Drc1p/Cps1p, a 1,3-β-Glucan Synthase Subunit, Is Essential for Division Septum Assembly in *Schizosaccharomyces pombe*

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ABSTRACT

Schizosaccharomyces pombe divides by medial fission through the use of an actomyosin-based contractile ring. A division septum is formed centripetally, concomitant with ring constriction. Although several genes essential for cytokinesis have been described previously, enzymes that participate in the assembly of the division septum have not been identified. Here we describe a temperature-sensitive mutation, *drc1-191*, that prevents division septum assembly and causes mutant cells to arrest with a stable actomyosin ring. Unlike the previously characterized cytokinesis mutants, which undergo multiple mitotic cycles, *drc1-191* is the first cytokinesis mutant that arrests with two interphase nuclei. Interestingly, unlike *drc1-191*, *drc1*-null mutants proceed through multiple mitotic cycles, leading to the formation of large cells with many nuclei. *drc1* is allelic to *cps1*, which encodes a 1,3-β-glucan synthase subunit. We conclude that Drc1p/Cps1p is not required for cell elongation and cell growth, but plays an essential role in assembly of the division septum. Furthermore, it appears that constriction of the actomyosin ring might depend on assembly of the division septum. We discuss possible mechanisms that account for the differences in the phenotypes of the *drc1-191* and the *drc1*-null mutants and also reflect the potential links between Drc1p and other cytokinesis regulators.

THE physical division of *Schizosaccharomyces pombe* is achieved by medial fission through the use of an actomyosin-based contractile ring. Late in anaphase, the actomyosin ring constricts and the division septum is deposited centripetally in coordination with ring constriction. The process of cytokinesis in *S. pombe* can be separated into at least five distinct steps: choice of the plane of cell division, assembly of the actomyosin ring, accumulation of F-actin patches at the division site, actomyosin ring constriction, and synthesis of the medial division septum (Gould and Simanis 1997). Genetic studies have identified mutants defective in a number of steps of this process (Nurse *et al.* 1976; Chang *et al.* 1996; Bahler and Pringle 1998; Bahler *et al.* 1998; Balasubramanian *et al.* 1998).

The genes *mid1*, *plo1*, and *pom1* are involved in selection of the division site (Chang *et al.* 1996; Sohrmann *et al.* 1996; Bahler and Pringle 1998; Bahler *et al.* 1998; Balasubramanian *et al.* 1998). Mid1p is nuclear during interphase and is a component of the actomyosin ring during mitosis and cytokinesis. Plo1p, a protein kinase, is thought to phosphorylate Mid1p at mitosis and cause the nuclear export of Mid1p to the cell cortex, where it might play a role in stabilizing the position of the actomyosin ring (Bahler *et al.* 1998). After the selection of the division site, the actomyosin ring is assembled. The genes *cdc3*, *cdc4*, *cdc8*, *cdc12*, *rng2*, *rng3*, *rng4*, *myo2*/*rng5*, and *act1* (collectively referred to as *rng* genes) are required for the assembly of the actomyosin ring (Marks *et al.* 1986; Chang *et al.* 1996; Ishiguro and Kobayashi 1996; Balasubramanian *et al.* 1998). The identification of the products of the *rng* genes as F-actin cytoskeletal proteins and their intracellular localization patterns suggest that the products of the *rng* genes interact to promote actomyosin ring assembly (for a description of the *rng* genes refer to Balasubramanian *et al.* 1997; Chang *et al.* 1997; Gould and Simanis 1997; Kitayama *et al.* 1997; May *et al.* 1997; Eng *et al.* 1998; Naqvi *et al.* 1999). In addition, proteins such as Myp2p and Imp2p, which are not essential for cell viability but are components of the actomyosin ring, have also been identified (Bezanilla *et al.* 1997; Motegi *et al.* 1997; Demeter and Sazer 1998). Following assembly of the actomyosin ring, the function of the ring component Cdc15p, a protein with SH3 and coiled-coil domains (Fankhauser *et al.* 1995), is required for assembly of F-actin patches at the division site (Balasubramanian *et al.* 1998).

Whereas the products of the *rng* genes and Cdc15p are components of the F-actin cytoskeleton important for cell division, a second group of genes (collectively referred to as the *sid* genes) that regulates cytokinesis has also been identified (Nurse *et al.* 1976; Marks *et al.* 1986; Fankhauser *et al.* 1995; Schmidt *et al.* 1997;

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Balasubramanian *et al.* 1998). The *sid* genes include *cdc7*, *cdc11*, *cdc14*, *sid1*, *sid2*, *spg1*/*sid3*, and *sid4*. The *cdc7* (Fankhauser and Simanis 1994), *sid1* (D. McCollum, unpublished results), and *sid2* genes encode protein kinases (Balasubramanian *et al.* 1998) and the *spg1*/*sid3* gene encodes a GTPase (Schmidt *et al.* 1997; Balasubramanian *et al.* 1998). Analysis of the products of the *sid* genes suggests that they act in a signaling cascade that controls septum deposition in response to signals originating from the spindle pole body (Sohrmann *et al.* 1998).

Although advances have been made in identifying proteins important for actomyosin ring positioning, assembly, and the regulation of septum formation, several key aspects of cytokinesis remain poorly understood in *S. pombe*. For example, the links between the enzymes required for assembly of the division septum and the previously identified proteins that regulate cytokinesis are unknown. In this article, we present evidence that *drc1*/*cps1*, which encodes a 1,3-β-glucan synthase (Ishiguro *et al.* 1997), is essential for assembly of the division septum, but not for cell elongation and cell growth. Drc1p appears to function downstream of the septum initiation defective gene products in effecting the assembly of the division septum.

MATERIALS AND METHODS

Media, reagents, yeast techniques, and cytological methods: The *S. pombe* strains used in this study and their relevant genotypes are shown in Table 1. Yeast cells were grown on YES medium or Edinburgh minimal medium (EMM) with appropriate supplements as described (Moreno *et al.* 1991). Genetic crosses were performed by mixing appropriate strains of opposite mating type on YPD medium, and recombinant strains were obtained by tetrad dissection. In general, yeast transformations with plasmid DNA were carried out by electroporation (Prentice 1992). In cases where cosmids were introduced into yeast cells, a spheroplast transformation method using lipofectin was used (Allshire 1990). FACS analysis was performed as described in the world wide web site of the laboratory of Dr. Susan Forsburg (http://pingu.salk.edu/users/forsburg/lab.html). Lipofectin was obtained from GIBCO (Bethesda, MD). Fluorescence microscopy methods used were essentially as described previously (Balasubramanian *et al.* 1997). Formaldehyde fixation was used for visualization of F-actin using rhodamine-conjugated phalloidin, and methanol fixation was used for the detection of microtubules, Myo2p (Naqvi *et al.* 1999), Cdc4p (McCollum *et al.* 1995), and stainings involving antibodies against the hemagglutinin (HA) epitope.

Isolation of the *drc1-191* mutant, molecular cloning of *drc1*$^+$, and mapping the *drc1-191* mutation: The *drc1-191* mutant was identified in a screen for mutants capable of diploidization following a short heat pulse as described elsewhere (Balasubramanian *et al.* 1998). *drc1-191*, a previously undescribed mutant defective in cytokinesis obtained from this screen, was capable of growth and colony formation at 24°, but died as a result of failed cytokinesis at 36°. A positional cloning strategy was employed to clone *drc1*$^+$. In short, *drc1-191* was mapped to chromosome 2 and was found to be located 1.1 cM from *cdc14*$^+$, to contig 14 based on the map of Garkavtsev and Mizukami (1997). Cosmids spanning contig 14 were donated by Drs. M. Yanagida (Kyoto University, Japan) and R. Gwilliam (Sanger Center, Cambridge, U.K.). The bacterial transposon Tn1000, contained as Tn1000 *his7*$^+$ *ars1* (Morgan *et al.* 1996), was a gift of Dr. P. Nurse, ICRF, London. Tn1000 was allowed to insert into these cosmids using standard techniques (Sambrook *et al.* 1989). Cosmids marked with the *his7*$^+$ gene were introduced into a *drc1-191 his7-366* strain and selected for histidine prototrophy. Transformants were subsequently tested for their ability to form colonies at 36°. One cosmid, c145, allowed the *drc1-191 his7-366* to form colonies at 36°. To identify the gene responsible for the rescue of *drc1-191*, transposon mutagenesis was employed and a bank of c145 with transposon insertions was generated and individual cosmids were introduced into *drc1-191 his7-366*. A cosmid containing an insertion at the presumed promoter region of the *cps1* gene, which encodes a 1,3-β-glucan synthase, was found to be incapable of rescuing *drc1-191*. Upon subcloning, the *cps1*$^+$ gene carried on the plasmid vector pUR19 (Barbet *et al.* 1992) was found to rescue *drc1-191*. Thus, *drc1* is allelic with *cps1*. The mutation in the *drc1-191* allele was mapped as follows: polymerase chain reaction was used to generate four PCR fragments from wild-type DNA that spanned the entire *drc1*$^+$ gene (Figure 5A). The primers used in the PCR reactions were as follows: (1) 3h-A655, 5′ ATTCATCATGGATCAGTAT TGGCGTGAAC 3′ and 3h-A2272c, 5′ AGCACCCAAAAATCT GATAGAATCTGCC 3′; (2) 3h-B2139, 5′ ATTTCACTCCGAC CTCAAAAACAGGTGC 3′ and 3h-B3612c, 5′ GCATCGAT CATTTGTACATATTCACCTC 3′; (3) 3h-C3388, 5′ GCAAAT TGCATATATGGATGAAGATCCTC 3′ and 3h-C4750c, 5′ AC TAATGCAAAGAGCAGTCAGCGTAATCC 3′; and (4) 3h-D4611, 5′ CACTATTGTATTCTCGATTCTCTGGACC 3′ and 3h-D5900c, 5′ GAAATAAGCATCCACATCACACAAATCC 3′. These PCR fragments were introduced individually into *drc1-191* and assessed for their ability to rescue the temperature-sensitive (ts) mutation by gene-conversion-mediated repair of the *drc1-191* mutation. The 5′ fragment (codons 1–450) rescued the *drc1-191* mutation. Five subfragments spanning this region found capable of rescuing *drc1-191* were introduced into *drc1-191* and the mutation was further mapped to lie between codons 150 and 300. DNA sequence determination of the corresponding region from the *drc1-191* mutant established that codon 277 was changed from GAT to AAT, resulting in the replacement of the aspartic acid residue at this position with an asparagine.

Construction and analysis of a *drc1*-null mutant: To analyze the phenotype of the *drc1*-null mutant, a DNA molecule was created in which over 80% of the *drc1*/*cps1* coding region was replaced with the *ura4*$^+$ gene (Figure 6A). A plasmid carrying this construction was linearized and introduced into the uracil auxotrophic diploid MBY494, of the genotype *ade6-M210*/*ade6-M216 leu1-32*/*leu1-32 his3-d1*/*his3-d1 ura4-D18*/*ura4-D18* h^+/h^-. Fifty uracil prototrophic colonies were screened by PCR and three were found to have undergone the expected gene replacement event. One of these, MBY507, of the genotype *drc1*$^+$/*drc1::ura4*$^+$ *ade6-M210*/*ade6-M216 leu1-32*/*leu1-32 his3-d1*/*his3-d1 ura4-D18*/*ura4-D18* h^+/h^- was used for further characterization of the *drc1*-null phenotype. MBY507 was plated on EMM plates lacking adenine and uracil until the diploid cells had sporulated, and spores were prepared from the mixture of asci and cells by treatment with glusulase. Spore germination experiments were carried out either in liquid EMM medium lacking uracil, to allow only spores bearing the *drc1::ura4*$^+$ allele to germinate, or in YES liquid medium as described in the legend to Figure 6C.

RESULTS

***drc1-191* blocks division septum formation:** In a screen for mutants defective in cytokinesis, we isolated

TABLE 1

List of *S. pombe* strains used in this study

Strain	Genotype	Reference
MBY17	*cdc3-124 leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY23	*cdc4-8 ura4-D18 h*$^+$	Our lab stock
MBY94	*cdc14-118 ade6-210 ura4-D18 h*$^+$	Our lab stock
MBY105	*cdc7-24 ade6-210 h*$^+$	Our lab stock
MBY106	*cdc11-123 ade6-210 h*$^+$	Our lab stock
MBY110	*cdc8-110 ade6-210 leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY113	*rng3-65 ade6-210 leu1-32 h*$^+$	Our lab stock
MBY152	*sid1-239 ade6-21x leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY159	*drc1-191 ade6-210 ura4-D18 lys1-131 h*$^-$	This study
MBY297	*mid1-18 ade6-21x leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY322	*sid4-A1 ade6-21x leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY338	*spg1-106 ade6-210 leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY358	*rng2-D5 ade6-21x leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY402	*cdc3-124 drc1-191 (MBY17 × MBY159)*	This study
MBY406	*spg1-106 drc1-191* (MBY338 × MBY159)	This study
MBY443	*myo2-E1 ade6-21x leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY451	*mid1-18 drc1-191* (MBY297 × MBY159)	This study
MBY455	*cdc11-123 drc1-191* (MBY106 × MBY159)	This study
MBY459	*sid1-239 drc1-191* (MBY152 × MBY159)	This study
MBY461	*sid2-250 drc1-191* (MBY250 × MBY159)	This study
MBY463	*sid4-A1 drc1-191* (MBY322 × MBY159)	This study
MBY469	*cdc8-110 drc1-191* (MBY110 × MBY159)	This study
MBY471	*cdc7-124 drc1-191* (MBY105 × MBY159)	This study
MBY483	*rng2-D5 drc1-191* (MBY358 × MBY159)	This study
MBY485	*rng3-65 drc1-191* (MBY113 × MBY159)	This study
MBY487	*cdc4-8 drc1-191* (MBY23 × MBY159)	This study
MBY493	*cdc14-118 drc1-191* (MBY94 × MBY159)	This study
MBY494	*ade6-M210/ade6-M216 leu1-32/leu1-32 his3-D1/his3-D1 ura4-D18/ura4-D18 h*$^+$*/h*$^-$	Our lab stock
MBY503	*sid2-250 ade6-216 leu1-32 ura4-D18 h*$^+$	Our lab stock
MBY506	*drc1-191 ade6-21x leu1-32 ura4-D18 his7-366 h*$^-$	This study
MBY507	*drc1::ura4*$^+$*/drc1*$^+$ *ade6/ade6 leu1/leu1 ura4/ura4 his3/his3 h*$^+$*/h*$^-$	This study
MSI5	*drc1-191 h*^{90}	Our lab stock

a temperature-sensitive mutant, *drc1-191*, that is defective in cell division. The *drc1-191* mutation was found to be recessive, since cells of the genotype *drc1*$^+$*/drc1-191* resembled wild-type cells and were capable of colony formation under conditions in which the *drc1-191* mutant was unable to form colonies (data not shown). To establish the stage of cytokinesis at which *drc1-191* cells are defective, we first stained cells incubated at permissive and restrictive conditions with calcofluor. Under permissive conditions (24°) *drc1-191* cells are capable of division and colony formation (Figure 1, 0 hr). Upon shift to the restrictive condition (36°) for 4 hr, however, cell proliferation was affected and cells arrested the cycle unable to form a division septum (Figure 1, 4 hr). In many cases, what appeared as an excessive deposition of cell wall material was detected at one or both ends of the cell. Upon prolonged incubation at 36°, cell morphology was affected severely and cells with cylindrical, spherical, and other abnormal morphologies accumulated (Figure 1, 8 hr). Although *drc1-191* cells were capable of proliferation at 24°, morphological abnormalities were associated with the *drc1-191* mutation even at the permissive temperature. We found that *drc1-191* cells were unable to maintain constant cell diameter and a cylindrical morphology. *drc1-191* cells were, however, not defective in initiating polarized growth (Figure 1, 0 hr, marked with arrows), since cell tips were approximately of the same diameter as wild-type cells. These observations established that *drc1-191* is defective in division septum deposition and is unable to maintain wild-type cell morphology.

Actomyosin rings are unusually stable in *drc1-191* mutant cells: Numerous studies have shown that the assembly of the division septum requires a functional F-actin cytoskeleton (Gould and Simanis 1997). We therefore stained heat-arrested *drc1-191* cells with rhodamine-conjugated phalloidin and 4′,6-diamidino-2-phenylindole (DAPI) to visualize the F-actin cytoskeleton and chromosomes, respectively. To clearly assess whether cells were in interphase or in mitosis, heat-arrested *drc1-191* cells were stained with TAT-1 antibodies to visualize the microtubule cytoskeleton. Under permissive conditions *drc1-191* cells resembled wild-type cells in that ~15–20% of the cells were binucleate and the rest were uninucleate (Table 2). F-actin was visualized in patches in interphase cells and in rings in cells undergoing mitosis

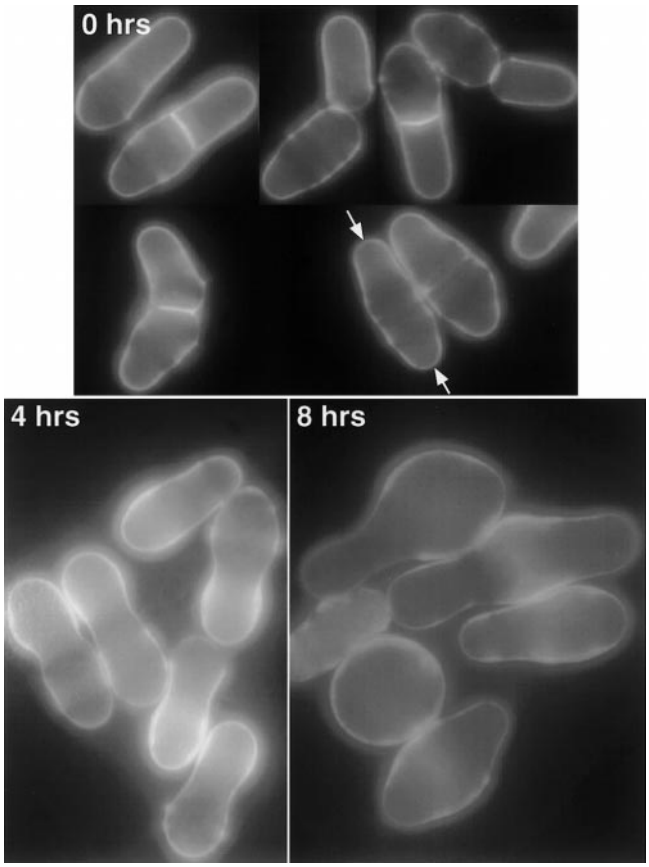


Figure 1.—*drc1-191* mutants are defective in division septum deposition and in maintenance of cell polarity. Samples were taken prior to shift to 36° (0 hr), as well as 4 and 8 hr after shift to 36°, fixed, and stained with calcofluor to visualize septa. Arrows point to the cell tips, whose diameter is comparable to that of wild-type cells.

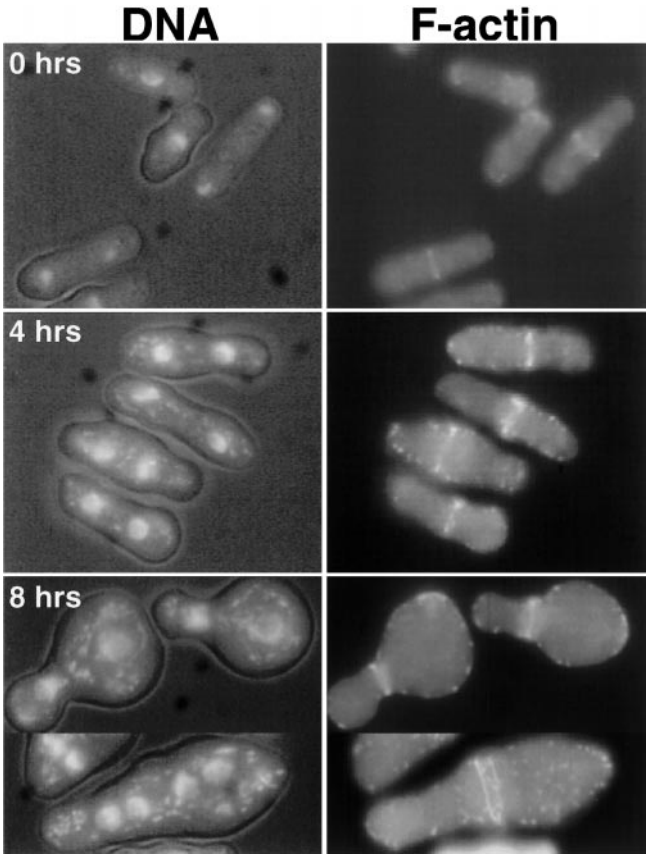


Figure 2.—*drc1-191* mutants arrest with stable actomyosin rings. *drc1-191* cells were grown at the permissive temperature (24°) to exponential phase and shifted to the restrictive temperature (36°). Samples were taken prior to shift to 36° (0 hr), as well as 4 and 8 hr after shift to 36°, fixed, and stained with rhodamine-conjugated phalloidin and DAPI to visualize F-actin and nuclei (labeled DNA), respectively.

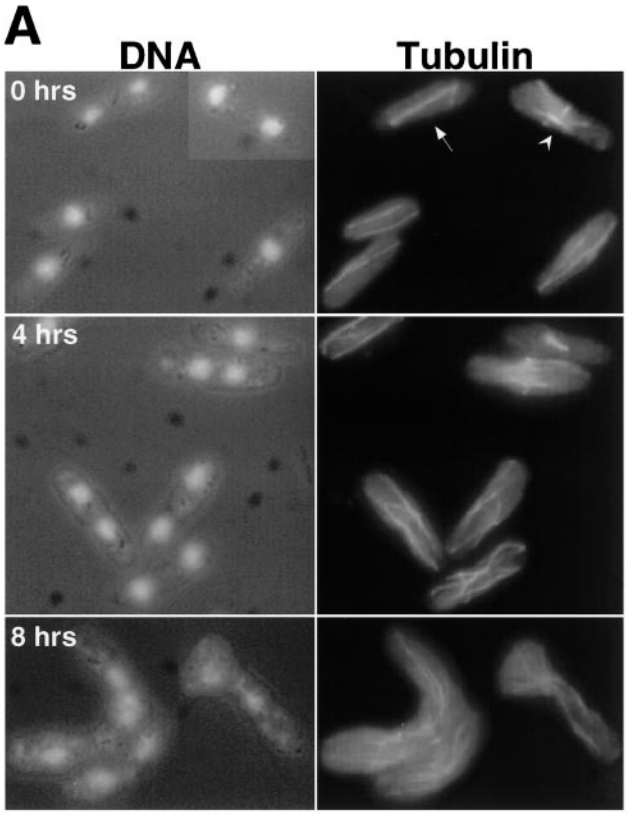
and cytokinesis (Figure 2, 0 hr). Under these conditions, as expected, most binucleate cells displayed a mitotic spindle (shown with an arrow in Figure 3A, 0 hr) and some binucleate cells displayed a postanaphase array of microtubules (shown with an arrowhead in Figure 3A, 0 hr). An interphase array of microtubules was visualized in all uninucleate cells that did not show detectable chromosome condensation.

TABLE 2

The percentage of uninucleate and binucleate cells of *drc1-191* and wild-type cells at 24° and after a 4-hr shift to 36°

	Uninucleate	Binucleate
24°		
wt	83.3	16.7
drc1-191	80.4	19.6
36°		
wt	75.3	24.7
drc1-191	18.3	81.7

Upon shift to the restrictive condition for 4 hr, 70–80% of *drc1-191* cells blocked with two interphase nuclei and F-actin was detected in a ring structure (Table 2 and Figure 2, 4 hr). The rest of the cells were found to be uninucleate. The unusually stable nature of the actomyosin ring in this mutant led to the gene name *drc1-191* (*d*efective in *r*ing *c*onstriction). Microtubule staining confirmed that the *drc1-191* cells were arrested in interphase since cells blocked predominantly either with interphase arrays of microtubules or with a postanaphase array of microtubules (Figure 3A, 4 hr). The medial ring in heat-arrested *drc1-191* cells also contained other components associated with the actomyosin ring such as Cdc4p and Myo2p (data not shown). This percentage of cells blocked with actomyosin rings is abnormally high, given that actomyosin rings are detected only in ~15% of asynchronously growing cells. Upon prolonged incubation at the restrictive temperature (Figure 2, 8 hr), cells assumed a variety of shapes and ~20% of cells were found to contain four nuclei with actomyosin rings, whereas the rest of the cells (80%) still contained only two interphase nuclei and detectable



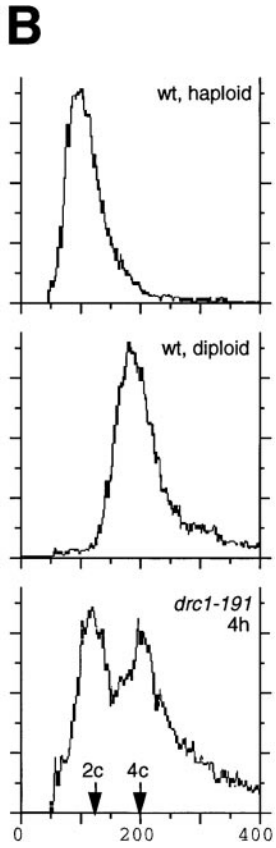


Figure 3.—(A) *drc1-191* mutants arrest with interphase or postanaphase microtubule configuration. (B) *drc1-191* mutants arrest predominantly with 4C DNA content. *drc1-191* cells were grown at the permissive temperature (24°) to exponential phase and shifted to the restrictive temperature (36°). Samples were taken prior to shift to 36° (0 hr), as well as 4 and 8 hr after shift to 36°, fixed, and stained either with TAT1 antibodies and DAPI to visualize microtubules and chromosomal DNA or with propidium iodide, and processed for FACS analysis. In the FACS analysis, a wild-type haploid and a wild-type diploid strain were used as controls for the 2C and 4C DNA peaks, respectively.

actomyosin rings. Again, microtubule staining confirmed the interphase status of the majority of cells (Figure 3A, 8 hr). These observations suggested that the execution of Drc1p function was important for actomyosin ring constriction and/or disassembly and septation.

Heat-arrested *drc1-191* cells also appeared to be incapable of substantial cell elongation when compared with other cytokinesis mutants. To rigorously test the effect of the *drc1-191* mutation on cell elongation, wild-type, *drc1-191*, and the cytokinesis-defective *cdc7-24* cells (Fankhauser and Simanis 1994) were grown at 24° and shifted to 36° for 6 hr and the length of 100 cells was measured (Table 3). Whereas 100% wild-type cells distributed in the 6–15 μm range, 44% of *drc1-191* cells were found to be distributed between 6 and 15 μm and the rest were distributed between 16 and 25 μm. By contrast, *cdc7-24* cells were distributed between 26–35 μm (10%), 36–45 μm (42%), and 46–55 μm (48%). This analysis clearly established that *drc1-191* was defective in substantial cell elongation.

TABLE 3

The length distribution of wild-type, *drc1-191*, and *cdc7-24* cells after a 6-hr shift to 36° from 24°

	Length (μm)				
	6–15	16–25	26–35	36–45	46–55
wt	100	0	0	0	0
drc1-191	44	56	0	0	0
cdc7-24	0	0	10	42	48

One hundred cells were scored in each case.

Given that *drc1-191* mutants blocked with two interphase nuclei and an actomyosin ring, we asked if the interphase nuclei in arrested *drc1-191* mutants had undergone DNA replication. To address this issue, the amount of DNA in *drc1-191* cells arrested at 36° for 4 hr was quantitated by FACS analysis. As controls, a wild-type haploid strain and a wild-type diploid strain were also quantitated in a similar manner. As shown in Figure 3B, peaks characteristic of 2C and 4C DNA were seen in asynchronously growing wild-type haploid and wild-type diploid strains, respectively, consistent with the fact that the G_1 and S phases of *S. pombe* are completed prior to cell division. Interestingly, consistent with the presence of 70% binucleate cells, ~70% of *drc1-191* cells accumulated a 4C DNA peak (based on integration of the 2C and 4C peaks), suggesting that under the arrest conditions they were not impaired for DNA replication but were incapable of entry into mitosis.

Cdc7p is localized asymmetrically at the *drc1-191* arrest point: To further characterize the phenotype of the *drc1-191* mutant, we investigated the localization of the septum-inducing Cdc7p-kinase (Fankhauser and Simanis 1994). Cdc7p localizes to the spindle pole body (SPB) in early mitosis and is then found only on one

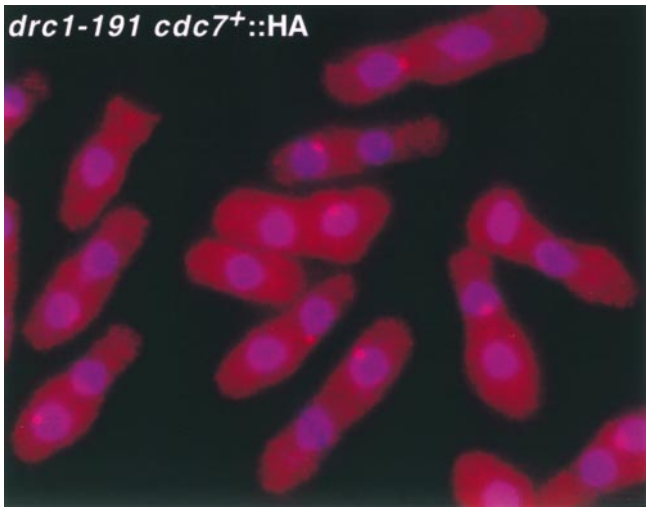


Figure 4.—Cdc7p is located at one SPB in *drc1-191* cells at the restrictive temperature. A *drc1-191 cdc7*-HA_3 strain grown at 24° was shifted to 36° for 4 hr, fixed, and stained with antibodies against the HA epitope and DAPI. Merged images with DNA in blue and Cdc7p in red are shown.

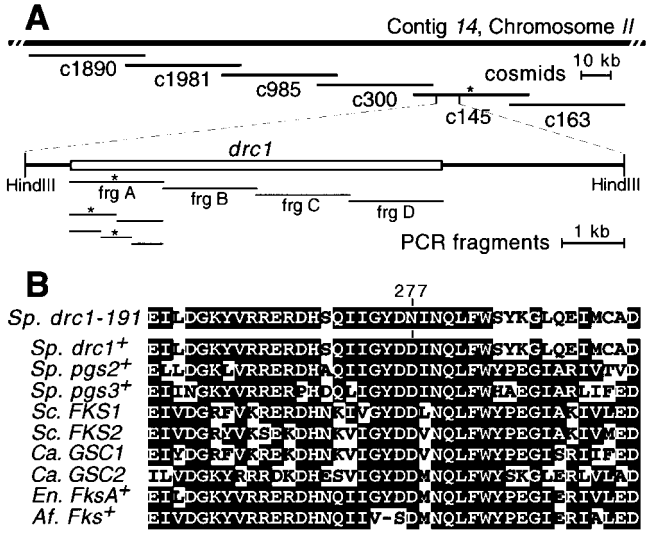


Figure 5.—Molecular cloning and analysis of *drc1*$^+$. (A) Positional cloning of *drc1*$^+$, identification of the *drc1* coding region, and mapping of the *drc1-191* mutation. *drc1-191* was found to be tightly linked to *cdc14-118*. Cosmids spanning this region were tagged with *his7*$^+$ and introduced into a *drc1-191 his7-366* strain. One cosmid, c145, was capable of rescuing *drc1-191*. An 8-kb *Hin*dIII-*Hin*dIII fragment from this cosmid was sufficient for the rescue of *drc1-191*. A previously described gene, *cps1*, which encodes a 1,3-β-glucan synthase, was found to reside in this rescuing fragment. The mutation in *drc1-191* was mapped as described in materials and methods. The overlapping DNA fragments that contain the mutation are marked with an asterisk. (B) Alignment of predicted amino-acid sequence of *drc1-191* with the corresponding region of other 1,3-β-glucan synthases. Sp., *S. pombe*; Sc., *Saccharomyces cerevisiae*; Ca., *Candida albicans*; En., *Emericilla nidulans*; and Af., *Aspergillus fumigatus*. The site of mutation is marked with "277" over the predicted amino-acid sequence of Drc1-191p.

SPB between anaphase B and completion of septum deposition. No distinct Cdc7p staining is visualized in uninucleate interphase cells and in cells that have completed septum formation (Sohrmann *et al.* 1998). To assess the localization of Cdc7p at the *drc1-191* blockpoint, we constructed a *drc1-191* strain whose chromosomal copy of the *cdc7* gene was tagged with three copies of the HA epitope. Cdc7-HA_3-tagged *drc1-191* cells were arrested at the restrictive temperature for 4 hr, fixed, and stained with antibodies against the HA epitope. At the *drc1-191* arrest point all binucleate cells were found to have Cdc7p staining localized at one SPB. Merged images of chromosomal staining with DAPI and Cdc7p staining with HA antibodies is shown in Figure 4. The *drc1-191* mutant, therefore, arrests at a point in the cell cycle where the septum-promoting Cdc7p is located on one SPB. Thus, the function of the actomyosin ring assembly proteins (products of the *rng* genes; required for actomyosin ring assembly) and the septum initiation proteins (products of the *sid* genes; required for onset of actomyosin ring constriction and septum deposition) appear to be executed normally in the *drc1-191* mutant.

***drc1* is allelic with *cps1* and encodes a 1,3-β-glucan synthase subunit:** To understand the molecular nature of Drc1p, we attempted to clone *drc1*$^+$ by complementation of the heat-sensitive colony formation defect of *drc1-191*. We failed to isolate *drc1*$^+$ from a number of plasmid-borne *S. pombe* genomic libraries. As an alternative, therefore, we utilized a positional cloning approach to isolate *drc1*$^+$. During the course of backcrosses performed following the mutagenesis we noticed that *drc1-191* was linked to the *leu1* and the *mat* loci, which are located near the centromere of chromosome II. In a cross between *leu1-32* and *drc1-191*, 36 parental ditypes (PD), 19 tetratypes (TT), and 1 nonparental ditype (NPD) were obtained, placing the *drc1* locus at a distance of 22.3 cM from *leu1*. From the same cross, we established that the *drc1* locus was 12.5 cM from the *mat* locus (24 PD: 8 TT: 0 NPD). Finally, *drc1* was found to be 1.1 cM from the *cdc14* locus (45 PD: 1 TT: 0 NPD). To isolate the *drc1*$^+$ gene, we then asked if cosmids spanning this region of the genome were capable of rescuing *drc1-191* for colony formation at 36°. Of the six cosmids tested, one cosmid, c145, allowed *drc1-191* to form colonies at 36° (Figure 5A). The reading frame conferring the Drc1$^+$ phenotype was identified by the isolation of a derivative of c145 following transposon mutagenesis that failed to rescue *drc1-191*. Analysis of DNA sequences flanking the transposon showed that the transposon had inserted upstream of a gene encoding a 1,3-β-glucan synthase subunit, previously identified as the product of the *cps1*$^+$ gene (Ishiguro *et al.* 1997). Proof that *drc1* was allelic with *cps1* was obtained in two ways. First, a plasmid carrying *cps1*$^+$ alone allowed *drc1-191* cells to form colonies at 36°. Second, a single base change (G to A transition) was detected in the *cps1* gene, when DNA sequence from *drc1-191* was analyzed.

This mutation caused the replacement of an aspartic acid residue at position 277, conserved in all 1,3-β-glucan synthases, by an asparagine residue (Figure 5B). Thus, we conclude that *drc1* is allelic with *cps1* and encodes a 1,3-β-glucan synthase subunit. Garnier analysis of the predicted amino acid sequence of Drc1p resulted in the identification of 14 potential transmembrane domains in this protein (data not shown). Sequence comparisons also identified two more genes in *S. pombe* that are predicted to encode 1,3-β-glucan synthase subunits, which we refer to as *pgs2* and *pgs3* (for *p*ombe *g*lucan *s*ynthase). Pgs2p (ORF SPAC24C9.07) and Pgs3p (ORF SPCC1840.02c) are ~55% identical to Drc1p/Cps1p.

***drc1/cps1* null mutants perform multiple nuclear cycles despite failed division septum deposition:** To test the phenotype resulting from the complete deletion of the *drc1* gene, we constructed a strain of the genotype *drc1::ura4/drc1*$^+$ (described in materials and methods and Figure 6A). Upon meiosis and sporulation, spores bearing the *drc1*-null allele were found to be capable of germination and establishing polarized growth, but were incapable of performing cytokinesis and did not maintain polarity (Figure 6B). Similar results were obtained when spores bearing the *drc1::ura4* allele were allowed to germinate on medium containing 1.2 m sorbitol, establishing that *drc1::ura4* spores were not defective in general cell wall assembly (Figure 6B). We conclude that the *drc1* gene product is not required for spore germination, polarity establishment, and cell elongation, but is required for division septum deposition and for maintenance of cell polarity.

To further characterize the terminal phenotype of the *drc1*-null mutants, *drc1::ura4* spores were germinated, fixed, and stained to visualize F-actin and nuclei. *drc1::ura4* mutants failed to form septa, although occasionally faint cell wall-like structures that did not stain with calcofluor were detected (shown with arrowhead in Figure 6C). Germinated *drc1::ura4* spores were capable of polarity establishment (shown with arrows in Figure 6C), but appeared to be incapable of polarity maintenance, causing them to become spherical and highly enlarged (Figure 6C). Interestingly, unlike the *drc1-191* mutant, *drc1::ura4* underwent multiple nuclear division cycles causing arrested cells to accumulate up to 32 nuclei. Note that cell 1 in Figure 6C has two interphase nuclei following failed cytokinesis and has actin patches at the cell ends. However, cell 2 in Figure 6C is in mitosis (as seen by the presence of condensed chromosomes) and contains an actomyosin ring. Thus, actomyosin rings assemble in the *drc1::ura4* mutants, but unlike the *drc1-191* mutant, they disassemble following mitosis.

It remained possible that the phenotype associated with the *drc1*-null mutant was a peculiarity associated with spore germination. To determine whether this was the case, spores arising from mating and meiosis of a *drc1-191* homothallic strain (spores of the genotype *drc1-191*) were germinated at 36°. After 24 hr of growth,

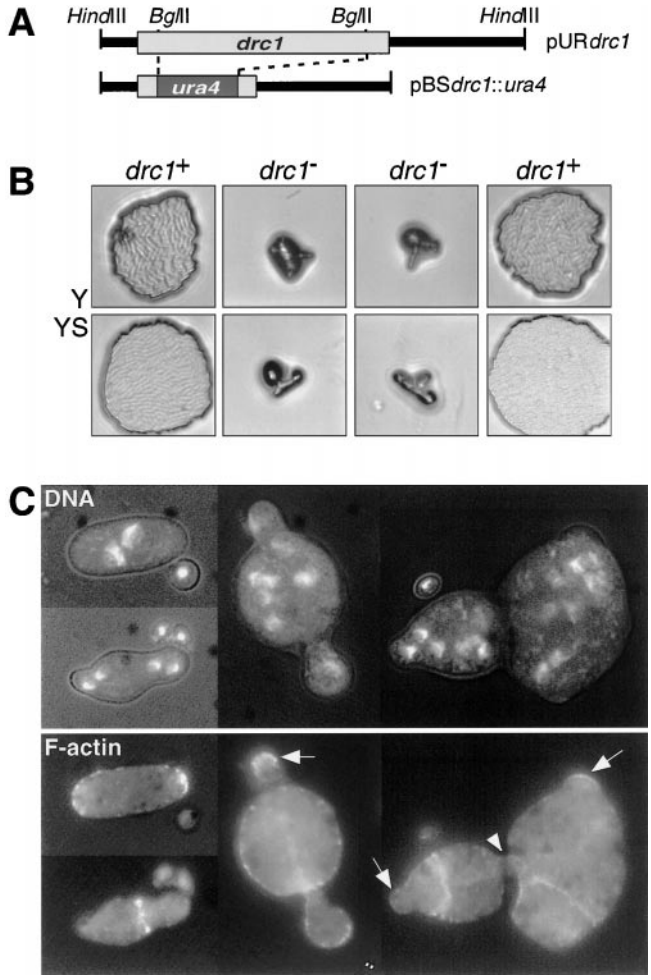


Figure 6.—(A) Restriction map of the *drc1::ura4* construction. Approximately 80% of the Drc1p coding region, contained between the two *Bgl*II sites, was deleted and replaced with a 1.8-kb *Bam*HI fragment carrying the *ura4*$^+$ gene. (B) Drc1p is essential for cell viability. Tetrads from a *drc1::ura4*$^+$/*drc1*$^+$ strain were dissected either on YES medium (Y) or YES medium containing 1.2 m sorbitol (YS) and incubated at 32°. (C) Terminal phenotype of *drc1*-null mutants. Spores prepared from the *drc1::ura4*$^+$/*drc1*$^+$ strain were inoculated in EMM lacking uracil (cells 1 and 2) or YES (the multinucleate cells), germinated for 18 hr (EMM) or 22 hr (YES), fixed, and stained with rhodamine-conjugated phalloidin and DAPI to visualize F-actin and nuclei, respectively.

similar to that observed with the vegetative *drc1-191* cells, germinated *drc1-191* spores were arrested predominantly with two nuclei (70%) and the rest had four nuclei (data not shown). We therefore conclude that in a strain depleted of Drc1p, septum assembly and cell polarity are affected, but nuclear cycles, assembly of actomyosin rings at mitosis, and disassembly of actomyosin rings at the end of mitosis happen normally.

Genetic interactions between *drc1-191* and mutations affecting actomyosin ring assembly and placement: To assess potential interactions between *drc1-191* and mutations causing defective actomyosin ring assembly, placement, and function, we crossed *drc1-191* to *mid1-18*, *cdc3-*

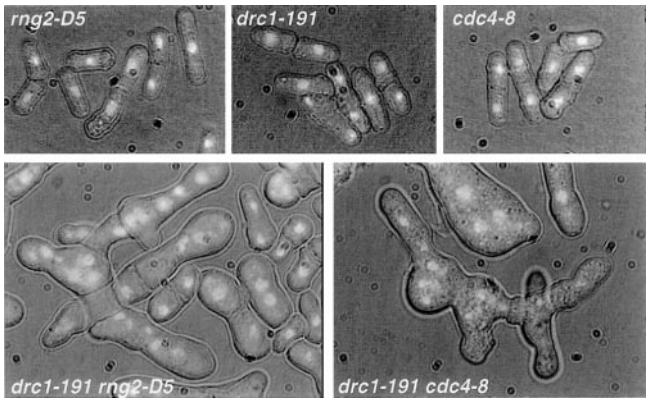


Figure 7.—*drc1-191* shows strong negative interactions with *cdc4-8* and *rng2-D5*. Cells of the genotypes *drc1-191*, *cdc4-8*, *rng2-D5*, *drc1-191 cdc4-8*, and *drc1-191 rng2-D5* were grown at 24° (permissive for *drc1-191*, *cdc4-8*, and *rng2-D5*), fixed, stained with DAPI to visualize the DNA, and photographed under Nomarski optic settings.

124, *cdc4-8*, *cdc8-110*, *cdc15-140*, *rng2-D5*, *rng3-65*, and *myo2-E1*. The *drc1-191* mutant showed strong negative interactions with *cdc4-8*, *myo2-E1*, and the *rng2-D5* mutants. The *drc1-191 myo2-E1* double mutant was unable to form colonies at 24°, a temperature at which both parental strains were capable of colony formation (data not shown). The *drc1-191 rng2-D5* and *drc1-191 cdc4-8* double mutants grew extremely poorly and showed cytokinesis defects at 24°, a temperature at which *rng2-D5* and *cdc4-8* single mutants grew healthily and resembled wild-type cells in morphology (Figure 7). In both double mutant combinations (*drc1-191 cdc4-8* and *drc1-191 rng2-D5*) highly elongated cells with multiple nuclei were seen frequently. Significant genetic interactions were not detected in other combinations. Based on the interactions with actomyosin ring mutants, we conclude that Drc1p might interact with other actomyosin ring components to effect septum assembly.

The *sid* group of mutations are epistatic to *drc1-191*: Previous studies have identified a large collection of mutants, referred to as the *sid* group of mutants, that are defective in septum deposition, even though F-actin rearrangements and nuclear cycles are not affected (Marks *et al.* 1986; Fankhauser *et al.* 1995; Balasubramanian *et al.* 1998). Thus, *sid* mutants accumulate multiple nuclei and assemble actomyosin rings during mitosis, which disassemble following mitosis. Mutations in seven known genes lead to a Sid phenotype (Marks *et al.* 1986; Schmidt *et al.* 1997; Balasubramanian *et al.* 1998). The major difference in the phenotype of *drc1-191* and the *sid* mutations is that the *drc1-191* mutant fails to elongate substantially (Table 3) and arrests with only two nuclei, whereas the *sid* mutants accumulate multiple nuclei and become highly elongated. To check for epistasis relationships, we combined *drc1-191* with ts *sid* mutations. Double mutants of the genotypes *cdc7-24 drc1-191*, *cdc11-119 drc1-191*, *cdc14-118 drc1-191*, *sid1-239 drc1-191*, *sid2-250 drc1-191*, *spg1-106 drc1-191*, and *sid4-A1 drc1-191* were constructed. To analyze the phenotypes of the double mutants, cells were grown to exponential phase, shifted to the restrictive temperature for 4 hr, fixed, and stained to visualize F-actin and DNA. The results obtained from characterizing one such double mutant, *cdc7-24 drc1-191*, are shown in Figure 8A. Interestingly, even though *drc1-191* arrested with two nuclei and stable actomyosin rings, the *cdc7-24 drc1-191* double mutants were indistinguishable from the *cdc7-24* single mutant (Figure 8A). Nuclear cycles continued in the double mutants leading to the formation of elongated cells with up to four nuclei. Furthermore, actomyosin rings were detected only in mitotic cells and F-actin patches at the cell ends were seen in interphase cells. All *sid drc1-191* double mutant combinations listed above resulted in similar phenotypes upon temperature shift (data not shown). We conclude that the *sid* mutant phenotype of elongated cells with multiple nuclei is epistatic to that of *drc1-191*. The double mutant analysis also suggested that the *drc1-191* mutant is not defective in cell elongation, but that the blockpoint of *drc1-191* prevents substantial cell elongation.

In the course of analysis of the *sid drc1-191* double mutants, we found that *drc1-191* showed a strong negative interaction with mutations in the *sid2*$^+$ gene, which encodes a protein kinase related to the budding yeast Dbf2p and Dbf20p kinases (Balasubramanian *et al.* 1998). At 24°, the permissive temperature for *sid2-250* and *drc1-191*, the double mutants grew very poorly. A high percentage of multinucleate cells that had failed to form a division septum were observed in the culture (Figure 8B). Thus, Sid2p might play an important role in the function of Drc1p.

DISCUSSION

Drc1p/Cps1p, a protein essential for division septum deposition, is a 1,3-β-glucan synthase subunit: Previous studies of cytokinesis have identified a large collection of gene products that regulate various aspects of actomyosin ring function and division septum deposition (Gould and Simanis 1997; Balasubramanian *et al.* 1998). However, the mechanisms that link the gene products controlling cytokinesis to the proteins that participate in the physical assembly of the division septum are not understood. The division septum is assembled in two distinct phases in *S. pombe*. First, a primary septum, which reacts strongly with calcofluor, is formed centripetally concomitant with actomyosin ring constriction. Subsequently, secondary septa are formed on either side of the primary septum, leading to the formation of a three-layered septum. Finally, the primary septum is degraded to liberate the two daughter cells, with the secondary septa being localized at the ends formed as a result of cell division (Johnson *et al.* 1989). The septa and cell wall of *S. pombe* are composed of three major

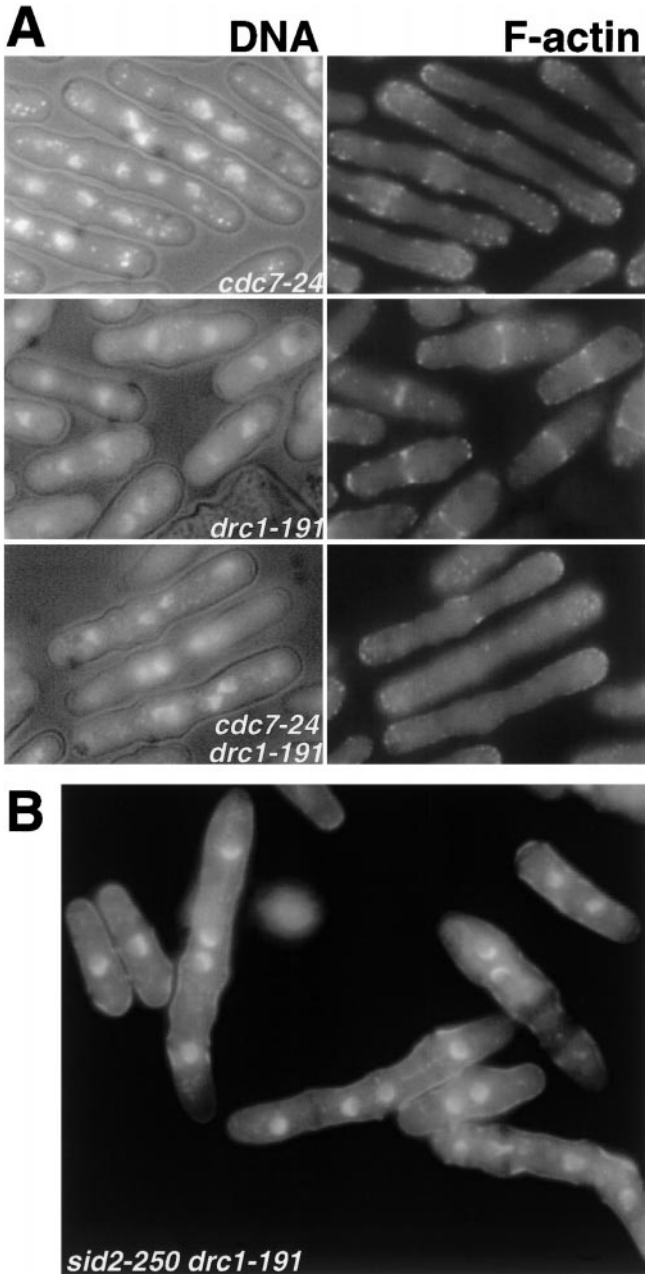


Figure 8.—Genetic interactions involving *drc1-191* and the *sid* genes. (A) *cdc7-24* is epistatic to the mitotic phenotype of *drc1-191*. Cells of the genotypes *cdc7-24*, *drc1-191*, and *cdc7-24 drc1-191* were grown at the permissive temperature (24°) to exponential phase and shifted to the restrictive temperature (36°). Samples were taken 4 hr after shift to 36°, fixed, and stained with rhodamine-conjugated phalloidin and DAPI to visualize F-actin and nuclei (labeled DNA), respectively. (B) Negative interaction between *drc1-191* and *sid2-250*. Cells of the genotype *drc1-191 sid2-250* were fixed and stained with DAPI to visualize nuclei.

types of polymers, 1,3-β-glucan, 1,3-α-glucan, and α-galactomannan. Thus, enzymes that participate in the assembly of these polymers should be important for cell wall assembly, division septum deposition, and cell polarity. Recently, characterization of *mok1/ags1*, a gene encoding an α-glucan synthase has been reported (Hochstenbach *et al.* 1998; Katayama *et al.* 1999). Mok1p localizes to the growing end(s) of interphase cells and to the medial region of the cell during septation consistent with a role for Mok1p in cell elongation, polarity, and septation (Katayama *et al.* 1999).

In this study we describe *drc1*, a gene allelic to the previously described gene *cps1*, which encodes a 1,3-β-glucan synthase subunit (Ishiguro *et al.* 1997). We have shown that Drc1p is essential for cell viability. Drc1p, together with Pgs2p and Pgs3p (J. Liu, H. Wang and M. K. Balasubramanian, unpublished observations) represent three proteins encoded in the *S. pombe* genome that are related to 1,3-β-glucan synthase subunits. Our identification of the amino-acid substitution in the temperature-sensitive mutant, *drc1-191*, should provide a basis to create similar mutations in other 1,3-β-glucan synthases and also for structure-function studies of these enzymes.

A number of studies have demonstrated the involvement of the Rho family of GTPases in regulation of 1,3-β-glucan synthases (Arellano *et al.* 1996; Drganova *et al.* 1996; Qadota *et al.* 1996; Nakano *et al.* 1997). Thus, a Rho GTPase pathway might regulate septum deposition in *S. pombe*.

Our characterization of *drc1-191* and *drc1*-null mutants has shown that Drc1p is essential for the assembly of the division septum. At least two lines of evidence suggest that Drc1p might not be involved in cell wall assembly during cell elongation. First, *drc1*-null mutants are capable of spore germination, assembling cell wall, and growth and become highly enlarged, suggesting that general cell wall assembly is not defective in cells lacking Drc1p. Second, double mutants of the genotype sid^{-} *drc1-191* are elongated and are phenotypically similar to the *sid* single mutants (Figure 8A). Thus, we conclude that Drc1p is required for division septum assembly, but not for cell elongation. It is likely that the other proteins related to 1,3-β-glucan synthases, such as Pgs2p and Pgs3p, participate in cell wall assembly for processes such as spore germination and cell elongation. *drc1*-null mutants assume a variety of cell morphologies. However, the diameter of cell tips of *drc1*-null mutants is similar to that of wild-type cells. Thus, Drc1p also appears to play a role in maintenance of cell shape, but not in the establishment of cell polarity. Again, Pgs2p and Pgs3p might be important for cell polarity establishment. Genetic interactions between the *sid* mutants and *drc1-191* demonstrate that the *sid* gene products, which regulate septum deposition, function upstream of Drc1p. Temperature-sensitive mutations, in particular Sid2p, which show a strong negative interaction with *drc1-191*, might play an important role in Drc1p function.

Actomyosin ring constriction/disassembly in *S. pombe*: The *drc1-191* mutant arrests with stable actomyosin rings. This is the first mutant that we are aware of that displays the phenotype of highly stabilized actomyosin

rings. Thus, it is possible that deposition of the division septum is important for disassembly / constriction of the actomyosin ring. Consistent with this idea, stable actomyosin rings are detected in reverting protoplasts treated with enzymes that degrade the cell wall. However, actomyosin rings constrict and division septa are laid down normally in reverting protoplasts not treated with cell wall-degrading enzymes (Jochova *et al.* 1991). Presently, it is unclear whether actomyosin constriction is powered by myosin II acting as a motor or whether cell wall secretion pushes the actomyosin ring, and the role of the actomyosin ring is only to guide and orient the direction of septum delivery. The previous studies do not address this question since Myo2p-ATPase (Myo2p is a type II myosin heavy chain) domain mutants fail to assemble proper actomyosin rings (Naqvi *et al.* 1999). Isolation and characterization of ts Myo2p ATPase mutants will resolve this issue.

Actomyosin rings are assembled at the onset of mitosis and disassembled during mitotic exit in *drc1*-null mutants. However, actomyosin rings are stable in *drc1-191* mutants at the restrictive temperature. These findings suggest that Drc1p might interact intimately with the ring to regulate its stability during septation. Thus, in mutants devoid of Drc1p, the actomyosin ring might simply collapse at the end of mitosis. Consistent with this, we have identified genetic interactions between *drc1-191* and mutations affecting Myo2p (Kitayama *et al.* 1997; May *et al.* 1997; Balasubramanian *et al.* 1998), Cdc4p, a light chain of Myo2p (Naqvi *et al.* 1999), and Rng2p, a protein related to IQGAP (Eng *et al.* 1998), all of which affect actomyosin ring function.

***drc1-191*, a novel cytokinesis mutant that arrests with only two nuclei:** The *drc1-191* mutant is the first cytokinesis mutant that we are aware of that blocks with two interphase nuclei. The previously characterized cytokinesis mutants were either defective in assembling proper actomyosin rings (*rng* mutants) or destabilized the actomyosin ring at the end of anaphase (*sid* mutants). By contrast, the *drc1-191* mutant arrests with a stable actomyosin ring. A possibility is that the presence of a stable actomyosin ring arrests cells at a point at which further cell elongation and cell mass increase are rendered inactive. Thus, arrested *drc1-191* cells might not grow sufficiently to allow the two interphase nuclei to undergo mitosis. An alternative possibility is that the presence of the actomyosin ring in an interphase cell (generated due to failed cytokinesis) might prevent entry of the two G_2 nuclei into the M phase. The findings that multiple rounds of mitoses occur in *drc1*-null mutants and *drc1-191 cdc7-24* double mutants is consistent with both possibilities, since actomyosin ring destabilization at the end of anaphase and cell growth occur in the *drc1*-null mutant as well as in the *drc1-191 cdc7-24* double mutant. Further quantitative and physiological studies of *drc1-191* single mutants and *drc1-191* in combination with other mutations affecting nuclear cycle progression will be necessary to firmly establish the molecular basis of arrest of the *drc1-191* mutants with two G_2 nuclei.

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