Novel Rho GTPase Involved in Cytokinesis and Cell Wall Integrity in the Fission Yeast Schizosaccharomyces pombe

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Received 9 December 2002/Accepted 18 February 2003

The Rho family of GTPases is present in all eukaryotic cells from yeast to mammals; they are regulators in signaling pathways that control actin organization and morphogenetic processes. In yeast, Rho GTPases are implicated in cell polarity processes and cell wall biosynthesis. It is known that Rho1 and Rho2 are key proteins in the construction of the cell wall, an essential structure that in Schizosaccharomyces pombe is composed of β-glucan, α-glucan, and mannoproteins. Rho1 regulates the synthesis of 1,3-β-D-glucan by activation of the 1,3-β-D-glucan synthase, and Rho2 regulates the synthesis of α-glucan by the 1,3-α-D-glucan synthase Mok1. Here we describe the characterization of another Rho GTPase in fission yeast, Rho4. rho4Δ cells are viable but display cell separation defects at high temperature. In agreement with this observation, Rho4 localizes to the septum. Overexpression of rho4Δ causes lysis and morphological defects. Several lines of evidence indicate that both rho4Δ deletion or rho4Δ overexpression result in a defective cell wall, suggesting an additional role for Rho4 in cell wall integrity. rho4Δ cells also accumulate secretory vesicles around the septum and are defective in actin polarization. We propose that Rho4 could be involved in the regulation of the septum degradation during cytokinesis.

Yeasts have been used as model systems to study morphogenesis because they undergo morphogenetic changes that require asymmetric cellular growth and actin cytoskeleton reorganization during their life cycle (42). Schizosaccharomyces pombe cells are rod shaped, grow mainly by elongation of their ends, and divide by binary fission after forming a centrally placed division septum. Cytokinesis and cell division occur through the action of a medial actomyosin-based contractile ring, where a septum of cell wall material is also formed. The establishment of the division plane is determined by the position of the premitotic nucleus. Mutations in the mid1Δ gene lead to misplaced septa, suggesting that Mid1 may couple the position of the nucleus with that of the division site (7, 48). When the division plane is established, the medial ring is formed; F-actin patches are subsequently recruited to the medial ring, probably directing the localization of proteins necessary for plasma membrane and cell wall biosynthesis during septation (reviewed in references 22 and 31). After assembly of the primary and secondary septa, the primary septum needs to be cleaved to separate the two cells. Little is known about how cleavage is achieved, but probably β-glucanases are needed, since 1,3-β-D-glucan is the main component of this structure (25).

In all eukaryotic organisms, Rho GTPases are key molecules in polarity processes (reviewed in references 34, 49, and 51). These small GTPases act as molecular switches that are turned on and off by binding to GTP or GDP. The GTP-bound form interacts with its effector molecules to perform its cellular functions. GTPases are positively regulated by GDP-GTP exchange factors and negatively regulated by GTPase-activating proteins (GAPs) and GDP dissociation inhibitors. In yeasts, Rho GTPases are responsible for the coordinated regulation of cell wall biosynthesis and actin cytoskeleton organization required to maintain cell integrity and polarized growth (2, 11).

S. pombe genome sequence analysis has revealed the existence of six Rho GTPases. cdc42Δ and rho1Δ are essential genes involved in polarity processes. The Rho1 GTPase was identified as a regulatory component of the 1,3-β-D-glucan synthase and is also required for maintenance of cell integrity and polarization of the actin cytoskeleton (4). Rho1 localizes to sites of polarized growth, the cell poles and the septum (3), and cells lacking this protein undergo lysis; this defect is not rescued by an osmotic stabilizer. Rho1-GTP also interacts with Pck1 and Pck2, the two fission yeast PKC homologues (5). The rho2Δ gene is not essential, but its overexpression is lethal. Rho2 also localizes to sites of polarized growth (24). Rho2-GTP interacts with Pck2 and acts as a positive regulator of Mok1, an essential 1,3-α-D-glucan synthase (13). Therefore, Rho1 and Rho2 are required for the synthesis of the two main cell wall polymers, 1,3-β-glucan and 1,3-α-D-glucan, respectively. rho3Δ characterization has been described recently; it is not essential, but cells lacking it grow poorly at 37°C. Rho3 interacts with the formin For3 and is involved in polarity processes (39). Nothing is known about the function of the other two Rho GTPases.

Here we describe the characterization of Rho4. Our results indicate that Rho4 is involved in cytokinesis in S. pombe. The rho4Δ gene is not essential; however, rho4 mutant cells are defective in cell separation at high temperature. Rho4 localizes to the septum but not to the cell poles. In addition, rho4Δ overexpression causes lysis prevented by sorbitol. All these data suggest that the Rho4 GTPase is involved in cell wall dynamics during cytokinesis.
TABLE 1. *S. pombe* strains used in this work

| Strain* | Genotype |
|---------|----------|
| PPG102... | h+ leu1-32 |
| PPG1541... | h+ leu1-32 ura4-D18 |
| PPG1580... | h+ leu1-32 ura4-D18 rho4:kanMX6 |
| PPG148... | ura4-D18 cdc25-22 |
| PPG1725... | ura4-D18 cdc25-22 rho4:kanMX6 leu1+::GFP-rho4* |
| PPG301... | h+ leu1-32 cdc3-19 |
| PPG327... | h+ leu1-32 mid1-366 |
| PPG1720... | h+ leu1-32 cdc15-140 |
| PPG1721... | h+ leu1-32 cdc16-116 |
| PPG305... | h+ leu1-32 cdc11-119 |
| PPG233... | h+ leu1-32 ura4-D18 cys1-12 |

*PPG148 is from Sergio Moreno’s laboratory, and PPG301, PPG327, PPG1720, and PPG1721 are originally from Paul Nurse’s laboratory.*

**MATERIALS AND METHODS**

Strains, growth conditions, and genetic methods. Fission yeast strains used in this study are listed in Table 1. Genetic methods and growth media were as described previously (38). Yeast transformations were performed by the lithium acetate method (27). Calcofluor sensitivity was analyzed on rich-medium (YES) plates buffered with 250 mM sodium phosphate (pH 6.0) and containing 0.5 mg of Calcofluor per ml. Echinocandin sensitivity was analyzed on YES plates containing 1 to 5 μg of echinocandin per ml. Cell lysis was visualized by staining cells with 0.001% methylene blue.

For overexpression experiments, cells were grown to mid-log phase in minimal medium (EMM) containing 15 μM thiamine. Then the cells were harvested, washed three times with water, and inoculated into fresh medium without thiamine at 25°C. They were then transferred back to 25°C. 

NcoI digestion of *S. pombe* Nrho4 was achieved using primers corresponding to the NcoI restriction sites of the pAS2 vector, creating the plasmid pAS2-Nrho4.

**Deletion and tagging of the rho4** gene. The complete ORF of rho4* was PCR-amplified from the starting codon to the stop codon. The PCR-amplified product was cloned into the integrative vector pJK148 (29), resulting in pJK148-GFP-rho4*. This plasmid was cut with NruI and integrated at the leu1 locus of strain PPG1541, which contains a rho4* null allele, to generate the strain PPG1580.

**Microscopy techniques.** For Calcofluor staining, exponentially growing *S. pombe* cells were harvested, washed once with water, and resuspended for 5 min at room temperature in water with Calcofluor at a final concentration of 50 μg/ml. After being washed twice with water, the cells were observed in a Leica DMRXA microscope. For Calcofluor and 4′,6-diamidino-2-phenylindole (DAPI) double staining, exponentially growing cells were fixed with 70% cold ethanol, harvested, and resuspended in phosphate-buffered saline containing 100 μg of DAPI per ml and 100 μg of Calcofluor per ml. The cells were visualized in a Leica DMRXA microscope. For nuclear staining of living cells, cells were incubated for 15 min in phosphate-buffered saline with Hoechst at a final concentration of 20 μg/ml.

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incubated with 100 U of Zymolyase 100T (Seikagaku Kogyo Co., Ltd.) or Quantazyme (Quantum Biotechnologies Inc.) for 36 h at 30°C. Aliquots without enzymes were included as controls. Samples were centrifuged, and the supernatant and washed pellet were counted separately. The supernatant from the Zymolyase reaction was considered β-glucan plus galactomannan, and the pellet was considered α-glucan. The supernatant from the Quantazyme reaction was considered β-glucan, and the pellet was considered α-glucan plus galactomannan.

**Measurement of 1,3-β-D-glucan synthase activity.** Cell extracts were prepared, and 1,3-β-D-glucan synthase activity was assayed as described previously (3) with some modifications. Basically, 3 × 10^7 cells were harvested, washed with 1 mM EDTA, and resuspended in 300 μl of the same buffer. Lysis was achieved in a Fast-Prep apparatus as described above. The resulting homogenates were collected by adding 30 ml of 50 mM Tris-HCl (pH 8.0) in 1 mM EDTA. Cell walls were removed by centrifugation for 5 min at 1,000 g. The supernatant was centrifuged for 30 min at 50,000 g, and the membrane pellet was resuspended in 0.5 ml of buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 30% glycerol. Protein concentration was determined by the Bio-Rad protein assay method. The amount that catalyzes the incorporation of 1 μmol of substrate (UDP-D-glucose) per minute at 30°C was considered a unit of activity.

**Other assays.** Glucanase sensitivity was evaluated by a procedure described previously (47). Wild-type cells overexpressing rho4+ or rho4G23V, or rho4T28N were grown for 12 h in EMM without thiamine at 32°C. Cells were harvested, washed in TE buffer, and resuspended at an OD600 of 1.0 in the same buffer containing 20 μg of Zymolyase-100T per ml. Cell suspensions were incubated at 30°C, and the OD600 was measured at different times. Acid phosphatase secretion was assayed as described previously (52).

**RESULTS**

**Rho4 is a novel GTPase of the Rho family.** Sequence analysis of the fission yeast genome revealed that in addition to the extensively studied cdc24+ and rho1+, and rho2+ genes, S. pombe possesses three other members of the Rho family of GTPases encoded by the SPAC23C4.08, SPAC16A10.04, and SPAC20H4.11c predicted ORFs, called rho3+, rho4+, and rho5+, respectively (http://www.genedb.org/genedb/pombe). rho3+ has recently been characterized (39). In this paper, we report the characterization of the predicted S. pombe ORF SPAC16A10.04 (Sanger Centre [http://www.sanger.ac.uk]), which we called rho4+. The rho4+ ORF was cloned by PCR using the appropriate primers and a S. pombe cDNA library (see Materials and Methods).

rho4+ encodes a protein of 203 amino acids with significant homology to GTPases of the Rho family (Fig. 1). Like all Rho proteins, Rho4 contains consensus amino acid sequences responsible for specific interaction with GDP and GTP and for GTPase activity. Additionally, Rho4 contains the CAAL sequence at its COOH terminus, which in other Rho proteins is posttranslationally modified by geranylgeranyl transferase I, allowing them to be targeted to the membranes in order to be functional (49). Rho4 displays 40 to 55% identity and 75 to 77% similarity over the entire length to the other S. pombe Rho proteins, being most closely related to Rho1. These proteins are highly conserved at their N termini but more divergent at their C termini (Fig. 1).

**Rho4 is required for cell separation.** To investigate the function of rho4+, the gene was deleted (see Materials and Methods). rho4Δ cells are viable at all temperatures; however, at high temperature (36 to 37°C), cells contain multiple septa (Fig. 2A). DAPI staining revealed that each compartment contains one nucleus, indicative of a defect in cell separation after septum assembly (Fig. 2B). Quantitation of the fraction of cells containing septa shows an accumulation of septated cells in the
FIG. 2. rho4Δ cells display cytokinesis defects at high temperatures. (A) Wild-type (PPG371) and isogenic (PPG1541) rho4Δ cells were grown at 37°C to the logarithmic phase. Cells stained with Calcofluor (right panels) and the corresponding differential interference contrast (DIC) images (left panels) are shown. (B) rho4Δ cells (PPG1541) were grown at 37°C and stained with DAPI and Calcofluor. Two representative multiseptated cells are presented. (C) Percentage of wild-type (PPG371) or rho4Δ (PPG1541) cells grown at 32 or 37°C containing a single septum or multiple septa. Cultures are in the logarithmic phase; longer periods of growth increase the fraction of cells with multiple septa in the rho4Δ strain (data not shown). (D) Wild-type (PPG371) and isogenic (PPG1541) rho4Δ cells were grown at 37°C to the logarithmic phase. Actin was stained with Alexa Fluor-phalloidin.
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rh4 mutant (Fig. 2C). Occasionally, some branched cells are observed, as described for other mutants with cell separation defects, such as sep1 or spl1 (31).

It is well known that Rho GTPases are involved in actin organization. To examine the localization of cortical F-actin in rh4Δ cells, Alexa Fluor-phalloidin staining was performed. In wild-type cells, cortical F-actin was localized as patches at the growing end(s) and the septation site, as reported previously (35). In contrast, in rh4Δ cells growing at 37°C, cortical F-actin is dispersed throughout the cell and is no longer polarized in cells without a septum but is properly polarized in septated cells (Fig. 2D). Actin is depolarized in 77% of the nonseptated rh4Δ cells compared with 35% of cells of the isogenic wild-type strain. This result suggests that, like other GTPases, Rho4 is also involved in actin organization.

**Rho4 localizes to the septum.** To determine the subcellular localization of Rho4, the protein was tagged with GFP at the N terminus and expressed from the rho4Δ promoter (see Materials and Methods). The N terminus was chosen because epitopes at the C terminus may affect posttranslational modification and localization of Rho proteins. A rho4Δ strain carrying GFP-rho4+ integrated in the genome has no cell separation defects at high temperatures, suggesting that the tagged protein is functional. Rho4 localization was examined in strains carrying GFP-rho4+ integrated in the genome and in strains transformed with a plasmid containing GFP-rho4+ (pBS2). In both cases, a similar localization pattern was observed (Fig. 3A and data not shown), although a stronger signal was detected when the plasmid was used.

GFP-Rho4 localizes to the region of polarized growth during cytokinesis and cell separation, the septum (Fig. 3A). First, it appears as a band at sites of the cell cortex where the septum is forming. This stage is very transient but was confirmed by using a synchronized culture of a cdc25-22 strain (data not shown). As the septum develops, the GFP-Rho4 signal extends further toward the center of the cell until it forms a single strong band across the cell. Finally, as cell separation begins by digestion of the primary septum, the GFP-Rho4 protein begins to disappear. These observations indicate that Rho4 is targeted to the developing septum early in the septation process and remains throughout cell separation. As shown in Fig. 3A, GFP-Rho4 localizes to the medial region after mitosis, when the two nuclei are perfectly separated. In contrast to the observations made for Rho1 and Rho2 (3, 24), no signal at the cell poles is observed for GFP-Rho4, indicating that Rho4 plays a more specific role in septation.

Rho4 can be visualized only in cells with a developing septum; therefore, we considered the possibility that Rho4 levels might be regulated in a cell cycle-dependent manner. To address this question, GFP-Rho4 was analyzed by Western blotting in a synchronous population of S. pombe cells. The cdc25-22 thermosensitive mutant, which arrests at the G2/M boundary, was used to synchronize the culture. Cells were arrested at the restrictive temperature and transferred to the permissive temperature, and septum formation was monitored. The first peak of synchronous septation took place at around 80 min. Western blot analysis of GFP-Rho4 using anti-GFP antibodies identified a specific band of around 46 kDa coincident with the predicted size for the GFP-Rho4 fusion protein (Fig. 3B). GFP-Rho4 was present throughout the cell cycle, and protein levels did not show any significant change. Thus, Rho4 is not a cell cycle-regulated protein, and protein levels do not appear to determine Rho4 function. There should be other proteins that regulate its specific localization to the septum at the right moment.

**Rho4 localization is defective in septation mutants.** To further investigate how localization of Rho4 is regulated, we analyzed its localization in different septation mutants. Mid1 is required for correct positioning of the medial ring (48), mid1-366 mutants at the restrictive temperature display misplaced septa. GFP-Rho4 is localized to these septa (Fig. 4A). This observation indicates that GFP-Rho4 localization is not dependent on Mid1 and that Rho4 can localize to the abnormally positioned septa.

A profilin mutant (cdc3-319) is defective in actin patch localization, does not have a medial ring, and displays a disorganized septum at the restrictive temperature (9). Cdc15 is an SH3 domain-containing protein; at the restrictive temperature, cdc15-140 mutants are impaired in the assembly of the medial ring and are unable to form a septum (19). GFP-Rho4 localizes properly in both cdc3-319 and cdc15-140 mutants at the permissive temperature; however, after the transfer to the restrictive temperature, no polarized localization corresponding to GFP-Rho4 could be observed (Fig. 4A). Western blot analysis demonstrated that similar levels of GFP-Rho4 were present in both cdc3-319 and cdc15-140 cells at the permissive or restrictive temperature (data not shown). These results suggest that the actomyosin ring could serve as a spatial landmark to target Rho4 to the division site.

Components of the septation initiation network (SIN) are essential for division septum formation after assembly of the actomyosin ring and accumulation of F-actin patches. Given that SIN mutants are defective in cytokinesis, localization of GFP-Rho4 in the SIN mutant cdc11-119 was analyzed. Cdc11 is a protein required for the localization of the known SIN components, except Sid4, to the spindle pole body (30). GFP-Rho4 localizes properly in the cdc11-119 mutant at the permissive temperature; however, after the transfer to the restrictive temperature, no polarized localization to the medial ring corresponding to GFP-Rho4 can be observed (Fig. 4A). This result suggests that the SIN signaling cascade is important for the assembly of Rho4 to the division site.

One of the proteins essential for division septum assembly is Cps1/Bgs1, a putative catalytic subunit of the enzyme 1,3-β-d-glucan synthase. The cps1Δ gene is essential and is required for cytokinesis (26, 32). At the restrictive temperature, cps1-12 mutants swell and finally lyse. We analyzed the localization of GFP-Rho4 in the cps1-12 mutant and found that even at the restrictive temperature, GFP-Rho4 was perfectly localized to the septum (Fig. 4A).

Cdc16 and Byr4 act as a GAP for the GTPase Spg1. At the restrictive temperature, cdc16-116 mutants form multiple well-oriented septa in one cell (21). GFP-Rho4 is localized to the septa of the cdc16-116 mutant (Fig. 4A). This observation indicates that GFP-Rho4 localization is not dependent on Cdc16 function and that Rho4 can localize to the septa even if they are supernumerary in the cell.

Taken together, these results suggest that localization of GFP-Rho4 to the septum is dependent on SIN signaling.

To independently test whether Rho4 localization depends...
on actin, cdc25-22 cells expressing GFP-Rho4 were treated with latrunculin A after release into mitosis. Latrunculin A sequesters monomeric actin and prevents actin polymerization. After the treatment, Rho4 was still localized to the septum, suggesting that actin is not required for the maintenance of Rho4 in the septum (Fig. 4B).

**rho4**^+_ overexpression causes cell lysis. It has been shown that overexpression of the rho1^+_ or rho2^+_ gene in *S. pombe* causes a loss of polarity; cells become round and have a very thick cell wall (4, 13), suggesting that Rho1 and Rho2 play a role in polarity processes and cell wall biosynthesis.

To study further the role of Rho4, we overproduced Rho4 to determine whether the effect was similar to rho1^+_ or rho2^+_ overexpression. rho4^+_ was cloned under the thiamine-repressible nmt1 promoter in the pREP3X vector (see Materials and Methods). After 16 to 18 h of induction, overexpression of rho4^+ caused the cells to lyse (Fig. 5). Cell lysis reached its maximum level (30%) at 20 h. rho4^+ overexpression causes lysis of individual cells more frequently than of separating cells. Interestingly, after longer overexpression (>24 h), cells that had not lysed became large and round, indicative of polarity or cell wall defects (Fig. 5).

It is known that Rho proteins are tightly regulated; they are active when bound to GTP and inactive when bound to GDP.

![Diagram](image-url)
To further analyze Rho4 function, we generated two site-specific mutations described in other GTPases (10). The G23V mutation should generate a Rho4 permanently bound to GTP (constitutively active Rho4), whereas the T28N mutation should generate a Rho4 permanently bound to GDP (constitutively inactive Rho4). Cells overexpressing rho4G23V under the nmt1 promoter also showed a lysis defect; most cells lyse during separation, suggesting a role for active Rho4 in this process.

**FIG. 4.** GFP-Rho4 localization in septation mutants. (A) Different septation mutants were transformed with a plasmid containing GFP-rho4 under its own promoter (pBS2), grown at the permissive temperature to the logarithmic phase, and then transferred to 37°C for 5 h. GFP-Rho4 localization is shown in the right panels, and the corresponding DIC images are shown in the left panels. GFP-Rho4 is properly localized to the septum in all mutants at the permissive temperature (data not shown). (B) cdc25-22 cells containing GFP-rho4 (PPG1725) were synchronized in G2 by incubation at 37°C for 3 h and allowed to enter mitosis by shifting to 25°C. After 90 min, GFP-Rho4 was localized to the septum in most cells. At this point, the cells were treated with either 50 μM LatA in dimethyl sulfoxide (DMSO) (right panels) or DMSO alone (left panels) for 2 min. DIC images, GFP-Rho4 localization, and actin staining using Alexa Fluor-phalloidin are presented.
FIG. 5. rho4+ overexpression causes cell lysis. Wild-type cells (PGI02) transformed with plasmid pREP3X, pREP3Xrhost, pREP3Xrho4G23V, or pREP3Xrho4T28N were grown at 32°C in EMM with 15 mM thiamine and then transferred to EMM without thiamine to induce expression of the rho4+ gene and its mutant alleles. Images of cells grown for 16 h (left panels) or 26 h (right panels) in the absence of thiamine are shown. Lysed cells (arrows) and round cells (asterisks) are indicated.

Levels of HA-Rho4, HA-Rho4G23V, and HA-Rho4T28N were analyzed during overexpression experiments by Western blot analysis using anti-HA antibodies. In all cases, the Rho4 proteins began to accumulate at 12 h and their levels peaked at around 16 to 20 h and began to diminish at 24 to 28 h (data not shown), in good agreement with the phenotypes observed.

Collectively, these results indicate that the effects of rho4+ overexpression are very different from those of rho1+ overexpression (4). Overexpression of wild-type rho4+ or the constitutively active rho4G23V has a lytic effect similar to that of overexpression of the rho1T20N dominant-negative allele, suggesting a different, and perhaps opposite, mechanism of action for Rho4 and Rho1.

The nmt1::rho4+, nmt1::rho4G23V, and nmt1::rho4T28N alleles were also integrated in the genome in a rho4Δ background. In the absence of thiamine, these strains showed similar phenotypes to those described above for nonintegrated plasmids (data not shown).

rho4+ overexpression or rho4+ deletion causes cell wall defects. Several lines of evidence suggest that the lytic phenotype conferred by an excess of Rho4 results from a defective cell wall. First, the growth defect and lysis can be partially rescued by addition of the osmotic stabilizer sorbitol (Fig. 6A). A sorbitol-remediable lysis phenotype has been described previously for mutants with a defective cell wall in both Saccharomyces cerevisiae and S. pombe (14, 43).

We also studied whether cells overproducing Rho4 exhibit cell wall defects by testing their sensitivity to cell wall inhibitors such as Calcofluor or echinocandin. Calcofluor binds mainly to the S. pombe septum and is toxic because it interferes with cell wall assembly. Echinocandin is an antibiotic that specifically inhibits the 1,3-β-glucan synthase. As shown in Fig. 6B, overexpression of either rho4+ or rho4G23V caused hypersensitivity to both agents, indicating that the cells display an abnormal cell wall. Surprisingly, overexpression of rho4T28N also resulted in hypersensitivity to Calcofluor but not to echinocandin. We also analyzed the sensitivity to Calcofluor in cells lacking rho4+. As expected, rho4Δ cells were more resistant to Calcofluor than were wild-type cells (Fig. 6C).

Cells overexpressing rho4+ or rho4G23V were more sensitive to Zymolyase 100T than were wild-type cells, an indication of cell wall integrity defects (Fig. 6D).

To corroborate the observations described above, we directly analyzed the incorporation of radioactive glucose into the walls of cells overexpressing rho4+ or rho4G23V in comparison to wild-type cells. As expected, there was a decrease in the amount of glucose incorporated into the walls of cells overexpressing rho4+ or rho4G23V, indicating that cell wall synthesis is diminished or that cell wall degradation is enhanced (data not shown). Measurement of the cell wall composition revealed that rho4+ overexpressing cells contained less cell wall material due to a 25% reduction of the β-glucan, the major polymer of the total cell wall (Fig. 6E). By contrast, rho4Δ cells grown at high temperature contained more cell wall (Fig. 6F).

In vitro measurement of the 1,3-β-d-glucan synthase activity of cells overexpressing rho4+ showed a reduction in 1,3-β-D-glucan synthase activity to 70% of that in wild-type cells (Fig. 6F). By contrast, 1,3-β-D-glucan synthase activity in rho4Δ cells grown at either 32 or 37°C was increased to approximately 130% compared to that in wild-type cells (Fig. 6F). Therefore,
FIG. 6. Cell wall-related defects in cells overexpressing or lacking rho4Δ. (A) Rho4+ overproduction cell lysis is rescued by sorbitol. Wild-type cells (PPG102) transformed with pREP3Xrho4+ (upper panels) and pREP3Xrho4G23V (lower panels) were grown for 16 h in EMM (left panels) or EMM plus 1 M sorbitol (right panels). Lysed cells appear dark due to staining with the vital dye methylene blue. (B) Cells overproducing Rho4 are sensitive to calcofluor. Wild-type cells (PPG102) transformed with plasmid pREP3X, pREP3Xrho4+, pREP3Xrho4G23V, or pREP3Xrho4T28N were grown at 32°C in EMM with 15 µM thiamine. The cells were washed, and fivefold serial dilutions starting with a cell suspension with an OD₆₀₀ of 20 were spotted in 4-µl drops onto EMM and EMM plus 0.5 mg of calcofluor per ml. The plates were incubated at 28°C for 3 days. (C) rho4Δ cells are resistant to calcofluor. Fourfold serial dilutions starting with log-phase wild-type cells (PPG371) and rho4Δ cells (PPG1541) at an OD₆₀₀ of 10 were spotted in 4-µl drops onto plates containing YES or YES plus 0.5 mg of Calcofluor per ml. The plates were incubated at 28°C for 3 days. (D) Cells overexpressing rho4Δ display Zymolyase 100T sensitivity. Sensitivity to Zymolyase 100T was measured in wild-type cells (PPG102) transformed with plasmids pREP3X, pREP3Xrho4+, pREP3Xrho4G23V, and pREP3Xrho4T28N grown for 12 h at 32°C in EMM without thiamine. Suspensions of cells at an OD₆₀₀ of 1.0 were incubated at 30°C in TE buffer containing 20 µg of Zymolyase 100T per ml, and the OD₆₀₀ was measured every 30 min. (E) The cell wall composition of wild-type cells (PPG102) transformed with plasmid pREP3X or pREP3Xrho4+, grown for 24 h in EMM without thiamine, and wild-type (PPG371) and rho4Δ (PPG1541) cells, grown at 37°C, is presented. [14C]glucose was added 4 h before harvesting. [14C]glucose radioactivity incorporated into each cell wall polysaccharide is presented. Values are the mean of three independent experiments. The standard deviation for total carbohydrate values is shown. (F) 1,3-β-D-Glucan synthase activity was measured in wild-type (PPG102) cells transformed with plasmid pREP3X or pREP3Xrho4+, and grown in EMM without thiamine for 16 h and wild-type (PPG371) and rho4Δ (PPG1541) cells grown at 32 or 37°C. Specific activity, as defined in Materials and Methods, is presented. wt, wild type.
there is a good correlation between in vitro and in vivo assays for cell wall integrity. All these results suggest that Rho4 plays a role in cell wall dynamics.

**rho4Δ cells accumulate secretory vesicles at high temperature.** The results presented in this paper show that rho4Δ cells are unable to separate at high temperature, rho4Δ overexpression causes lysis, and Rho4 localizes to the septum. Taken together, these results suggest that one possible function for Rho4 is the regulation of some cell wall-degradative activity required for cell separation. It has recently been proposed that the *S. pombe* exocyst, required for cell separation, might carry wall-degradative activities (52). It is possible that Rho4 participates in the polarized delivery of the secretory vesicles. To test this hypothesis, rho4Δ cells were examined by electron microscopy; the cells were fixed with permanganate, which permits the visualization of the cell wall structure and the secretory vesicles (rarely observed in wild-type cells).

rho4Δ cells grown at high temperature displayed a thicker septum with less well-defined layers than did wild-type cells (Fig. 7). This observation corroborates the cell wall defects found for rho4Δ cells by using other approaches. Interestingly, rho4Δ cells show a clear accumulation of vesicles compared to wild-type cells (Fig. 7). These vesicles are stained intensively after permanganate fixation and most probably represent post-Golgi secretory vesicles (16). The vesicles are more dispersed than those accumulated in the exocyst mutants, which appear clustered (52).

To test whether rho4 mutants are defective in secretion, we...
monitored the transport of a well-known secreted protein, acid phosphatase, through the *S. pombe* secretory pathway. The activity of secreted acid phosphatase was assayed in the culture supernatant. No differences between wild-type and rho4Δ cells were observed at either 28 or 36°C (data not shown), indicating that Rho4 is not required for general secretion.

**DISCUSSION**

It has been described that Rho1 and Rho2 are GTPases involved in cell wall biosynthesis (2). We have characterized a new *S. pombe* Rho GTPase, named Rho4, which is also implicated in cell wall dynamics but seems to play a completely different role from the one proposed for Rho1 or Rho2.

Our results indicate that rho4Δ cells display cytokinesis defects at high temperatures. Consistent with a possible role in cell separation, Rho4 specifically localizes to the septum. Interestingly, other Rho proteins, such as Rho1 and Rho2, localize to all sites of growth: cell poles and septum. Cdc42 localizes mainly to the septum, similarly to Rho4, but it is also targeted to the periphery of the cell and internal membranes (37). We did not detect localization of Rho4 at the poles or at any other point of the cell periphery. However, we cannot rule out the possibility that Rho4 is also present in other cellular regions, being too diffused or being present at concentrations too low to be detected by fluorescence microscopic methods.

We have shown that Rho4 protein levels do not vary significantly throughout the cell cycle. Interestingly, the Rho4 protein is clearly localized only during septum formation, suggesting a tight temporal and spatial regulation. One possibility is that Rho4 is retained in some internal compartment and that some modification of the protein drives its localization to the septum. Alternatively, it could be targeted to the division site after other proteins have been delivered there. Using mutants defective in septation, we have shown that localization of Rho4 could be SIN pathway dependent. Similarly, localization of Csp1, the putative glucan synthase responsible for septum formation, to the division site is dependent on the actomyosin ring and the SIN pathway (15, 33). Interestingly, the fact that the protein is present in the cell throughout the cell cycle suggests that Rho4 may perform additional functions to those at the division septum. Moreover, nonseptated rho4Δ cells do not polarize actin patches to the poles, suggesting that Rho4 participates in actin organization.

Septum formation needs cell wall synthesis, but cell separation needs cell wall degradation. It is well known that Rho1 and Rho2 are involved in cell wall synthesis (see Introduction). Curiously, Rho4 appears to play an opposite role to that of Rho1. In contrast to overexpression of the rho1Δ and rho2Δ genes, rho4Δ overexpression causes cell lysis. Moreover, the multiseptation phenotype generated by deletion of rho4Δ is also observed in some cells overexpressing rho1Δ (4) or lacking a Rho1-GAP (12, 40).

The lysis caused by Rho4 overproduction is due to cell wall defects because it is rescued by sorbitol and because cells are hypersensitive to Calcofluor and Zymolyase 100T, incorporate less glucose into the cell wall, and have a lower level of 1,3-β-D-glucan synthase activity. By contrast, rho4Δ cells are resistant to Calcofluor, display increased amounts of cell wall, and have higher levels of 1,3-β-D-glucan synthase activity. These cell wall defects could be due to an interference of the overproduced Rho4 with regulators required for other Rho proteins, such as Rho1. In addition, Rho4 could be acting as a negative regulator of the glucan synthase once the septum is formed.

It has been proposed that hyperactivation of Rho3 and Rho4 in *S. cerevisiae* by deletion of its GAP Rgd1 alters the secretory pathway and/or the actin cytoskeleton and diminishes the activation of the cell integrity pathway, causing cell lysis (18). Additionally, RHO5 deletion results in an increased activity of the Pkc1-dependent signal transduction pathway (46). A similar explanation could be possible for the lysis caused by rho4Δ overexpression in *S. pombe*. Unfortunately, there is not a well-defined integrity pathway in the fission yeast (41), but Pck1 or Pck2 overproduction does not rescue the lysis of cells overexpressing rho4Δ, suggesting that low levels of these kinases, involved in cell integrity, are not responsible for the lysis (our unpublished results).

We propose that, in contrast to Rho1, which regulates cell wall synthesis, Rho4 could be involved in the regulation of cell wall degradation. The primary septum is formed mainly by β-glucan, and so β-glucanases must be essential for septum degradation. Supporting this hypothesis, it has been described recently that an endo-β-1,3-glucanase, named Eng1, is required for the dissolution of the primary septum during cell separation in *S. pombe* (36). Thus, Rho4 might be required for the activity of β-glucanases such as Eng1. Another possibility is that Rho4 may be required for the proper localization of the glucanases or that Rho4 is involved in the machinery needed for secretion of these enzymes (see below). It has been shown that Rho proteins are related to the exocyst complex in *S. cerevisiae* (23); Rho3 physically interacts with Exo70, and Rho1 interacts with Sec3 (1, 23). Thus, Rho1 and Rho3 regulate cell polarity by simultaneously directing the rearrangement of the actin cytoskeleton and polarized delivery and fusion of secretory vesicles to specific sites on the cell surface (23). It has been recently shown that the exocyst complex in *S. pombe* is required mainly for cell separation (52). Similarly to Rho4, the exocyst proteins Sec6, Sec8, Sec10, and Exo70 localize to the division site, and this localization is dependent on the actomyosin ring. We have shown by electron microscopy that rho4Δ cells, like sec8-1 cells, accumulate vesicles at the restrictive temperature. sec8-1 mutants accumulate 60- to 100-nm vesicles, clustered in the vicinity of the septa, that most probably represent post-Golgi secretory vesicles unable to fuse with the plasma membrane (52). However, rho4Δ is not an essential gene, suggesting that it is not required for general secretion. In fact, we have shown that secretion of the acid phosphatase is not affected in rho4Δ cells. A possible explanation is that Rho4 could be required for delivery and/or fusion of a subset of secretory vesicles containing hydrolytic enzymes, such as glucanases, to the septum. In fact, preliminary results suggest that rho4Δ cells growing at high temperatures display less glucanase activity secreted to the medium (our unpublished results). It is well established that in *S. cerevisiae* there are subsets of secretory vesicles containing different cargos and that there are proteins regulating each subset; for example, Chs5 and Chs6 are involved in the polarized transport of the Chs3 chitin synthase, but they are not part of the general secretion machinery (45, 50).

It has also been demonstrated that actin cytoskeleton defects
in S. cerevisiae cause the accumulation of vesicles in the cell (28). Thus, an alternative explanation for the accumulation of vesicles in rho4Δ cells could be the defect in actin polarization observed in these cells. Further experiments are required to demonstrate the role of this GTPase.

ACKNOWLEDGMENTS

Carlos R. Vázquez de Aldana and Henar Valdivievo provided critical comments on the manuscript. We thank Cristina Durán and Manuel Arellano for technical help. We thank Paul Nurse and Sergio Moreno for antibodies and strains. Carlos Belinchón provided technical help with electron microscopy.

T.M.C. was supported by a fellowship from the Spanish Ministerio de Educación and 13P from the CSIC. This work was supported by grants IFD97-1507-C02-01 and BIO-2001-1531 from the Comisión Interministerial de Ciencia y Tecnología, Spain and SA026/02 from Junta de Castilla y León.

REFERENCES

1. Adamo, J. E., G. Rossi, and P. Brennewald. 1999. The Rho GTPase Rho3 has a direct role in actin that is distinct from its role in actin polarity. Mol. Biol. Cell 10:4121–4133.

2. Arellano, M., P. M. Coll, and P. Pérez. 1999. Rho GTPases in the control of cell morphology, cell polarity, and actin localization in fission yeast. Microsc. Res. Tech. 47:51–60.

3. Arellano, M., A. Durán, and P. Pérez. 1997. Localization of the Schizosaccharomyces pombe rho1 GTPase and its involvement in the organization of the actin cytoskeleton. J. Cell Sci. 110:2547–2555.

4. Arellano, M., A. Durán, and P. Pérez. 1996. Rho1 GTPase activates the (1-3)β-glucan synthase and is involved in Schizosaccharomyces pombe morphogenesis. EMBO J. 15:4584–4591.

5. Arellano, M., H. Valdivievo, T. Calonge, P. M. Coll, A. Durañón, and D. Millan. 1995. Schizosaccharomyces pombe protein kinase C homologues, pck1p and pck2p, are targets of rho1p and rho2p and differentially regulate cell morphology, cell polarity, and actin localization in Schizosaccharomyces pombe. Mol. Biol. Cell 6:163–177.

6. Auble, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Marians, R. E. Randall, I. M. Hagan, and K. S. Sheldrick. 1995. Transformation of yeast cells lacking the Rho3/Rho4-GAP Rgd1p in Schizosaccharomyces pombe. Yeast 11:149–159.

7. Humbel, B. M., M. Konomi, T. Tukagi, N. Kamasawa, S. A. Ishijima, and M. Osumi. 2001. In situ localization of β-glucans in the cell wall of Schizosaccharomyces pombe. Yeast 18:433–444.

8. Ishiguro, J., A. Saitou, A. Durán, and J. C. R. Ribas. 1997. csp1Δ, a Schizosaccharomyces pombe gene homolog of Saccharomyces cerevisiae FKS genes whose mutation confers sensitivity to cyclopentin A and papulacandin B. J. Bacteriol. 179:7653–7662.

9. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163–168.

10. Karpova, T. S., E. L. Elkin, N. S. Moesker, J. M. Schmitz, H. P. Schmitz, and J. J. Heinisch. 2001. The Cdc42p GTPase is targeted to the site of cell division in the yeast cell division cycle of Schizosaccharomyces pombe. Mol. Biol. Cell 12:4129–4131.

11. Krapp, A., S. Schmidt, E. Cano, and V. Simanis. 1999. Analysis of the cdc15+ gene provides evidence for a septation checkpoint in Schizosaccharomyces pombe. Mol. Gen. Genet. 262:163–172.

12. Lina, J., X. Tang, H. Wang, and S. B. Offenreiter, M. K. 2002. The localization of the integral membrane protein Cps1p to the cell division site is dependent on the actomyosin ring and the septation-inducing network in Schizosaccharomyces pombe. Mol. Biol. Cell 13:898–1000.

13. Mackay, D. J., and A. Hall. 1999. Rho GTPases. J. Biol. Chem. 273:20685–20697.

14. Marks, J., and J. S. Hyams. 1985. Localization of F-actin through the cell division cycle of Schizosaccharomyces pombe. Eur. J. Cell Biol. 39:235–242.

15. Martín-Cuadrado, A. B., E. Dueñas, M. Sipiczki, C. R. Vázquez de Aldana, and F. Del Redo. 2003. The endo-β-1,3-glucanase eng1p is required for disorganization of the primary septum during cell separation in Schizosaccharomyces pombe. J. Cell Sci. 116:1689–1698.

16. Merla, A., and D. I. Johnson. 2000. The Cdc42p GTPase is targeted to the site of cell division in the fission yeast Schizosaccharomyces pombe. Int. J. Cell Biol. 79:69–77.

17. Moreno, S. A., R. Norte, and M. Snyder. 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194:795–823.

18. Nakano, K., J. Imai, R. Araí, A. Toh-e, Y. Matsui, and I. Mabuchi. 2002. The small GTPase Rho3 and the diaphanous/formin For3 function in polarized cell growth in fission yeast. J. Cell Sci. 115:4629–4639.

19. Nakano, K., T. Mutoh, and I. Mabuchi. 2001. Characterization of GTPase-activating proteins for the Rho-family small GTPases in fission yeast Schizosaccharomyces pombe. Genetics 156:1031–1042.

20. Pérez, P. B., S. Santos, and P. M. Coll. 2002. Yeast; cell wall integrity pathway. In: L. Dekker, ed.), Protein kinase C. Landes Bioscience, Georgetown, Tex.

21. Puyne, D., and A. Bretscher. 2000. Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. J. Cell Sci. 113:365–375.

22. Riba, A., C. M. Díaz, A. Durán, and P. Pérez. 1999. Isolation and characterization of Schizosaccharomyces pombe mutants defective in cell wall (1-3)β-glucan synthase. J. Bacteriol. 173:4354–4362.

23. Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 4th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

24. Santos, B., and S. Macy. 1997. Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myp2p. J. Cell Biol. 136:95–110.

25. Schacher, H. P., S. Hupfer, and J. I. Hepler. 1998. Rho5p downregulates the yeast cell integrity pathway. J. Cell Biol. 115:319–318.

26. Shiozaki, K., and P. Russell. 1995. Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature 378:739–743.

27. Sohrmann, M., C. Fankhauser, C. Brudbeck, and V. Simanis. 1996. The
dmf1/mid1 gene is essential for correct positioning of the division septum in fission yeast. Genes Dev. 10:2707–2719.

49. Takai, Y., T. Sasaki, Tanaka, and T. Matozaki. 2001. Small GTP-binding proteins. Physiol Rev. 81:153–208.

50. Valdivia, R. H., D. Baggott, J. S. Chuang, and R. W. Schekman. 2002. The yeast clathrin adaptor protein complex 1 is required for the efficient retention of a subset of late Golgi membrane proteins. Dev. Cell. 3:283–294.

51. Van-Aelst, L., and C. D’Souza-Schorey. 1997. Rho GTPases and signalling networks. Genes Dev. 11:2295–2322.

52. Wang, H., X. Tang, J. Liu, S. Trautmann, D. Balasundaram, and D. B. McCollum, M. K. 2002. The multiprotein exocyst complex is essential for cell separation in Schizosaccharomyces pombe. Mol. Biol. Cell 13:515–529.