diaphanous/formin family proteins have recently been highlighted since they seem to play an important role in organizing the contractile ring during cytokinesis in various

cells. *Drosophila* diaphanous (Castrillon and Wasserman, 1994), *S. pombe* Cdc12 (Chang et al., 1997), *S. cerevisiae* Bni1p (Kohno et al., 1996), *Aspergillus nidulans* SepA (Harris et al., 1997), and *Caenorhabditis elegans* Cyk1 (Swan et al., 1998) are proteins responsible for the cytokinesis in these organisms. mDia has been shown to localize to the cleavage furrow of dividing Swiss 3T3 cells (Watanabe et al., 1997). It must be noted that some of these proteins has been considered to be controlled by Rho family proteins: mDia and Bni1p bind active forms of RhoA and Rho1p, respectively. *S. pombe* has another diaphanous/formin protein Fus1 which is essential for the mating conjugation (Petersen et al., 1995; Petersen et al., 1998). Fus1 is expressed when the cells are nitrogen-starved and is localized to the tip of the mating projection. This formin protein does not appear to function in polarized growth of the vegetative cell.

Here, we identify Rho3 and demonstrate that it interacts with diaphanous/formin family protein For3 in fission yeast. Analyses of mutants and overexpression phenotypes show that these proteins function cooperatively in regulation of both actin and MT cytoskeletons in polarized cell growth.

Materials and Methods

Strains, media and genetic techniques

The *S. pombe* strains used in this study are listed in Table 1. The media used have been described previously (Moreno et al., 1991). Complete medium YEA and minimum medium EMM were used for growing the *S. pombe* strains. MEA was used for induction of conjugation and sporulation. All plates contained 2% agar. Standard procedures for *S. pombe* genetics were followed (Alfa et al., 1993; Moreno et al., 1991).

Isolation of fission yeast *rho3*$^+$

The budding yeast *rho3* null cells (*YMR504*), which had been rescued by the expression of the budding yeast *RHO4* under the *GAL7* promoter (Matsui and Toh-e, 1992b), were transformed with plasmids containing the fission yeast cDNA under the control of the budding yeast *ADC1* promoter, and spread on glucose plates. After incubation at 30°C, the growing colonies were restreaked on glucose plates. Plasmids were recovered from the clones which grew well on the glucose plates, and were introduced into *rho3* null cells to test the ability to complement the defect.

DNA manipulation and cloning of genomic *rho3*$^+$

Standard methods were used for the DNA manipulations (Sambrook et al., 1989). The procedures for the isolation of *rho3*$^+$ genomic DNA clones by plaque hybridization using *rho3*$^+$ cDNA as a probe were previously described (Nakano and Mabuchi, 1995; Nakano et al., 1997).

Gene disruption

To disrupt *rho3*$^+$, we replaced the *Sna*I-*Eco*RV region in *Eco*RI 3.6 kb genomic fragment containing *rho3*$^+$ with *ura4*$^+$. A fragment produced by digestion with *Hinc*II was used to transform a diploid constructed by mating *JY741* and *JY746* strains. Correct integration was verified by Southern blotting.

To disrupt *for3*$^+$, we replaced the *Spe*I-*Hpa*I region in *Sma*I-*Pst*I 6.2 kb fragment containing *for3*$^+$ with *ura4*$^+$. A fragment produced by digestion with *Sma*I and *Sac*I was used for gene disruption as mentioned above. *for3Δ*FH12C, *for3Δ*FH2C, and *for3Δ*C were constructed by inserting *ura4*$^+$ at 1463, 2589, and 3572 bp in the open reading frame of *for3*$^+$, respectively.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
JY1	*h*$^-$	Lab. stock
leu1-32	*h*$^-$ *leu1-32*	Lab. stock
JY333	*h*$^-$ *ade6-M216 leu1-32*	Lab. stock
JY741	*h*$^-$ *ade6-M216 leu1-32 ura4D18*	Lab. stock
JY746	*h*$^+$ *ade6-M210 leu1-32 ura4D18*	Lab. stock
KNR302	*h*$^-$ *ade6-M216 leu1-32 ura4D18 rho3::ura4*$^+$	This study
KNR303	*h*$^+$ *ade6-M210 leu1-32 ura4D18 rho3::ura4*$^+$	This study
KNF302	*h*$^-$ *ade6-M216 leu1-32 ura4D18 for3::ura4*$^+$	This study
KNF303	*h*$^+$ *ade6-M210 leu1-32 ura4D18 for3::ura4*$^+$	This study
KNF312	*h*$^-$ *ade6-M216 leu1-32 ura4D18 for3Δ*FH12C*::ura4*$^+$	This study
KNF322	*h*$^-$ *ade6-M216 leu1-32 ura4D18 for3Δ*FH2C*::ura4*$^+$	This study
KNF332	*h*$^-$ *ade6-M216 leu1-32 ura4D18 for3Δ*C*::ura4*$^+$	This study
KRF332	*h*$^-$ *ade6-M216 leu1-32 ura4D18 rho3::ura4*$^+$ *for3::ura4*$^+$	This study
cdc3	*h*$^-$ *leu1-32 ura4D18 cdc3-124*	Lab. stock
cdc8	*h*$^-$ *leu1-32 ura4D18 cdc8-110*	Lab. stock
cdc12	*h*$^-$ *leu1-32 ura4D18 cdc12-112*	Lab. stock

Gene expression in fission yeast

S. pombe expression vectors pREP1, pREP41 or pREP81 (Forsburg, 1993; Maundrell, 1993) were used. pREP1 has the strongest promoter, while pREP81 the weakest. The expression of exogenous genes from these plasmids was repressed by 4 μM thiamine in the medium.

Two-hybrid system

We used the two-hybrid system in which expression of the *HIS3* reporter gene was utilized to detect a positive interaction between two proteins as described previously (Vojtek et al., 1993). pBTMrho1Q64L, pBTMrho3, pBTMrho3Q71L, or pBTMcdc42Q61L contained cDNA encoding the full length of each protein, in which the Cys in the CAAX-motif was mutated to Ser in order to prevent isoprenylation of the C-terminus. pGADfor3N contained amino acid residues 149-488 of For3.

Electrophoresis, transfer onto PVDF membranes and western blotting

Soluble and insoluble protein fractions were prepared from exponentially growing cultures, subjected to SDS-PAGE, and transfered onto PVDF membranes as descrived previously (Nakano et al., 1997). For western blotting, the PVDF membrane was first reacted with anti-budding yeast Rho3p antibody (Imai et al., 1996) and then Rho3 was detected with secondary antibodies coupled to alkaline phosphatase (Santa Cruz Co.).

Microscopy

Cells were fixed and processed for immunofluorescence microscopy as described previously (Alfa et al., 1993) using anti-budding yeast Rho3 antibodies, anti-HA antibodies (Y-11; Amersham Pharmacia Biotech), or a monoclonal anti-tubulin antibody (TAT1; a kind gift from K. Gull). Staining of the cells with Calcofluor, Rhodamine-phalloidin, or Bodipy-phallacidin (Molecular Probes, Eugene, OR) was performed as described previously (Alfa et al., 1993; Arai et al., 1998). The stained cells were viewed by two approaches. Conventional images were obtained using a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Jena, Germany) equipped with a Plan Apochromat ×63 lens and photographed on Kodak T-MAX ASA 400 films. Three-dimensional (3D) reconstitution was performed using a Delta Vision system (Applied Precision, Issaquah, WA) attached to an Olympus IX-70-SIF fluorescence microscope equipped with a UplanApo ×100 lens (Olympus, Tokyo, Japan). The images were captured and processed as described previously (Motegi et al., 2001).

Electron microscopy

Cells were pre-fixed with 2.5% glutaraldehyde dissolved in 0.1 M sodium phosphate buffer (pH 7.0) at room temperature for 1 hr and then post-fixed with 1.5% potassium permanganate dissolved in distilled-deionized water at 4°C overnight. They were embedded, thin-sectioned, and examined as described previously (Nakano et al., 1997).

Results

Identification of Rho3 in fission yeast

In order to identify the Rho3p-counterpart in fission yeast, we screened fisson yeast cDNA clones that suppress the growth defect of the *rho3* null budding yeast cells when overexpressed (see Materials and Methods). We identified 11 clones, 10 of which encoded Rho3 (accession NO. D83723). The fission yeast Rho3 contains GTP-binding and hydrolysis domains, together with a C-terminal CAAX motif similar to other Rho family small GTPases. Computer search of the deduced amino acid sequence showed that Rho3 is 65.4% identical to the budding yeast Rho3p, 50.0% to fission yeast Rho1, 49.7% to human RhoA (Yeramian et al., 1987), 44.0% to fission yeast Rho2 (Nakano and Mabuchi, 1995) and 44.5% to fission yeast Rho4 (K. Nakano, T. Mutoh, R. Arai and I. Mabuchi, unpublished).

rho3 null cells showed defects in cell morphology and septation

rho3 null cells were normal in both the growth and the shape of the cells at 25°C (Fig. 1). However, they grew poorly and displayed aberrant shapes at 37°C; majority of the cells were dumpy while some were elongated and multi-septated. We also found that these defects in growth and shape of the *rho3* null cells were suppressed by addition of 1M sorbitol in the medium (Fig. 1A and data not shown). Furthermore, almost all the *rho3* null cells were multi-septated at 34°C but were still able to grow. These results suggest that Rho3 may be involved in controlling cell shape and septation. These defects in the *rho3* null cells were suppressed by expression of the budding yeast *RHO3* (data not shown), indicating that the function of Rho3p is conserved between these organisms. In contrast, a high dose of the fission yeast Rho1, Rho2, Rho4, or Cdc42 did not suppress the defects of the *rho3* null cells (data not shown). Thus the function of Rho3 in fission yeast is probably different from that of these Rho family proteins.

Next, we examined distribution of F-actin and MTs in the *rho3* null cells (Fig. 1C). At 25°C there was no difference in F-actin- or MT-staining between the *rho3* null cells and the wild-type cells. However, at 37°C F-actin patches were delocalized all over the cell cortex and the cytoplasmic MTs were abnormally oriented. In addition, binucleate *rho3* null cells without an F-actin ring were occasionally observed, suggesting that cytokinesis was defective in these cells.

Rho3 localizes to the cell periphery

The fission yeast Rho3 was specifically recognized by antibodies raised against the budding yeast Rho3p (Imai et al., 1996) (Fig. 2A). Almost all the Rho3 was detected in the 10,000 ***g*** pellet of the cell homogenate. Thus, Rho3 may exist predominantly as a membrane-bound form like other Rho family small GTPases in fission yeast (Nakano et al., 1997; Hirata et al., 1998).

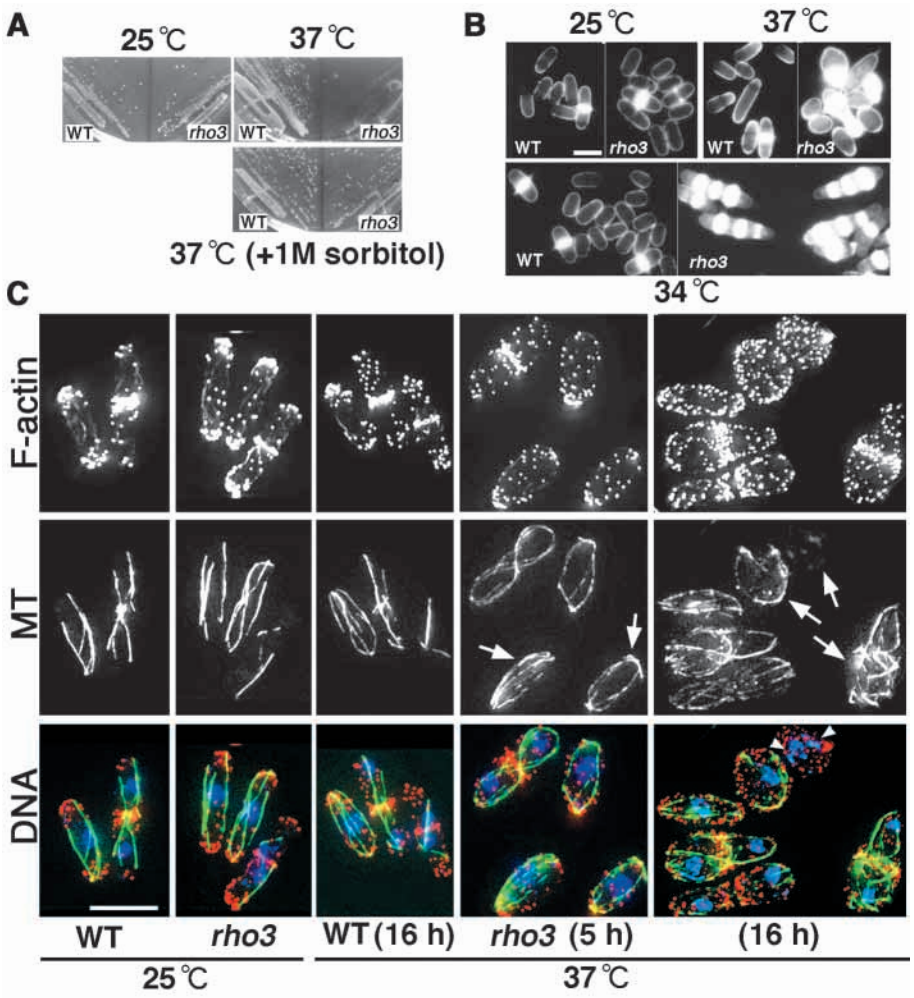

Fig. 1. Aberrant morphology of *rho3* null cells. (A) Growth of wild-type cells (WT) and *rho3* null cells (*rho3*) on a YEA plate and on YEA containing 1 M sorbitol at the indicated temperatures for 3 days. (B) Wild-type cells and *rho3* null cells grown in YEA medium at the indicated temperatures for 16 hours were stained with Calcofluor. (C) Wild-type cells and *rho3* null cells grown in YEA medium at 25 or 37°C for 5 or 16 hours were stained with Rhodamine-phalloidin (red in merged images), TAT-1 (green in merged images), and DAPI (blue in merged images). Deconvoluted 3D images are shown. Arrows indicate cells with abnormal organization of cytoplasmic MTs. Arrowheads indicate nuclei in a cytokinesis-defective binucleate cell. Bars, 10 μm.

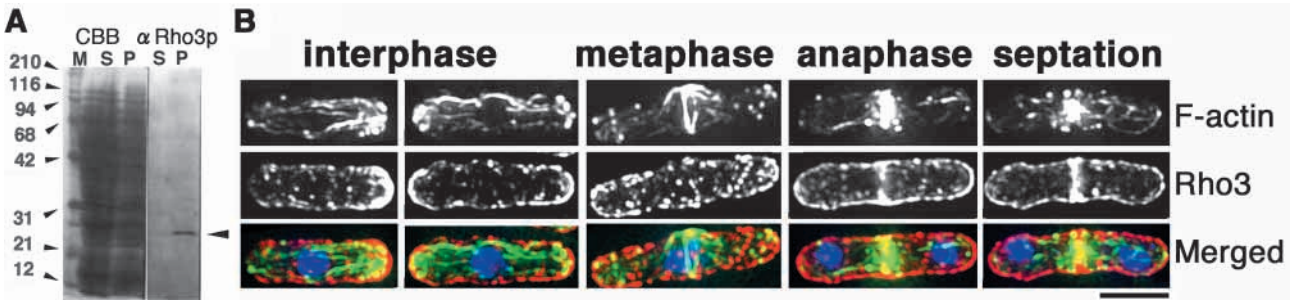

Fig. 2. Localization of Rho3. (A) Rho3 was detected in the insoluble fraction of the cell homogenate. Homogenates of wild-type cells grown in YEA medium at 30°C were spun at 10,000 ***g*** to obtain the supernatant (S) and pellet (P) fractions. Each fraction was run on an SDS-15% polyacrylamide gel and subjected to CBB staining or immunoblot analysis using anti-Rho3p antibodies. Molecular weight markers (M) were run on the left lane: trypsin inhibitor (21 k), carbonic anhydrase (31 k), actin (42 k), BSA (68 k), phosphorylase b (94 k), β-galactosidase (116 k), and myosin heavy chain (210 k). The arrowhead on the right indicates a band of Rho3. (B) Immunofluorescent localization of Rho3. *rho3* null cells containing pREP81Rho3 grown in EMM medium without thiamine at 30°C were fixed and stained with Bodipy-phallacidin (green in merged images), anti-Rho3p antibodies (red in merged images), and DAPI (blue in merged images). Deconvoluted 3D images are shown. No Rho3 signal was detected in the cells grown in the presence of thiamine (data not shown). Bar, 5 μm.

Next, we investigated cellular localization of Rho3 by immunofluorescence microscopy. Localization of Rho3 in the wild-type cells was very similar to that in the *rho3* null cells containing pREP81Rho3 (see below). However, the fluorescence signal in the wild-type cells was too weak to be processed for 3-d imaging (data not shown). Thus, we showed images of the latter cells in Fig. 2B. Rho3 was localized to the cell periphery and relatively concentrated around the growing ends of interphase cells and the mid-region of mitotic cells. The Rho3 staining overlaped with that of F-actin at the very tips of the cell and the septum, but the staining patterns were different: the Rho3 staining was continuous, while F-actin was present as patches. Accumulation of Rho3 at the middle cortex of the cell began at metaphase, progressed during anaphase, and persisted at the septum even during cytokinesis when the F-actin ring contracted. This suggests that Rho3 is not a component of the F-actin ring.

Overexpression of constitutively active Rho3Q71L

Overexpression of Rho3Q71L in the wild-type cells partially inhibited cell growth and produced both abnormal dumpy cells and mini-cells after removal of thiamine (Fig. 3). This effect seemed to be caused by high activity of Rho3Q71L, since both cell growth and cell shape were not affected by overexpression of wild-type Rho3 (data not shown). The mini-cells frequently had no nucleus (Table 2) indicating that cell division sometimes occurred at an eccentric position of the cell. Staining of F-actin in mitotic cells showed that formation of the F-actin ring was incomplete although accumulation of F-actin took place, and that the position of the F-actin accumulation was often not at the middle of the cell. Electron microscopy showed that the Rho3Q71L-overexpressing cells formed aberrant septum: they were often incomplete, not symmetric with respect to the long axis of the cell, and formed at an eccentric position. Thus, Rho3 is likely to be involved in controlling both cell shape and septation in fission yeast.

For3 binds to Rho3

A novel gene encoding the diaphanous/formin family protein *for3*$^{+}$ has recently been reported (Feierbach and Chang, 2001). As activated Rho binds to an N-terminal region of some diaphanous/formin family proteins (reviewed by Wasserman, 1998), we examined whether any of the Rho family proteins bind to the N-terminal region of For3 (For3N, amino acid

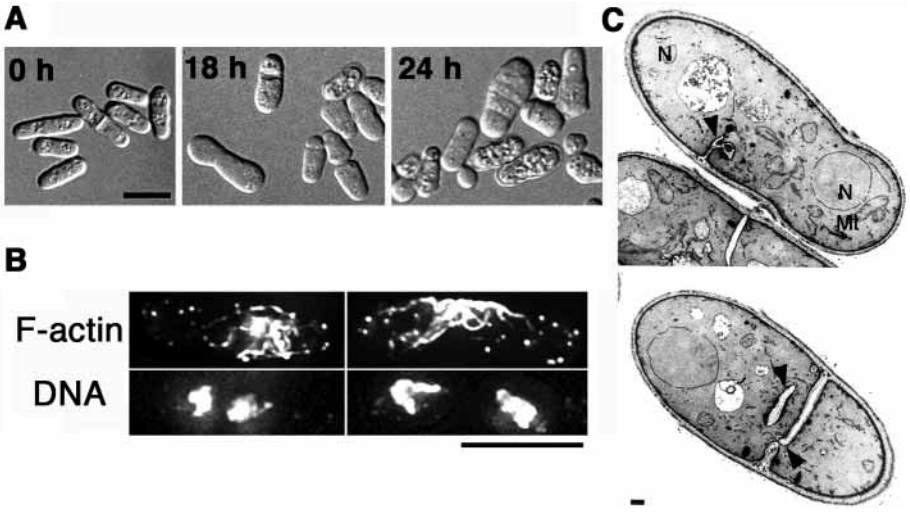

Fig. 3. Phenotype of Rho3Q71L-overexpressing cells. (A) Overexpression of Rho3Q71L produced large dumpy cells and mini-cells. Wild-type cells carrying pREP1Rho3Q71L were grown at 30°C in EMM without thiamine for 18 or 24 hours. DIC images are shown. (B) F-actin ring was abnormally formed in Rho3Q71L-overexpressing cells. The cells (18 hours in A) were fixed and stained with Rhodamine-phalloidin (top) and DAPI (bottom). Deconvoluted 3D images are shown. Bars, 10 μm. (C) Electron micrographs of Rho3Q71L-overexpressing cells (18 hours in A). Arrowheads indicate abnormally formed septa. N, nucleus. Mt, mitochondria. Bar, 0.2 μm.

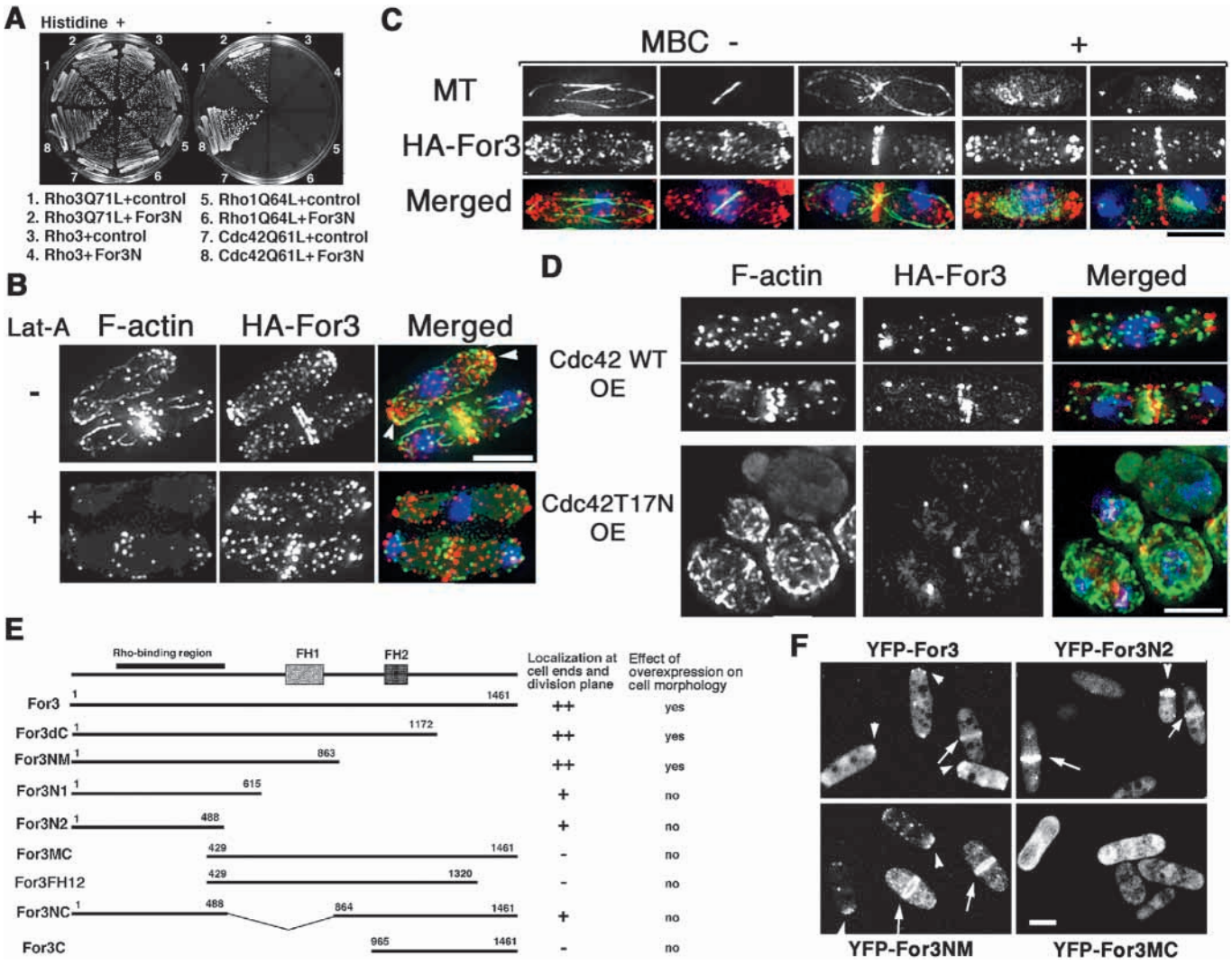

Fig. 4. Characterization of For3. (A) The N-terminus of For3 binds directly to constitutively active Rho3. *L40* cells expressing the indicated proteins were cultured on an SD plate with (+) or without (–) histidine at 30°C for 3 days. 'Control' means empty vector. (B,C) Localization of HA-For3. *for3* null cells containing pREP41HA-for3 were grown in EMM without thiamine at 30°C for 18 hours. Before (–) and after (+) treatment with 10 μM Lat-A or 100 μM MBC for 10 minutes, the cells were fixed and stained with Bodipy-phallacidin or TAT-1 (green in merged images), anti-HA antibody Y-11 (red in merged images), and DAPI (blue in merged images). Deconvoluted 3D images are shown. Arrowheads in B indicate colocalization of HA-For3 with F-actin. No HA-For3 signal was detected in the cells grown with thiamine (data not shown). (D) Localization of HA-For3 in *rho3 cdc42* double-deficient cells. *rho3* null cells containing pREP41HA-for3 and pREP1cdc42 or pREP1cdc42T17N were grown in EMM without thiamine at 25°C for 20 hours and were fixed and stained with Bodipy-phallacidin (green in merged images), Y-11 (red in merged images), and DAPI (blue in merged images). Deconvoluted 3D images are shown. (E) Summary of the functional analysis of For3 and truncated proteins. (F) Localization of For3 and truncated proteins using their YFP fusion proteins. Wild-type cells containing pREP1YFP-for3, REP1YFP-for3NM, pREP1YFP-for3N2, or pREP1YFP-for3MC were grown in EMM containing thiamine until early log phase at 25°C. Arrowheads and arrows indicate the signal of YFP at the cell ends and the division site, respectively. Bars, 10 μm.

Table 2. Expression of dominant active Rho3Q71L caused a defect in cytokinesis

	Formation of F-actin ring (%)			Number of nuclei (%)			
	Normal	Abnormal	Mis-positioned	1 or 2 (with F-actin ring)	2 (without F-actin ring)	>2	0
Control	100	0	0	100	0	0	0
Rho3Q71L	29.4	38.6	32.0	67.8	28.0	0.8	3.4*

Wild-type cells containing pREP1 (control) or pREP1rho3Q71L were grown in the absence of thiamine for 20 hours at 30°C. About 150 mitotic cells or 500 cells were counted for the observation of the F-actin ring or the nucleus, respectively.

*All of these cells were mini cells.

residues 149-488) using the two-hybrid system (Fig. 4A). A positive two-hybrid interaction was detected between For3N and Rho3Q71L or constitutively active Cdc42Q61L, but not between For3N and constitutively active Rho1Q64L. Wild-types of both Rho3 and Cdc42 also did not show binding to For3N (Fig. 4A and data not shown). Thus, it is possible that For3 functions as a target protein for both Rho3 and Cdc42.

Localization of For3

We observed cellular localization of For3 by tagging it with an HA epitope (Fig. 4B,C). HA-For3 was localized as dots to the ends of the interphase cells and to the middle of dividing cells. Only a few For3 dots colocalized with F-actin patches at the cell ends. The For3 dots did not colocalize with F-actin cables. During early mitosis, the For3 dots seemed to encircle the middle of the cells as a single ring. However, they were gradually separated from the F-actin ring as double rings along the septum as cytokinesis proceeded (Fig. 4B,C). When the cells were treated with Latrunculin A (Lat-A) in order to disrupt F-actin structures (Spector et al., 1983), the For3 dots were partially delocalized (Fig. 4B). Thus, F-actin is likely to be involved in establishment of the polarized localization of the For3 dots. In addition, For3 did not seem to colocalize with cytoplasmic MTs as well throughout the cell cycle (Fig. 4C). Moreover, the localization of For3 was maintained after disrupting MTs by treatment with methyl benzimidazole carbamate (MBC) (De Brabander et al., 1976) (Fig. 4C). Thus, MTs may not play an important role in the localization of For3.

Next, we asked whether Rho3 or Cdc42 functions in the localization of For3. First, For3 was localized properly in the *rho3* null cells (data not shown). Then, we examined localization of HA-For3 in the *rho3* null cells overexpressing wild-type Cdc42 or dominant negative Cdc42T17N, respectively (Fig. 4D). Although F-actin patches were delocalized in the *rho3* null cells overexpressing Cdc42, HA-For3 was localized as dots to the ends of interphase cells and to the middle of dividing cells. On the other hand, overexpression of Cdc42T17N caused rounding up of the cell, delocalization of cortical F-actin patches and disappearance of the HA-For3 dots (Fig. 4D).

Furthermore, we investigated a region of For3 molecule, which is required for its localization by expressing For3 and For3-truncated proteins both fused to YFP (an yellow-green variant of the *Aequorea victoria* green fluorescent protein) (Fig. 4E,F). YFP-For3, YFP-For3NM, YFP-For3N1, or YFP-For3N2 showed a localization pattern similar to HA-For3. In contrast, YFP-For3MC diffused in cytoplasm throughout the cell cycle. Thus, the N-terminal region of For3 is essential for the cellular localization, but other regions are not.

Overexpression of For3 induced formation of thick MT bundles

We examined effect of overexpression of For3 or its truncated proteins in the wild-type cells (Fig. 5A). Inhibition of cell growth was seen when For3, For3dC, or For3NM was expressed. On the other hand, the truncated clone lacking the N-terminal region or the FH1 domain did not interfere with the cell growth. In addition, overexpression of For3 inhibited the growth of the *rho3* null cells as well as that of the wild-type

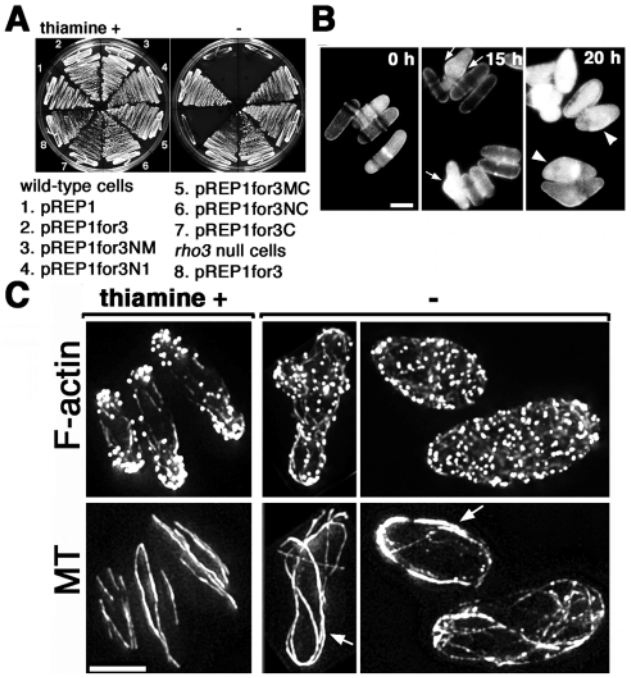

Fig. 5. Overexpression of For3. (A) Effects of overexpression of For3 or truncated proteins on cell viability. Wild-type cells or *rho3* null cells containing indicated vectors, respectively, were grown on an EMM plate with (+) or without (–) thiamine at 25°C for 4 days. (B) Effect of For3 overexpression on cell shape. Wild-type cells containing pREP1for3 grown in EMM without thiamine for the indicated periods were stained with Calcofluor. Arrows indicate bulges. Arrowheads indicate swollen cells. (C) MT bundles were formed in For3-overexpressing cells. The cells (0 and 20 hours in B) were fixed and stained with Rhodamine-phalloidin (top) and TAT-1 (bottom). Deconvoluted 3D images are shown. Arrows indicate thick bundles of cytoplasmic MTs. Bars, 5 μm.

cells, suggesting that this inhibition occurred independently of Rho3.

Calcofluor staining showed that For3-overexpressing cells formed a bulge in the middle of the cell, and then the cells became swollen (Fig. 5B): 7.3% of these cells displayed the bulge 15 hours after removal of thiamine, while after 20 hours 23% were bulged and 28% were swollen. Moreover, the overexpression of For3 seemed to interfere with septation: 21% of the cells were binucleate without septation at 20 hours after the thiamine removal, while such cells were only 1.1% in the presence of thiamine.

We examined organization of F-actin and MTs in the For3-overexpressing cells (Fig. 5C). F-actin patches were scattered all over the cell cortex. Cytoplasmic MTs formed long bundles which did not end at the cell tips, but lined the cell membrane at the tips and further elongated to the other side of the cell, in contrast to cells not overexpressing For3. Thus, For3 may be involved in controlling organization of both F-actin and MTs in fission yeast.

For3 is involved in controlling cell morphology in fission yeast

for3 null cells were viable (Fig. 6A), and were slightly dumpy or bent (Fig. 6B, Table 3). Cytoplasmic MTs were often

Table 3. Rho3 and For3 cooperatively control cell shape

	Normal (%)	Dumpy or swollen	Bent
Wild-type cells	100	0	0
rho3 null cells	97.8	2.2	0
for3 null cells	64.0	30.4	5.6
rho3for3 double null cells	15.5	72.0	12.5

Cells were grown in YEA medium at 25°C. More than 300 cells were observed for each cell type.

abnormally organized in interphase cells (Fig. 6C): they tended to be disordered in the dumpy cells, or curved and located on a convex side of the bent cells. F-actin patches were moderately randomized. It has very recently been reported that interphase *for3* null cells lack F-actin cables (Feierbach and Chang, 2001). However, we were able to find F-actin cables in the interphase *for3* null cells although they were reduced in number and short in length as compared with those in the wild-type cells (Fig. 6D). In mitotic cells especially during metaphase, F-actin cables were frequently seen (Fig. 6C). F-actin ring was organized normally in the middle of anaphase cells (Fig. 6C).

We have recently reported that overexpression of an actin-binding domain of fission yeast fimbrin Fim1 induces formation of thick F-actin cables by stabilizing pre-existent F-actin cables (Nakano et al., 2001b). We thus compared effect of overexpression of this domain (Fim1A2) in the *for3* null cells to that in wild-type cells (Fig. 6E). It was found that thick F-actin cables were formed by overexpressing Fim1A2 even in the absence of For3. However, these cables were not straight but curly and often entangled with each other. For3 may play a role in formation of straight F-actin cables in interphase cells. Moreover, we investigated a genetic interaction between For3 and Cdc8 (tropomyosin) since Cdc8 is essential for formation of F-actin cables probably by stabilizing F-actin (Arai et al., 1998). However, no synthetic effect was seen in *for3 cdc8* double mutant cells (data not shown). Furthermore, overexpression of Cdc8 did not suppress the defect of the organization of the F-actin cables in *for3* null cells. Thus, For3 may control the organization of the F-actin cables in a way different from that of Cdc8.

Next, we investigated a domain of For3 molecule which is involved in controlling organization of F-actin and MTs. *for3*$^+$ gene in *for3Δ*FH12C, *for3Δ*FH2C, or *for3Δ*C strain has an insertion of *ura4*$^+$ so that expression of downstream region from amino acid residues 489, 864, or 1192, respectively, is disrupted. Moreover, we transformed *for3* null cells with pREP1 containing various *for3*-truncates listed in Fig. 4E. Expression of a full length of For3 suppressed the defect of the *for3* null cells (Fig. 4B,C). In contrast, none of the cells expressing For3-truncated proteins displayed normal organization of actin cytoskeleton and cytoplasmic MTs (Fig. 6F and data not shown). Therefore, each region of For3 is likely to be necessary for its function. Interestingly, it was found that many interphase F-actin cables were oriented at random direction in the *for3* null cells containing pREP1For3MC (Fig. 6Fb). This result is unlikely to be caused by extraexpression of For3MC since overexpression of For3MC in wild-type cells does not significantly affect organization of actin cytoskeleton (data not shown). Thus, it is suggested that For3 lacking the N-terminal region retains the ability to induce formation of F-actin cables. Moreover, expression of neither For3FH12 nor For3C induced formation of F-actin cables in the *for3* null cells (Fig. 6F). Therefore, both the FH1 domain and the C-terminus of For3 are likely to play important role in formation of the F-actin cables.

We examined a genetic interaction between Rho3 and For3 by making double null cells. The *rho3 for3* double null cells showed severe growth and morphological defects as compared with each single null cells: majority of the cells were swollen including almost round cells (Table 3, Fig. 6G). The double null cells displayed abnormal organization of F-actin and MTs: F-actin patches were delocalized all over the cell surface, and the cytoplasmic MTs were curved and sometimes formed a basket-like structure especially in the round cells. Thus, the defect caused by the depletion of For3 was enhanced in the absence of Rho3.

Next, we examined whether or not depletion of For3 suppresses the effect of overexpression of Rho3Q71L. It was found that the overexpression of Rho3Q71L in *for3* null cells caused a defect in the organization of the F-actin ring similar to that in the wild-type cells. Thus, Rho3Q71L can also control actin organization through another pathway which does not include For3. Furthermore, we examined whether the defects caused by the depletion of Rho3 or For3 were suppressed by an increased expression of For3 or Rho3, respectively. No suppressing effect was seen in either case. In addition, defects in organization of both actin cytoskeleton and MTs in *for3* null cells were not suppressed by overexpression of Cdc42.

Lastly, we tested whether or not For3 shares function with other *S. pombe* diaphanous/formins. It has previously been reported that Cdc12 functions in the assembly of the F-actin ring and both genetically and physically interacts with Cdc3 (profilin) (Chang et al., 1997). We thus examined the phenotypes of *for3 cdc12-112* and *for3 cdc3-124* double mutants. No synthetic effect was found between For3 and either of these proteins (data not shown). Furthermore, the *for3* null cells were able to conjugate and sporulate under nitrogen starvation (data not shown), indicating that For3 is not essential in the process of cell conjugation. Since *fus1*$^+$ is essential for the conjugation (Petersen et al., 1995), it is unlikely that For3 exert the function identical to that of Fus1. Thus, the functions of the three diaphanous/formin family proteins, namely Cdc12, Fus1, and For3, are probably different from each other in fission yeast.

Discussion

Rho3 and For3 are involved in controlling polarized growth of the cell

Rho3 is a functionally conserved member of the Rho family small GTPase: it is most similar to budding yeast Rho3p, and Rho3 can be replaced by Rho3p in the fission yeast cells. Since other fission yeast Rho proteins did not complement the growth defect of *rho3* null cells, Rho3 may have activities distinct from them. Rho3 deletion caused a defect in maintaining the polarized cell growth: *rho3* null cells displayed a dumpy shape at 37°C. This defect may be caused by alteration of the organization of the cytoskeleton since F-actin patches were delocalized and the cytoplasmic MTs were abnormally organized in these cells.

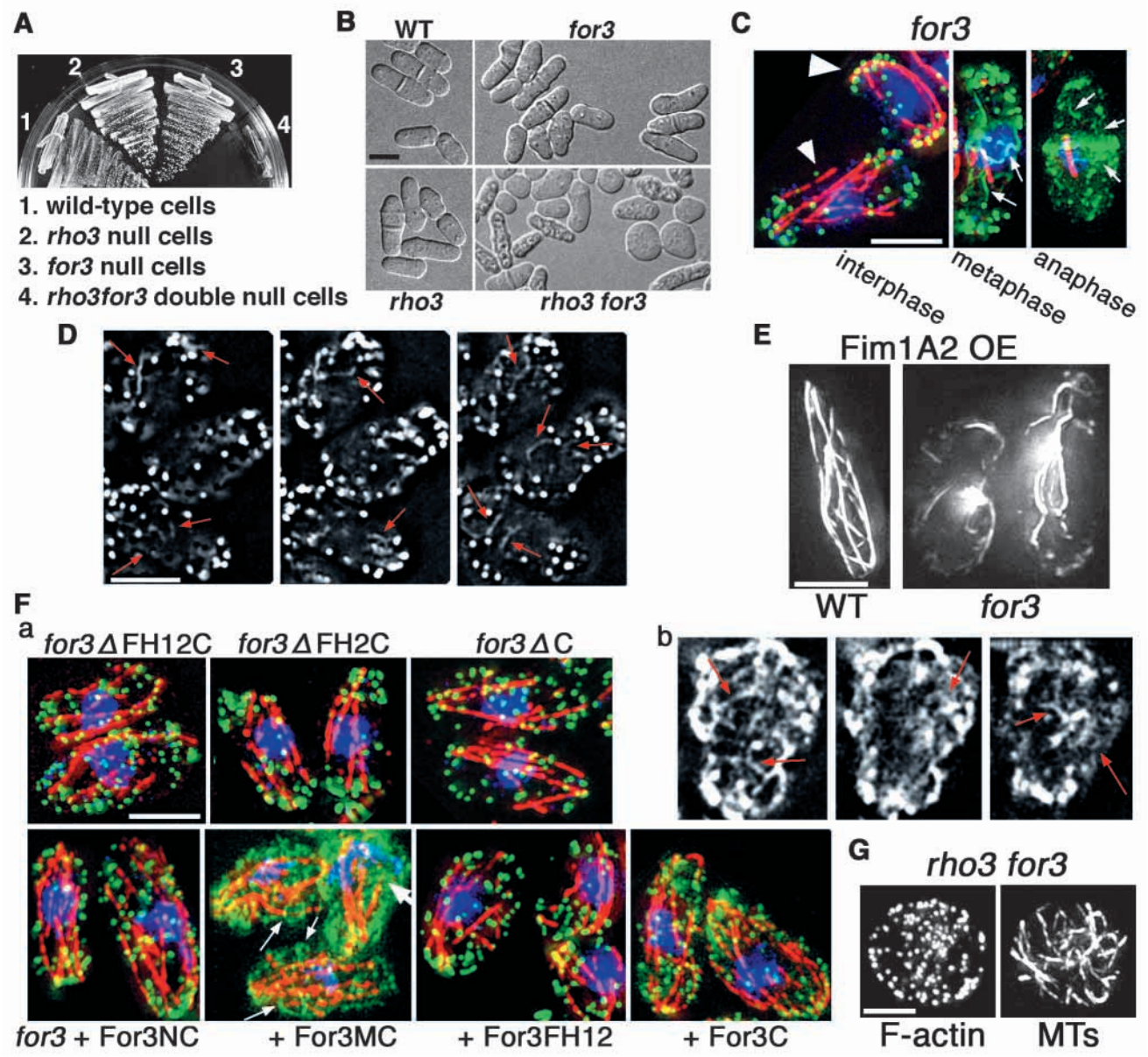

Fig. 6. Phenotype of *for3* null cells. (A) Viability of *for3* null cells. Indicated cells were grown at 25°C for 4 days on a YEA plate. (B) Aberrant morphology of *for3* null cells and *rho3 for3* double null cells. Cells grown in YEA medium at 25°C. DIC images are shown. (C) Defects in actin cytoskeleton and MTs in *for3* null cells. *for3* null cells grown in YEA medium at 25°C were stained with Bodipy-phallacidin (green), TAT-1 (red), and DAPI (blue). Deconvoluted 3D images are shown. Small arrowhead indicates disorganized cytoplasmic MTs in dumpy cells. Large arrowhead indicates curved MTs located on a convex side of a bent cell. Arrows indicate F-actin cables. (D) Interphase F-actin cables in *for3* null cells. *for3* null cells grown in YEA medium at 25°C were stained with Bodipy-phallacidin and DAPI. The pictures represent deconvoluted Z-axis sections (top, middle, and bottom sections from left to right) of three interphase cells. Arrows indicate F-actin cables. (E) Formation of thick F-actin cables by overexpression of Fim1A2. Wild-type cells or *for3* null cells containing pREP1fim1A2 were grown in EMM without thiamine for 18 hours at 25°C. F-actin images are shown. (F) Observation of cells expressing For3-truncated proteins. (a) Deconvoluted 3D images of *for3Δ*FH12C cells, *for3Δ*FH2C cells, *for3Δ*C cells, and *for3* null cells containing pREP1for3NC, pREP1for3MC, pREP1for3FH12, or pREP1for3C are shown. The cells were stained as in C. (b) F-actin images of a *for3* null cell containing pREP1for3MC indicated by the large arrow in a. The pictures represent deconvoluted Z-axis sections (top, middle, and bottom sections from left to right). (G) A *for3 rho3* null cell grown in YEA medium at 25°C was stained with Bodipy-phallacidin (left), and TAT-1 (right). Deconvoluted 3D images are shown. Bars, 5 μm.

For3 binds to active forms of both Rho3 and Cdc42 but not to that of Rho1. Cdc42 has been reported to play an important role in establishing cell polarity: *cdc42* deficient cells completely lose the polarity and show delocalized F-actin patches (Miller and Johnson, 1994) and basket-like cytoplasmic MTs (K.N. and I.M., unpublished). *for3* null cells were a little dumpy and also showed delocalization of F-actin patches and abnormal organization of cytoplasmic MTs. Therefore, For3 is likely to function through interaction with Rho3 and/or Cdc42 in the control of polarized growth of the

cell. Since the *rho3 for3* double null cells showed defects severer than those seen in each of the single null cells such that round cells appeared, both Rho3 and For3 play important roles in the polarized growth in the fission yeast cells.

Increased expression of For3 did not suppress the defect of the *rho3* null cells. This suggests that Rho3 may have another target protein(s) which functions in a cooperative manner with For3. In budding yeast, Rho3p directly interacts with type V myosin Myo2p (Robinson et al., 1999). As the fission yeast type V myosin, Myo4/Myo52, plays an important role in the polarized cell growth (Motegi et al., 2001; Win et al., 2001), it is possible that Rho3 may function in the cell morphogenesis through both For3 and Myo4. In addition, the budding yeast Rho3p genetically interacts with Bem1p, Boi1p and Boi2p (Matsui and Toh-e, 1992b; Bender et al., 1996; Matsui et al., 1996). Bem1p directly interacts with Boi1p, Boi2p, Cdc24p (Peterson et al., 1994) and Ste20p protein kinase (Leeuw et al., 1995). These proteins function in the polarized growth of the budding yeast cell. Recently, it has been reported that Scd1/Ral1, Scd2/Ral3, Shk1/Pak1/Orb2, and Pob1, which are homologous to budding yeast Cdc24p, Bem1p, Ste20p, and Boi1p/Boi2p, respectively, play important roles in polarized cell growth in fission yeast (Chang et al., 1994; Marcus et al., 1995; Ottilie et al., 1995; Sawin et al., 1998; Toya et al., 1999). Thus, it may also be possible that Rho3 functions cooperatively with these proteins in addition to For3.

Localization of For3

For3 was localized as dots at the ends of interphase cells which remained in the absence of either F-actin patches or cytoplasmic MTs. Thus, the For3 localization in the cell cortex is independent of both actin cytoskeleton and cytoplasmic MTs. Since active forms of both Rho3 and Cdc42 bind For3 and these proteins localize to the cytoplasmic membrane at the ends of interphase cells [present study (Merla and Johnson, 2000)], it is likely that For3 associates with these Rho GTPases during the polarized growth. This is consistent with the fact that the N-terminal region of For3, including the Rho-binding region, was essential and sufficient for its cellular localization. Actually, For3 was delocalized in *rho3* null cells overexpressing dominant negative Cdc42T17N. The N-terminal region of For3 is also required for its function (see below). Therefore, cortical localization of For3 may be important for its cellular function.

For3 is involved in organization of both actin cytoskeleton and microtubules

Both the gene disruption and the overexpression experiments strongly suggested that For3 may control organization of both F-actin and cytoplasmic MTs. It was demonstrated that the N-terminal half of For3 including both the Rho-binding region and the FH1 domain was required for manifestation of the overexpression effect. The FH1 domain of the diaphanous/formin proteins is considered to control the organization of the actin cytoskeleton by interacting with the G-actin-binding protein, profilin (Wasserman, 1998). Indeed, fission yeast Cdc12 has been suggested to function in assembling the F-actin ring by interacting with Cdc3 (Chang et al., 1997). However, we were not able to detect any interaction between For3 and Cdc3. Interestingly, it has very recently been reported that the budding yeast diaphanous/formin family protein Bni1p stimulates polymerization of actin in vitro (Pruyne et al., 2002; Sagot et al., 2002a). Thus, it is possible that For3 controls the actin cytoskeleton in a direct manner in *S. pombe* cells.

It is not clear how overexpressed For3 induced the formation of thick MT bundles. The overexpressed For3 was scattered all over the cell cortex as large dots but was not localized to the thick MT bundles (K.N. and I.M., unpublished). Therefore, For3 may influence organization of MTs in an indirect manner. It is possible that For3 in the cell cortex interacts with other proteins which control cytoplasmic MTs. It would be important to identify proteins that associate with the N-terminal half of For3 in order to clarify the mechanisms of regulation of the microtuble cytoskeleton by For3.

We also demonstrated that the C-terminal region of For3 is required for its function. Both *for3Δ*FH2C cells and *for3Δ*C cells were defective in organization of actin cytoskeleton and cytoplasmic MTs like *for3* null cells. Moreover, For3MC exerted an ability to induce formation of F-actin cables. Interestingly, it has very recently been reported that the FH2 domain of Bni1p plays an important role in formation of actin cables in budding yeast (Evangelista et al., 2002; Sagot et al., 2002b). Moreover, it has also been suggested that mDia in HeLa cells is possible to control both actin cytoskeleton and MTs through the FH1 and FH2, respectively (Ishizaki et al., 2001). Therefore, the FH2 domain of For3 is possible to control the cytoskeletal organization in the fission yeast. In addition, our results indicate that the downstream region of the FH2 domain is required for the function of For3. It has been reported that the C-terminal region of Bni1p binds to Bud6p/Aip3p (Evangelista et al., 1997). A protein homologous to Bud6p/Aip3p has been identified and revealed to be involved in establishment of cell polarity in *S. pombe* (Glynn et al., 2001; Jin and Amberg, 2001). Therefore, it is possible that For3 interacts with Bud6/Aip3 for controlling the cytoskeletal organization.

Rho3 is also involved in cytokinesis

Rho3 was localized at the division site during mitosis and septation. The *rho3* null cells formed multi-septated cells above 34°C and some of the *rho3* null cells fail to undergo cytokinesis at 37°C. Thus, Rho3 may be involved in controlling cytokinesis. Interestingly, overexpression of Rho3Q71L interfered with proper formation of the F-actin ring both in its shape and positioning. It has been considered that the position of nucleus is important for determining the division site in fission yeast (Tran et al., 2001). However, the nucleus was correctly located in the Rho3Q71L-overexpressing cells. Therefore, the control of Rho3 activity may be required for positioning the cell division plane.

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