Localization of the (1,3)β-D-glucan synthase catalytic subunit homologue Bgs1p/Cps1p from fission yeast suggests that it is involved in septation, polarized growth, mating, spore wall formation and spore germination

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Summary

Schizosaccharomyces pombe **Bgs1p/Cps1p has been identified as a putative (1,3)β-D-glucan synthase (GS) catalytic subunit with a possible function during cytokinesis and polarized growth. To study this possibility, double mutants of *cps1-12* and *cdc* septation mutants were made. The double mutants displayed several hypersensitive phenotypes and altered actin distribution. Epistasis analysis showed mutations prior to septum synthesis were dominant over *cps1-12*, while *cps1-12* was dominant over the end of septation mutant *cdc16-116*, suggesting Bgs1p is involved in septum cell-wall (1,3)β-D-glucan synthesis at cytokinesis. We have studied the in vivo physiological localization of Bgs1p in a *bgs1*Δ strain containing a functional *GFP-bgs1*$^+$ gene (integrated single copy and expressed under its own promoter). During vegetative growth, Bgs1p always localizes to the growing zones: one or both ends during cell growth and contractile ring and septum during cytokinesis. Bgs1p localization in *cdc* septation mutants indicates that Bgs1p needs the medial ring and septation initiation network (SIN) proteins to localize properly with the rest of septation components. Bgs1p localization in the actin mutant *cps8-188* shows it depends on actin localization. In addition, Bgs1p remains polarized in the mislocalized growing poles and septa of *tea1-1* and *tea2-1* mutants. During the meiotic process of the life cycle, Bgs1p localizes to the mating projection, to the cell-to-cell contact zone during cell fusion and to the neck area during zygote formation. Also, Bgs1p localization suggests that it collaborates in forespore and spore wall synthesis. During spore germination, Bgs1p localizes first around the spore during isotropic growth, then to the zone of polarized growth and finally, to the medial ring and septum. At the end of spore-cell division, the Bgs1p displacement to the old end occurs only in the new cell. All these data show that Bgs1p is localized to the areas of polarized cell wall growth and so we propose that it might be involved in synthesizing the lineal (1,3)β-D-glucan of the primary septum, as well as a similar lineal (1,3)β-D-glucan when other processes of cell wall growth or repair are needed.**

Key words: Fission yeast, Cell wall, Glucan, Septation, Polarized growth, 1,3-β-D-glucan synthase

Introduction

The cell wall is a structure external to the plasma membrane that is present in Mycota. This structure provides mechanical strength and osmotic support to these organisms. Its integrity is vital for the fungal cell, participating in morphogenetic and differentiation processes during the fungal life cycle, which require polarized cell growth and microtubules and actin cytoskeleton reorganizations (Brunner and Nurse, 2000; Chang, 2001; Goode et al., 2000; Mata and Nurse, 1998).

In the fission yeast *Schizosaccharomyces pombe*, the major cell wall structural components are two glucose homopolymers, (1,3)β-D-glucan with 2-4% (1,6)β-D-branches and (1,3)α-D-glucan with 7% (1,4)α-D-bonds, constituting 50-54% and 28-32% of total cell wall polysaccharides, respectively. A third glucan component, a highly branched (1,6)β-D-glucan, accounts for about 2% of total cell wall polysaccharides (Kopecka et al., 1995; Manners and Meyer, 1977). Chitin is another important structural component in many fungal cell walls. Its presence in fission yeast remains controversial, but a chitin synthase gene, *chs1*$^+$, and the corresponding enzymatic activity, has recently been described to be important for spore formation (Arellano et al., 2000). Galactomannan is a non-structural cell wall polymer, linked to proteins to form glycoproteins and representing 9-14% of cell wall polysaccharides (Manners and Meyer, 1977). (1,3)α-D-glucan synthesis has been attributed to the essential gene *ags1*$^+$ (=*mok1*$^+$), described to encode a putative catalytic subunit of the enzyme (Hochstenbach et al., 1998; Katayama

et al., 1999). There are four more *mok* genes, none of them is essential (Katayama et al., 1999) and their role in cell wall biosynthesis has not been clarified yet.

The (1,3)β-D-glucan is a major contributor to the cell wall framework. On this matter, it is relevant to emphasize the only new antifungal agents under clinical trials are the echinocandins (Tkacz and DiDomenico, 2001), whose mode of action, apparently similar to other antifungal drugs, the papulacandins (Castro et al., 1995), is not well known although both clearly interfere with (1,3)β-D-glucan synthesis.

The enzyme involved in (1,3)β-D-glucan synthesis in yeast is the (1,3)β-D-glucan synthase (GS), which uses UDP-glucose as substrate, producing a de novo lineal (1,3)β-D-glucan (Cabib et al., 1998). The activity requires at least two components, a catalytic and a regulatory subunit (Kang and Cabib, 1986; Ribas et al., 1991). The latter is the Rho1p GTPase, which drives the activation of GS by GTP (Arellano et al., 1996; Drgonová et al., 1996; Qadota et al., 1996). This GTPase plays a fundamental role in many morphogenetic processes, particularly in organization of actin cytoskeleton and genesis of active growing regions (Arellano et al., 1999; Cabib et al., 1998; Cabib et al., 2001).

In budding yeast, two highly homologous genes, *FKS1* and *FKS2*, encode two putative GS catalytic subunits, which are differentially expressed depending on the cycle or growth conditions (Inoue et al., 1995; Mazur et al., 1995; Zhao et al., 1998). Single disruption of either *FKS1* or *FKS2* is viable, but the double disruption is lethal (Mazur et al., 1995). A third *FKS* gene, found in the *Saccharomyces cerevisiae* genome, appears to be nonfunctional.

In *S. pombe* four *FKS* homologues have been found. The first identified gene coding for a putative GS catalytic subunit was cloned by complementation of the *cps1-12* mutant, hypersensitive to a spindle poison, and named *cps1*$^+$ (Ishiguro et al., 1997). Later on, it was also isolated by complementation of mutants defective in septum formation (Le Goff et al., 1999b; Liu et al., 1999) and implicated in the coordination between cytokinesis and cell cycle, being part of a Wee1p-dependent septation checkpoint (Le Goff et al., 1999b; Liu et al., 2000b). During the *S. pombe* genome sequencing project, three other open reading frames (ORF), whose predicted amino acid sequence share high identity (50-60%) with Cps1p, have appeared. As other genes already were designated *cps* (Ishiguro and Uhara, 1992), the *cps1*$^+$ homologues were named in a common agreement as *bgs* (for beta 1,3-glucan synthesis), and therefore *cps1*$^+$ has been renamed *bgs1*$^+$. *bgs2*$^+$ is essential for the sexual phase of the life cycle. Its expression is induced during sporulation and GS activity is diminished in sporulating *bgs2*Δ/*bgs2*Δ diploids (Martin et al., 2000). Bgs2p localizes to the ascospore periphery and is required for correct spore wall maturation and survival (Liu et al., 2000a; Martin et al., 2000). Finally, to date nothing is known about the role of the other two homologue genes, *bgs3*$^+$ and *bgs4*$^+$.

While in *Saccharomyces cerevisiae* there are only two redundant FKS activities for (1,3)β-D-glucan synthesis, it is very interesting to notice that *S. pombe* contains four different Bgs proteins (and perhaps the corresponding GS isozymes), to apparently synthesize the same cell wall component. What is the function and regulation of all of these proteins? Previous results have pointed to the idea that Bgs1p plays a role in septum assembly (Ishiguro et al., 1997; Le Goff et al., 1999b; Liu et al., 1999). Fission yeast cytokinesis starts with medial ring formation and contraction. As the medial ring grows centripetally, an intense Calcofluor white-stained material is concomitantly extruded to the lumen of the invaginated plasma membrane. Eventually, a disk can be visualized between the membranes of both daughter cells, which is called, by analogy to the budding yeast system, the primary septum (Schmidt et al., 2002). Subsequently, new cell wall material, the so-called secondary septum, is laid down at both sides of the primary septum, giving rise to a complete three-layered septum structure (Johnson et al., 1973). In addition, a recent study describes the in situ localization of different β-D-glucans in *S. pombe* cell wall by using colloidal-gold labeled specific antibodies (Humbel et al., 2001). (1,6)β-D-glucan-branched (1,3)β-D-glucan is localized forming filamentous structures all over the cell wall non-dense layer and in all the septum. (1,6)β-D-glucan appears in the same cell wall non-dense layer, but always close to the outer galactomannan layer, and in the secondary septum. A linear (1,3)β-D-glucan is visible only at the primary septum, as detected by a monoclonal antibody that also specifically detects the linear (1,3)β-D-glucan (callose) that constitutes the cell plate in plant cells (Hong et al., 2001).

In this paper, we show genetic and epistatic interactions found between *cps1-12* mutant and several *cdc* septation mutants, suggesting that Bgs1p (Cps1p) is involved in the septum cell-wall synthesis process. In an attempt to elucidate further the Bgs1p function, we have studied Bgs1p localization during the *S. pombe* life cycle. In contrast to a recent report (Liu et al., 2002), in which it is stated that Bgs1p localizes exclusively to the cell division site, we have found that Bgs1p localizes first to the growing poles and moves to the contractile actomyosin ring during septation, remaining in the septum and reappearing later on at the poles, before the two daughter cells separate. In addition, we show that Bgs1p is not only restricted to that role, but it is also present in the meiotic phase of the life cycle, localizing to the mating projection, cell-to-cell contact zone of fused cells, neck of zygote, and to or around prospore and spore. Finally, during spore germination, Bgs1p localizes first, around the spore and next, to the zone of polarized growth. All these data suggest that Bgs1p is present and could play a role in every process of the *S. pombe* life cycle in which synthesis of (1,3)β-D-glucan takes place.

Materials and Methods

Strains and culture conditions

S. pombe strains used in this work are listed in Table 1. The *bgs1*Δ strain 519 containing an integrated copy of *GFP-bgs1*$^+$, was made by transforming the strain 437 (*bgs1*Δ pJG25, *his3*$^+$ selection) with *Hin*dIII-cut pJR98 (*leu1*$^+$ selection, see below), which will direct its integration at the *Hin*dIII site adjacent to *bgs1*Δ*::ura4*$^+$, at position –661 of the *bgs1*$^+$ promoter sequence. This strain contains *GFP-bgs1*$^+$ and *bgs1*Δ sequences separated by pJK148 plasmid backbone. In order to eliminate pJG25, to analyze the GFP-Bgs1p functionality and to confirm that integration was in the *bgs1*$^+$ promoter sequence, this strain was crossed with strain 285 (Leu$^-$, Ura$^-$, His$^-$) and tetrads analysis was performed. Leu and Ura phenotypes always appeared linked and segregating 2+:2–. The His phenotype showed that pJG25 was lost in most of the clones, as a consequence of the sporulation process. The obtained *GFP-bgs1*$^+$ *bgs1*Δ strain displays a wild-type phenotype at any tested condition

Table 1. Fission yeast strains used in this study

Strain	Genotype	Source
33	972 h$^-$	P. Munz*
38	968 h^{90}	P. Munz*
40	*leu1-32* h$^+$	J. C. Ribas
265	*ura4-Δ18* h$^+$	J. C. Ribas
285	*leu1-32 ura4-Δ18 his3-Δ1* h$^+$	This study
470	*leu1-32 ura4-Δ18 his3-Δ1* h^{90}	This study
317	*leu1-32*/*leu1-32 ura4-Δ18*/*ura4-Δ18 his3-Δ1*/*his3-Δ1 ade6-M210*/*ade6-M216* h$^-$/h$^+$	This study
CP1-1	*cps1-12* h$^-$	J. Ishiguro
CP1-2	*cps1-12* h$^+$	J. Ishiguro
253	*cps8-188* h$^-$	This study
276	*cdc3-6* h$^-$	NCYC†
277	*cdc14-118* h$^-$	NCYC†
278	*cdc15-140* h$^-$	NCYC†
279	*cdc16-116* h$^-$	NCYC†
573	*cdc14-118 leu1-32* h$^-$	This study
575	*cdc15-140 leu1-32* h$^-$	This study
581	*cdc16-116 ura4-Δ18* h$^-$	This study
CP962	*cps1-12 cps8-188* h$^+$	This study
280	*cps1-12 cdc3-6* h$^+$	This study
281	*cps1-12 cdc14-118* h$^-$	This study
282	*cps1-12 cdc15-140* h$^+$	This study
283	*cps1-12 cdc16-116* h$^+$	This study
635	*mid1-366 leu1-32* h$^+$	P. Nurse‡
583	*tea1-1 leu1-32* h$^-$	P. Nurse‡
585	*tea2-1 cdc11-119 leu1-32* h$^-$	P. Nurse‡
387	*leu1-32*/*leu1-32 ura4-Δ18*/*ura4-Δ18 his3-Δ1*/*his3-Δ1 ade6-M210*/*ade6-M216 bgs1$^+$*/*bgs1Δ::ura4$^+$* h$^-$/h$^+$	This study
390	*leu1-32 ura4-Δ18 his3-Δ1 ade6-M? bgs1Δ::ura4$^+$* h$^-$ pCP1	This study
437	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4$^+$* h$^-$ pJG25	This study
511	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4$^+$* h$^-$ pJR92	This study
513	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4$^+$* h$^-$ pJR93	This study
519	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^-$	This study
520	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
588	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h^{90}	This study
641	*mid1-366 leu1-32 ura4? his3-Δ1 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^-$	This study
690	*cdc3-6 leu1-32 ura4? bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
616	*cdc11-119 leu1-32 ura4? bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^-$	This study
628	*cdc14-118 leu1-32 ura4? bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
611	*cdc15-140 leu1-32 ura4? bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
612	*cdc16-116 leu1? ura4-Δ18 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
688	*cps8-188 leu1-32 ura4? bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^-$	This study
613	*tea1-1 leu1-32 ura4? his3-Δ1 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
614	*tea2-1 leu1-32 ura4? bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study
618	*tea2-1 cdc11-119 leu1-32 ura4? his3-Δ1 bgs1Δ::ura4$^+$* P$_{bgs1^+}$*::GFP-bgs1$^+$:leu1$^+$* h$^+$	This study

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and expresses GFP-Bgs1p at the physiological level, from a single integrated *GFP-bgs1$^+$* gene controlled from its own promoter.

Standard complete yeast growth medium (YES), selective media (EMM) supplemented with the appropriate amino acids (Alfa et al., 1993) and sporulation medium (SPA) (Egel, 1984) have been described. The drugs papulacandin B (Ciba-Geigy, fused to create Novartis) and echinocandin LY280949 (Lilly) were added to complete (YES) medium, at concentrations ranging from 1.0 to 15 μg/ml. General procedures for yeast culture and genetic manipulation were carried out as described (Alfa et al., 1993; Moreno et al., 1991).

Escherichia coli strains DH5α and DH10B (Life Technologies) were used for routine propagation of plasmids as described (Sambrook et al., 1989), and CJ236 (Bio-Rad) was used for site-directed mutagenesis. Bacterial LB, 2xYT and TB media, supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin when appropriate, and standard transformation methods (Sambrook et al., 1989), have been described.

Plasmids and DNA techniques

pCP1 is the 8.04 kb *Hin*dIII-*Hin*dIII DNA fragment containing the *bgs1$^+$* (*cps1$^+$*) gene sequence cloned into the *Hin*dIII site of the *S. pombe-E. coli* shuttle vector pAL-KS$^+$ (pBluescript KS$^+$ with *ars1$^+$* and *S. cerevisiae LEU2* selection) (Ishiguro et al., 1997; Tanaka et al., 2000).

pJR16 is pCP1 with *Not*I from the multiple cloning site destroyed (*Not*I cut and Klenow filled). pJR19 is pJR16 with the *Apa*I site from the multiple cloning site destroyed and with an *Apa*I site inserted by site-directed mutagenesis just after the *bgs1$^+$* TAA stop codon. pJR20 is pJR19 with a *Not*I site inserted just before the ATG start codon of *bgs1$^+$*. pJR24 is pJR20 cut with *Not*I-*Apa*I to eliminate the *bgs1$^+$* ORF and with the *ura4$^+$* gene from pBluescript SK$^+$-*ura4$^+$* (Moreno et al., 2000) cut with *Not*I-*Apa*I inserted. This plasmid was used for PCR amplification of a *bgs1$^+$* disruption cassette, as described below.

pJR17 is pJR16 with a *Spe*I site inside the *bgs1$^+$* ORF destroyed by site-directed mutagenesis and making no change in the amino acid

sequence of Bgs1p. pJR18 is pJR17 with a *Spe*I-*Spe*I deletion, which removes the 0.79 kb 3′-end fragment of the cloned *bgs1*$^+$ sequence. pJR22 is pJR18 with a 9 bp insertion containing a *Not*I site just after and a *Spe*I site before the ATG initiation codon of *bgs1*$^+$ ORF. pJR23 is pJR18 with a 9 bp insertion containing a *Not*I site just before and a *Spe*I site after the TAA termination codon of *bgs1*$^+$ ORF. pJG20 is pJR19 with a *Hpa*I site inserted before the *bgs1*$^+$ start codon. pJG25 is pJR1-81XH (81X version of *nmt1*$^+$ thiamine-repressible promoter and *S. pombe his3*$^+$ selection) (Moreno et al., 2000) cut with *Nru*I-*Apa*I and with the *Hpa*I-*Apa*I *bgs1*$^+$ ORF sequence from pJG20 inserted. The resulting *S. pombe* strain *bgs1*Δ pJG25 displayed wild-type phenotype even in the presence of thiamine, which represses the regulatable *nmt1*$^+$ promoter to a very low expression level (Moreno et al., 2000).

pJR92 (pAL-*bgs1*$^+$-*GFP*) is pJR23 with an *Apa*I-*Hin*dIII DNA fragment obtained from PCR amplification of the 1.57 kb sequence just 5′ to the *Hin*dIII site of the promoter sequence (nucleotides –2229 to –661) of the cloned *bgs1*$^+$ fragment of pCP1, and also inserted in frame, just before the *bgs1*$^+$ TAA stop codon, a 0.74 kb *Not*I-*Not*I fragment from pKS$^+$-*GFP* containing a modified GFP-encoding sequence, GFP2-5, which has the changes S65T, V163A, I167T and S175G (Fernandez-Abalos et al., 1998). Similarly, pJR93 (pAL-*GFP*-*bgs1*$^+$) is pJR22 with the same *Apa*I-*Hin*dIII DNA fragment as in pJR92 and the 0.74 kb *Not*I-*Not*I fragment from pKS$^+$-*GFP* inserted in frame after the *bgs1*$^+$ ATG start codon. pJR98 (pJK-*GFP*-*bgs1*$^+$) is the integrative plasmid pJK148 (*S. pombe leu1*$^+$ selection) (Keeney and Boeke, 1994) with the *Apa*I-*Xba*I *GFP*-*bgs1*$^+$ fragment from pJR93. pJR108 (pJK-*bgs1*$^+$-*GFP*) is pJK148 with the *Apa*I-*Xba*I *bgs1*$^+$-*GFP* fragment from pJR92. pJR98 was used to create stable *GFP*-*bgs1*$^+$ strains, expressed at physiological level in a *bgs1*Δ background.

Site-directed mutagenesis (Kunkel, 1985) was carried out with the Bio-Rad Muta-Gene kit. DNA sequencing was with a Sequenase kit (Amersham Pharmacia Biotech). Ligation of DNA fragments was carried out as described (Moreno et al., 2000). Plasmid DNA was introduced into *S. pombe* cells by an improved LiAc method (Gietz et al., 1995). Other DNA manipulations were carried out essentially as described (Sambrook et al., 1989).

Gene disruption

bgs1$^+$ gene disruption was performed in the Leu$^-$ Ura$^-$ His$^-$ diploid strain 317. The disruption cassette was made by PCR amplification of a 3.9 kb DNA fragment from pJR24, which has completely eliminated the *bgs1*$^+$ coding sequence and contains 0.55 kb of *bgs1*$^+$ promoter and 5′ sequence, the 1.76 kb *ura4*$^+$ sequence and 1.6 kb of *bgs1*$^+$ terminator and 3′ sequence. Correct deletion of *bgs1*$^+$ ORF was confirmed by PCR analysis using a combination of oligonucleotides external and internal to the disruption fragment, either for the 5′ end or the 3′ end of both *bgs1*$^+$ and *ura4*$^+$ sequences (data not shown). Tetrads dissection of the *bgs1*$^+$/*bgs1*Δ strain showed that only the two Ura$^-$ clones of each tetrad were viable, confirming the essential function of Bgs1p, at least for spore germination, as described for a partial *bgs1*$^+$ disruption strain (Liu et al., 1999). Haploid *bgs1*Δ strains were obtained by standard random spore analysis of a *bgs1*$^+$/*bgs1*Δ diploid strain transformed with the *bgs1*$^+$-carrying plasmid pCP1 and selection of Leu$^+$ (pCP1), Ura$^+$ (*bgs1*Δ), His$^-$, Ade$^-$ clones.

Enzyme preparation and (1,3)β-D-glucan synthase assay

Cell extracts and GS assay were essentially as described previously (Ishiguro et al., 1997). Early logarithmic phase cells grown at 28°C were washed with buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM β-mercaptoethanol), suspended in 100 μl of buffer A and broken with glass beads in a FastPrep FP120 apparatus (Savant; BIO 101) (a 15 second pulse at a speed of 6.0). Broken material was diluted with buffer A, the cell debris was removed by low speed centrifugation and the supernatant was centrifuged at 48,000 ***g*** for 30 minutes at 4°C. The pellet was resuspended in buffer A containing 33% glycerol and stored at –80°C. Standard GS assay mixture contained 5 mM UDP-D-[^{14}C]glucose (4×10^4 cpm/200 ηmoles) and enzyme (20-40 μg protein) in a total volume of 40 μl. All reactions were carried out in duplicate and data for each strain were calculated from three independent cultures.

Sensitivity of cells to cell-wall-digesting enzyme complex

Sensitivity of cells to Novozym 234 (Novo Industries) was measured essentially as described elsewhere (Ishiguro et al., 1997). The log-phase cells were washed with 50 mM citrate-phosphate buffer (pH 5.6) and incubated at 30°C with continuous shaking in the same buffer containing Novozym 234 (30 μg/ml). The residual absorbance of the cell suspensions was monitored at 600 nm every hour.

Fluorescence microscopy

For F-actin visualization, cells grown in YES liquid medium were fixed with 4% formaldehyde (EM-grade, Polysciences) and stained with rhodamine-conjugated phalloidin (R-415, Molecular Probes) as described (Alfa et al., 1993). For Calcofluor white fluorescence, early-logarithmic phase cells grown in YES liquid medium were visualized directly by adding a solution of Calcofluor white to the sample (50 μg/ml final concentration) and using the appropriate UV filter. For nuclei and cell wall staining, early-logarithmic phase cells grown in YES liquid medium were fixed with cold 70% ethanol, centrifuged, resuspended in PBS containing 10 μg/ml Calcofluor white and 20 μg/ml Hoechst (No. 33258, Sigma), and visualized with the same UV filter. GFP was directly visualized from early-logarithmic phase cells by using the appropriate UV filter. Calcofluor white or Hoechst and GFP fluorescence do not interfere with each other and therefore, they can simultaneously be used to visualize the same cells. Images were obtained using a Leica HC (Germany) fluorescence microscope, type 020-523.010. For phase contrast and Calcofluor white-fluorescence a PL APO 40×/0.75 PH2 objective, a Leica DC 100 digital camera and the Leica DC 100 V2.51 software were used. For GFP, Calcofluor white and Hoechst fluorescence a PL APO 63×/1.32 OIL PH3 objective, a Sensys (Photometrics) digital camera and the Leica QFISH V2.3a software were used.

Results

Epistatic interactions localize Bgs1p function during the septum synthesis process

In a previous study (Ishiguro et al., 1997), the cloning of the GS homologue *bgs1*$^+$ by complementation of the *cps1-12* thermosensitive mutant was described. The fact that the mutant cells grown at 37°C in the presence of sorbitol became multiseptated and branched suggested that Bgs1p could be involved in septation and polarity processes. Later on, other laboratories also proposed a role for Bgs1p during septation (Le Goff et al., 1999b; Liu et al., 2000b; Liu et al., 1999).

In order to study this possibility further, nine double mutants of *cps1-12* with some of the numerous septation mutations, *cdc3-6*, *cdc4-8*, *cdc7-24*, *cdc8-134*, *cdc11-136*, *cdc12-112*, *cdc14-118*, *cdc15-140* and *cdc16-116*, representative of different septation steps (reviewed by Gould and Simanis, 1997; Le Goff et al., 1999a), were constructed. Four double mutants, *cps1-12* with *cdc3-6*, *cdc14-118*, *cdc15-140* and *cdc16-116*, were much more sensitive to Novozym 234 than the parental single mutants (data not shown). In addition, the former four double mutants were more

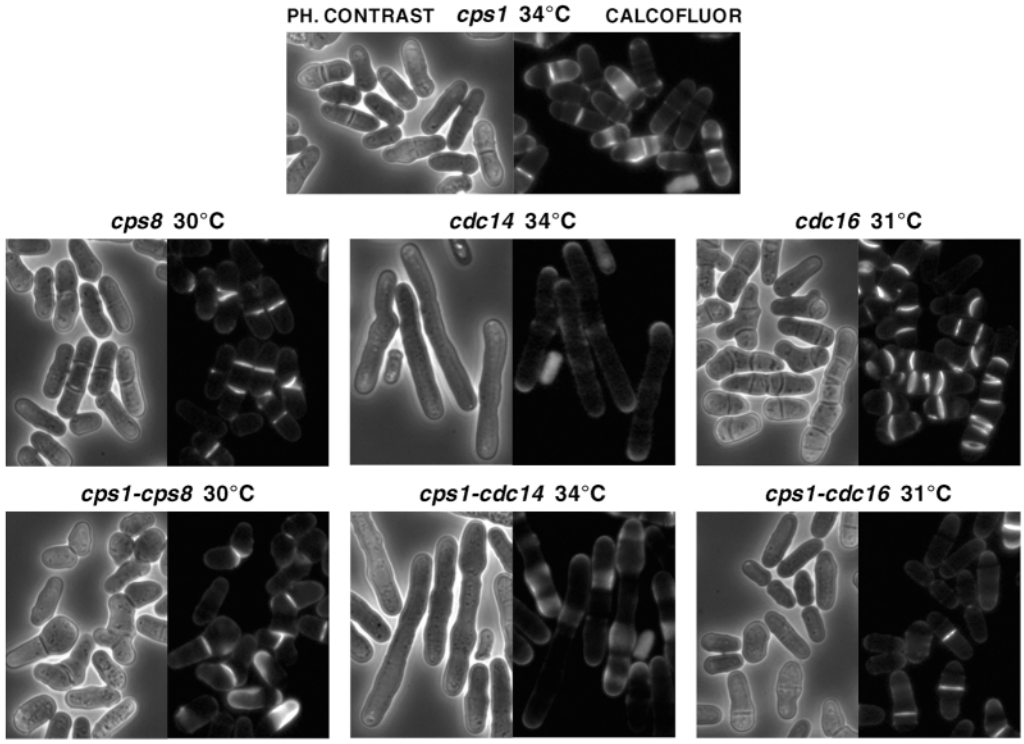


Fig. 1. Genetic interactions of *S. pombe cps1-12* mutant with the actin mutant *cps8-188* and with *cdc* septation mutants. Phase-contrast and Calcofluor white UV staining micrographs of log-phase cells grown on YES liquid medium at 25°C and shifted to different temperatures. Cells are shown grown at the temperature that reveals in each case the most apparent difference between single and double mutant phenotypes (8 hours at 30°C for *cps8-188*, 8 hours at 31°C for *cdc16-116* and 6 hours at 34°C for *cdc14-118* single and double *cps1-12* mutants). The phenotype of the *cps1-12 cps8-188* double mutant is more aggravated than that of the corresponding single mutants, while the phenotype of the *cps1-12 cdc14-118* double mutant resembles that of the *cdc14-118* single mutant, and the phenotype of the *cps1-12 cdc16-116* double mutant is similar to that of the *cps1-12* single mutant. The *cps1-12* single mutant cells were grown at 34°C, but a weaker phenotype was obtained at 30 or 31°C. Cells were centrifuged, suspended in 50 μg/ml Calcofluor white and directly observed for phase-contrast and Calcofluor white staining.

thermosensitive, either in the absence or in the presence of 1.2 M sorbitol, and exhibited abnormal cell morphology and actin distribution at conditions in which the single mutants showed no alteration (data not shown). Moreover, a double mutant of *cps1-12* with the actin mutant *cps8-188* (Ishiguro and Kobayashi, 1996) was found to be more thermosensitive (data not shown) and to have abnormal cell morphology at conditions in which the single mutants were essentially normal (Fig. 1). Furthermore, the four *cdc* and *cps8-188* double mutants with *cps1-12* presented variable increased hypersensitivities to the cell wall and membrane disturbing detergent SDS (Bickle et al., 1998; Igual et al., 1996) and to the GS inhibitors papulacandin B and echinocandin, and a slightly altered GS activity (from 70% to 120% wild-type activity, data not shown). All these data suggest a certain genetic interaction of actin and septation mutants with *cps1-12*, which are somehow increasing its cell wall-related phenotypes and altering its role in GS activity.

cps1-12 single and double mutants were also analyzed for their morphology phenotypes at different temperatures. The cells were visualized by phase-contrast and by Calcofluor white UV staining, which preferentially binds to cell wall material, probably the (1,3)β-D-glucan, of septum and growing zones. At the corresponding temperature, an epistatic interaction between *cps1-12* and these *cdc* mutants (Fig. 1) was found. Three of the mutants, *cdc3-6*, *cdc14-118* and *cdc15-140*, are involved in steps prior to the beginning of septum synthesis (Gould and Simanis, 1997; Le Goff et al., 1999a). In these double mutants, the dominant phenotype was that of the *cdc* mutant (Fig. 1 and data not shown). This result is in agreement with that described for double mutants of other *cps1* mutant alleles and some *cdc* mutants involved in steps prior to septum synthesis (Le Goff et al., 1999b; Liu et al., 1999). The last mutant, *cdc16-116*, is involved in stopping septum synthesis, and in this case, the dominant double mutant phenotype was that of *cps1-12* (Fig. 1). The same result was obtained in the presence of 1.2 M sorbitol (data not shown). Therefore, these results suggest that Bgs1p is involved in the septum synthesis process, acting in a window between the beginning and end of septum synthesis, in agreement with the proposed function of Bgs1p as a possible GS catalytic subunit (Ishiguro, 1998; Ishiguro et al., 1997).

Bgs1p localizes to the septum during cytokinesis and to one or both poles during cell growth

As the genetic results seem to implicate Bgs1p in the synthesis of septum cell wall, we were interested in knowing the physiological localization of Bgs1p along the cell cycle. GFP-Bgs1p, but not Bgs1-GFP, expressed either from multicopy or integrative plasmids restored completely the wild-type phenotype to a *bgs1*Δ strain and the GFP fluorescence appeared localized as well. Therefore, a *bgs1*Δ strain containing an integrated *GFP-bgs1*$^+$ copy (single copy, own promoter and absence of original *bgs1*$^+$ gene) was chosen for Bgs1p localization studies.

Calcofluor white staining shows a marked fluorescence at one pole during monopolar growth and at both poles when the cell grows in a bipolar fashion. Somewhat unexpectedly, Bgs1p was found localized not only to the septum but also to the growing ends along the mitotic cycle, overlapping the Calcofluor white staining (Fig. 2A). Initially Bgs1p

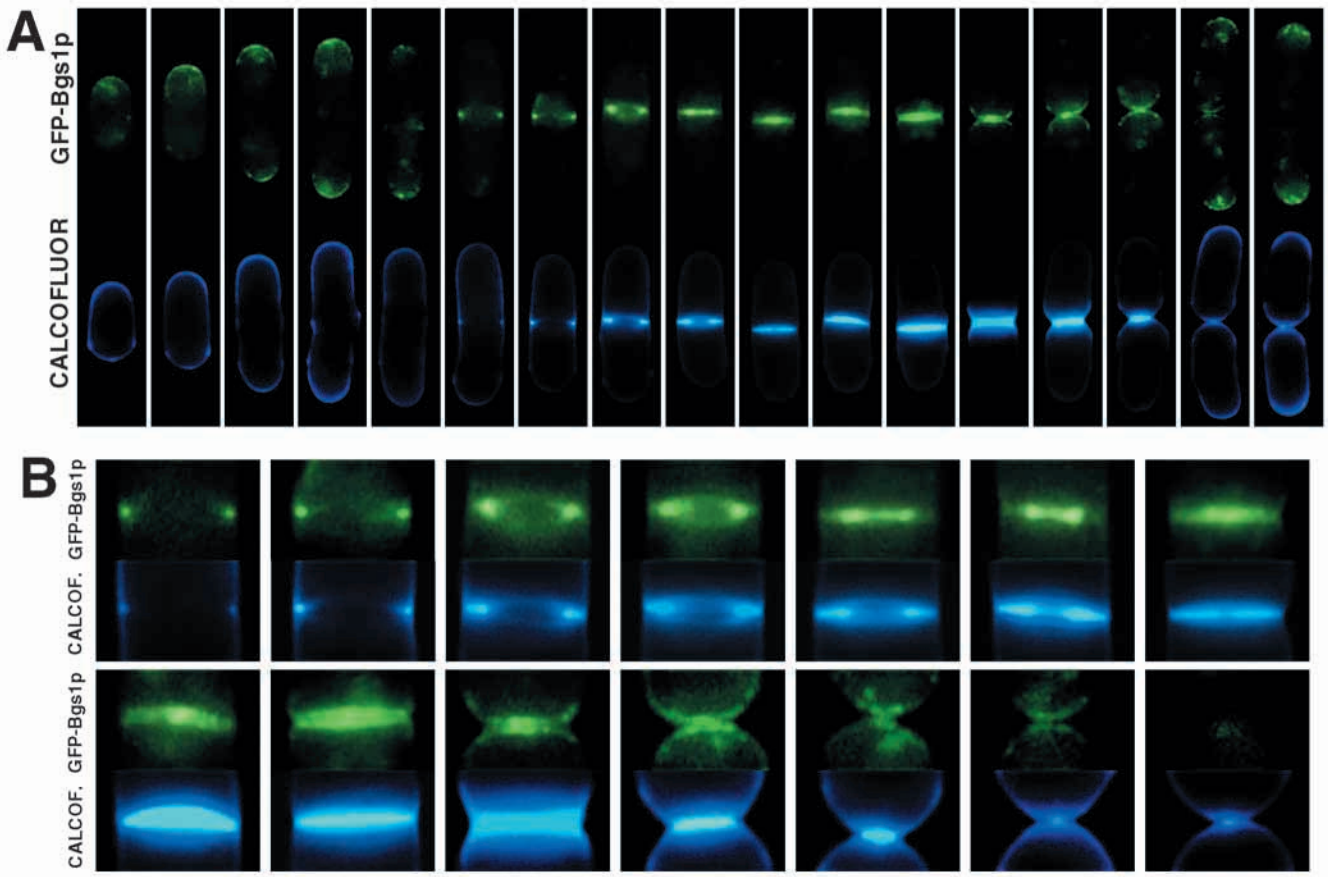


Fig. 2. Bgs1p localizes to the growing areas, to one or both poles, and to the contractile ring and septum. (A) Calcofluor white staining and GFP-Bgs1p localization along the mitotic cell cycle. (B) Detail of Calcofluor white staining and GFP-Bgs1p localization to the contractile ring and along the plasma membrane during septum formation. Early-logarithmic phase cells (*GFP-bgs1*$^{+}$ *bgs1*Δ), grown on YES liquid medium at 28°C, were visualized for GFP and Calcofluor white staining. Calcofluor white was added at 50 μg/ml following immediate examination of the cells. Cells representative of each cell cycle step were selected and aligned to show a cell cycle progression.

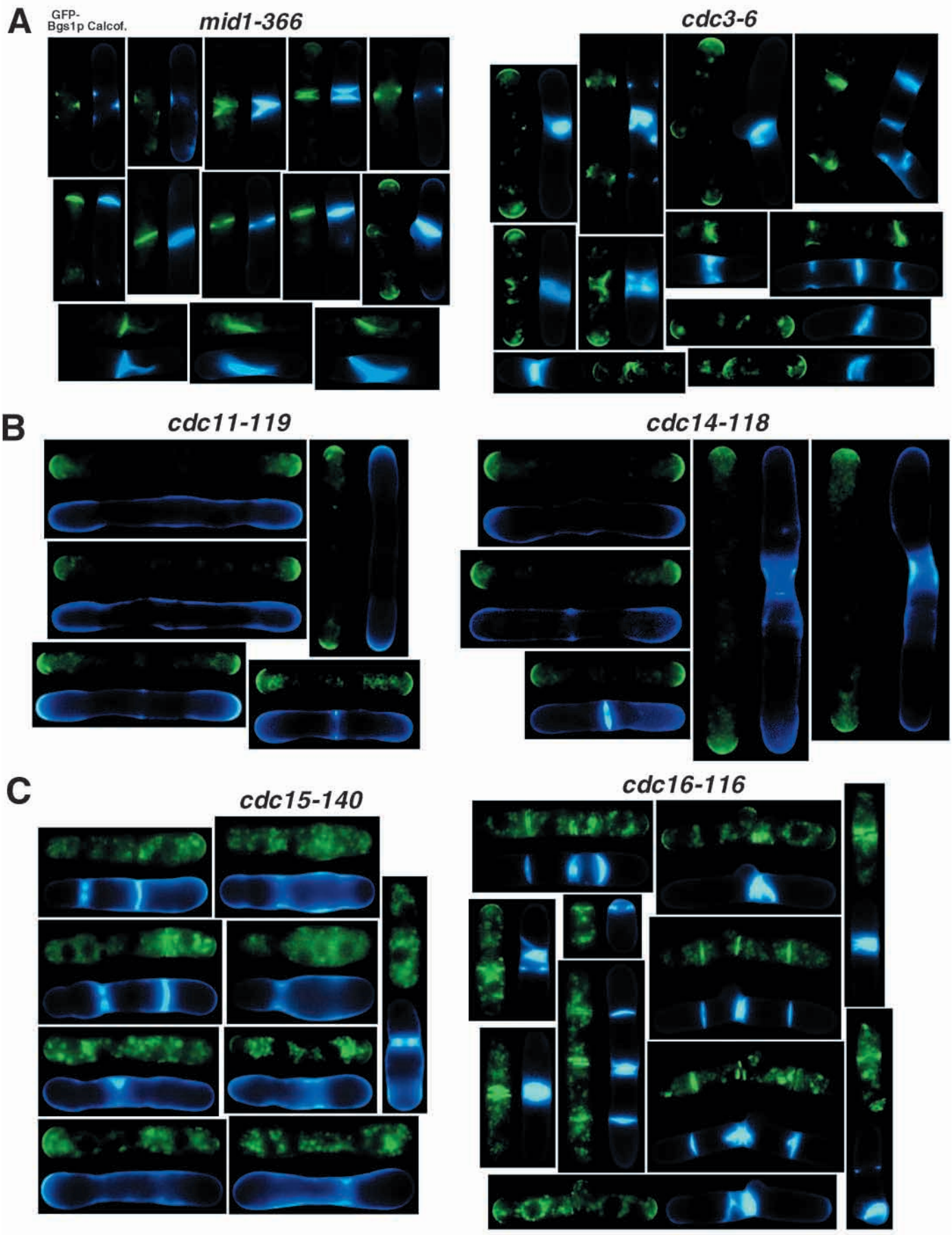
accumulates at one pole, then it also appears at the opposite pole and finally, the GFP disappears from both poles and localizes to the middle of the cell, where it concentrates into a narrow ring. The GFP signal is strongest where the ring is intersected by the focal plane, and it moves centripetally with the inner edge of the growing septum. A fainter signal remains along the invaginated membrane where new Calcofluor white-stained cell wall material has been made (Fig. 2B). When the cell-wall septum is completed, the GFP appears in two separated bands. During cell division Bgs1p remains at this pole. Finally, before the two daughter cells separate and the Calcofluor white staining disappears, Bgs1p changes its localization to the old end of both cells, which are then ready to initiate the monopolar growth (Fig. 2A,B). Our Bgs1p localization results differ from those recently published by other authors (Liu et al., 2002), in which Bgs1p is detected only to the division site. Possible reasons why they find Bgs1p exclusively localized to the cell division site are given in the Discussion. In summary, Bgs1p localization correlates with septation and polarized cell growth, suggesting that Bgs1p is responsible for the synthesis of both a specific (1,3)β-D-glucan that constitutes the fission yeast primary septum and part of the (1,3)β-D-glucan that is made at the poles during cell growth.

Bgs1p septum localization depends on the medial ring and the septation initiation network

In order to know whether Bgs1p localization at the middle of the cell is dependent on the presence and/or localization of the medial ring, several septation mutants were made in a *bgs1*Δ *GFP-bgs1*$^{+}$ background and GFP-Bgs1p localization was analyzed (Fig. 3).

Mid1p is essential for proper positioning of the medial ring, arriving at the zone before other ring proteins (Bahler et al., 1998; Paoletti and Chang, 2000; Sohrmann et al., 1996). In *mid1* mutants, actin ring and septum are positioned at random locations and angles on the cell surface (Chang et al., 1996). The *mid1-366* mutant grown at the restrictive temperature showed that ring and septum alterations were coincident with

Fig. 3. Bgs1p localization during septation depends on the medial ring formation and localization, and on proteins that form the septation initiation network. Calcofluor white staining and GFP-Bgs1p localization in different *cdc* septation mutants. *GFP-bgs1*$^{+}$ *bgs1*Δ mutant cells were grown as in Fig. 2, shifted to 37°C for 3 hours (*mid1-366*) or 4 hours (*cdc3-6*, *cdc11-119* and *cdc14-118*) or to 32°C for 5 hours (*cdc15-140* and *cdc16-116*), and visualized for GFP and Calcofluor white staining. Cells representative of the different mutant phenotypes are shown.


A
GFP-
Bgs1p Calcof.
mid1-366
cdc3-6
B
cdc11-119
cdc14-118
C
cdc15-140
cdc16-116

Bgs1p localization and with the Calcofluor white-stained cell wall material (Fig. 3). Cdc3p, or profilin, is essential for medial ring assembly (Balasubramanian et al., 1994). The *cdc3-6* mutant grown at the restrictive temperature is defective in septum formation but not in cell elongation (Nurse et al., 1976), accumulating Calcofluor white-stained cell wall material at the site of septum formation (Ishiguro et al., 2001). In this mutant, Bgs1p was detected at the poles and/or coincident with many of the cell wall structures synthesized at the middle of the cell (Fig. 3). Cdc15p is essential for cell division. The mutants have impaired the actin ring formation or form unstable ring structures, and display disorganized actin patches during mitosis (Balasubramanian et al., 1998; Chang et al., 1996; Fankhauser et al., 1995). The *cdc15-140* mutants have thick or diffuse cell wall depositions at both sides of the cell, making aberrant structures resembling the septa but in which Bgs1p was never present. As the mutants may continue growing, Bgs1p was sometimes present at one cell pole. Part of Bgs1p also remained in ring or septum structures made during the temperature shift of the mutant and before expressing the thermosensitive phenotype (Fig. 3). Cdc11p (Balasubramanian et al., 1998; Krapp et al., 2001; Mitchison and Nurse, 1985; Nurse et al., 1976) and Cdc14p (Fankhauser and Simanis, 1993; Guertin et al., 2000) are essential for septum formation. The *cdc11-119* and *cdc14-118* mutations block cytokinesis but assembly of the actomyosin ring or accumulation of actin patches at late anaphase during mitosis or at the cell ends during interphase is not affected. In *cdc11-119* and *cdc14-118*, Bgs1p localized only to the poles, coincident with cell growth and new cell wall synthesis. Some mutant cells presented Calcofluor-stained ring or septum structures and *cdc14-118* also showed some diffuse cell wall synthesis at the middle of the cell, in a septum-like manner, but Bgs1p was never present in any of these structures (Fig. 3). Cdc16p is required for negative regulation of septum formation, by forming a two-component GAP for Spg1p GTPase (Cerutti and Simanis, 1999; Fankhauser et al., 1993; Furge et al., 1998). The *cdc16-116* mutant fails to turn off septation, making multiple septa in the cell (Minet et al., 1979). Bgs1p appeared at the poles and at many of the multiple septa (Fig. 3). In addition, Bgs1p localization in *cdc15-140* and *cdc16-116* was affected, increasing the fluorescence background inside the cell and making Bgs1p localization undetectable at longer incubations at the restrictive temperature (data not shown). In summary, the contractile ring is necessary but not sufficient for Bgs1p localization to the middle of the cell. The proteins that form part of the septation initiation network (SIN), such as Cdc11p and Cdc14p, are also necessary for its localization.

Next, we wondered whether the SIN proteins are necessary not only for stable Bgs1p localization to the medial ring, but also for Bgs1p displacement from the tips to the medial ring during mitosis. For that purpose, Calcofluor white and nuclei staining and GFP-Bgs1p were simultaneously analyzed in the same cells. In wild-type cells, Bgs1p was localized to both cell ends until the end of anaphase, when nucleus division is completed and the nuclei are separated at both cell poles. Next, Bgs1p disappeared from the poles and two GFP dots appeared at the middle of the cell. Finally, two Calcofluor-stained dots appeared overlapping the bright GFP signals (data not shown). If SIN signaling is not necessary for Bgs1p displacement from the tips, the SIN mutants will show post-anaphase Bgs1p displacement from the tips to the contractile ring and its reappearance at the tips when end growth resumes. Alternatively, if SIN signaling is necessary, Bgs1p will never be detected at the medial ring. Analysis of *cdc11-119* and *cdc14-118* mutants after 2, 4 and 6 hours of growth at the

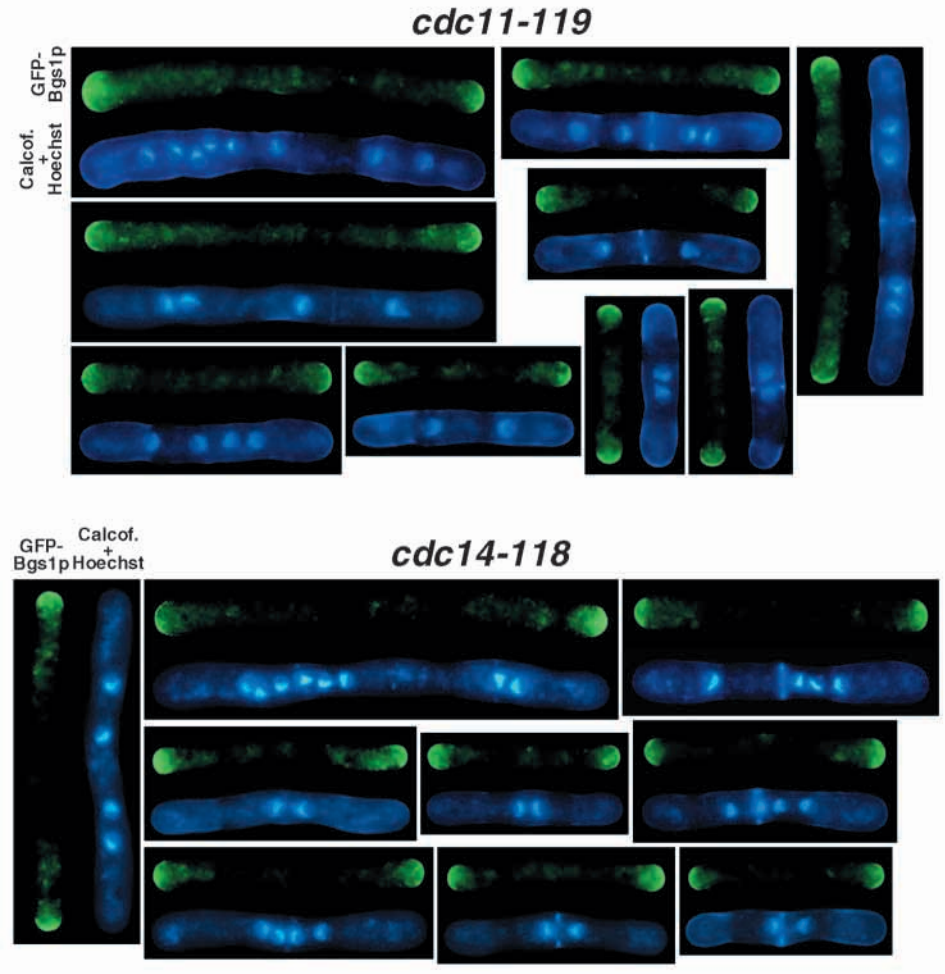


Fig. 4. Bgs1p does not localize to the contractile ring during or after mitosis in the septation initiation network mutants *cdc11-119* and *cdc14-118*. *GFP-bgs1*$^{+}$ *bgs1*Δ mutant cells were grown as in Fig. 2, shifted to 37°C and collected at different times (2.5, 3.5, 4.5 and 5.5 hours). Cells were ethanol fixed and analyzed for GFP and Calcofluor white/Hoechst staining. Cells representative of the mutant phenotypes at different times, including cells displaying a partial septum made before expressing the mutant phenotype, are shown.

restrictive temperature, containing two, four and eight nuclei after completion of one, two or three mitotic cycles, showed that Bgs1p never relocated to the medial ring, even in those cells that contained a partial septum structure (Fig. 4). Bgs1p was detected in the medial ring only at the beginning, before expressing the thermosensitive phenotype, and at late times, when the mutants escaped the phenotype and started to make complete septa (data not shown). Therefore, SIN signaling is required for Bgs1p displacement from the tips to the contractile ring.

The actin cytoskeleton is essential for correct Bgs1p localization

F-actin localizes to either the growing tips or the medial region of dividing cells (Marks et al., 1986), where new cell wall material is being made, and overlaps Bgs1p localization along the cell cycle. In addition, the *cps1-12* mutant showed genetic interactions with the actin mutant *cps8-188*. Therefore, we were interested in analyzing whether the actin cytoskeleton is involved in correct Bgs1p localization. In the *cps8-188* mutant grown at 37°C, the actin remains polymerized but the dots are randomly distributed throughout the cell and the middle ring structure is absent (Ishiguro and Kobayashi, 1996). The cells are rounded and enlarged, and some of them display a septum but do not divide. As the mutant increased its phenotype of random actin distribution, Bgs1p localization extended from poles or septum along the plasma membrane until it was located all around the cell (Fig. 5). The cells also presented areas of more intense GFP fluorescence in septum or cell periphery, coincident with regions of delocalized cell wall synthesis. Besides, the cells showed a substantial GFP-Bgs1p accumulation inside the cell. The *cps8-188* mutant grown at 32°C displays an intermediate phenotype of enlarged and aberrant cells that can still form aberrant contractile ring structures. GFP-Bgs1p was localized as a broad band in cell poles and septum simultaneously, and in regions of aberrant cell wall accumulation. Bgs1p was also present in abnormal contractile ring and septum structures, and part was dispersed inside the cell (Fig. 5).

The actin structures were disrupted in the profilin mutant *cdc3-6* after 5 hours of growth at 37°C. The cells showed aberrant septa and cell wall structures, but Bgs1p never localized there. Instead, it appeared delocalized in the cytoplasm as a low GFP background (data not shown). In summary, first Bgs1p localization is dependent on the presence of a polymerized actin cytoskeleton, and second the specific Bgs1p localization depends on the correct localization of polymerized actin.

Bgs1p localization is altered in the polarity establishment mutants *tea1-1* and *tea2-1*

Bgs1p not only localizes to the septum but also to the poles during both monopolar and bipolar growth. Tea1p and Tea2p are end markers, required to establish the correct growth site and therefore, they might also be responsible for Bgs1p localization during polarized growth. For that purpose, GFP-Bgs1p was analyzed in strains *tea1-1*, *tea2-1* and *tea2-1 cdc11-119* (Fig. 6). *tea1-1* and *tea2-1* mutants do not recognize the old end after mitosis, becoming branched by activating a new incorrectly positioned growing site, and the double mutant *tea2-1 cdc11-119* activates several new growing ends (Browning et al., 2000; Mata and Nurse, 1997; Verde et al., 1995). In the *tea1-1* and *tea2-1* mutants, Bgs1p appeared localized to the incorrect new growing end. During mitosis, the mutants also showed mislocalization of contractile ring and septum, often initiating ring contraction from three different points and dividing the cell into three compartments. In all

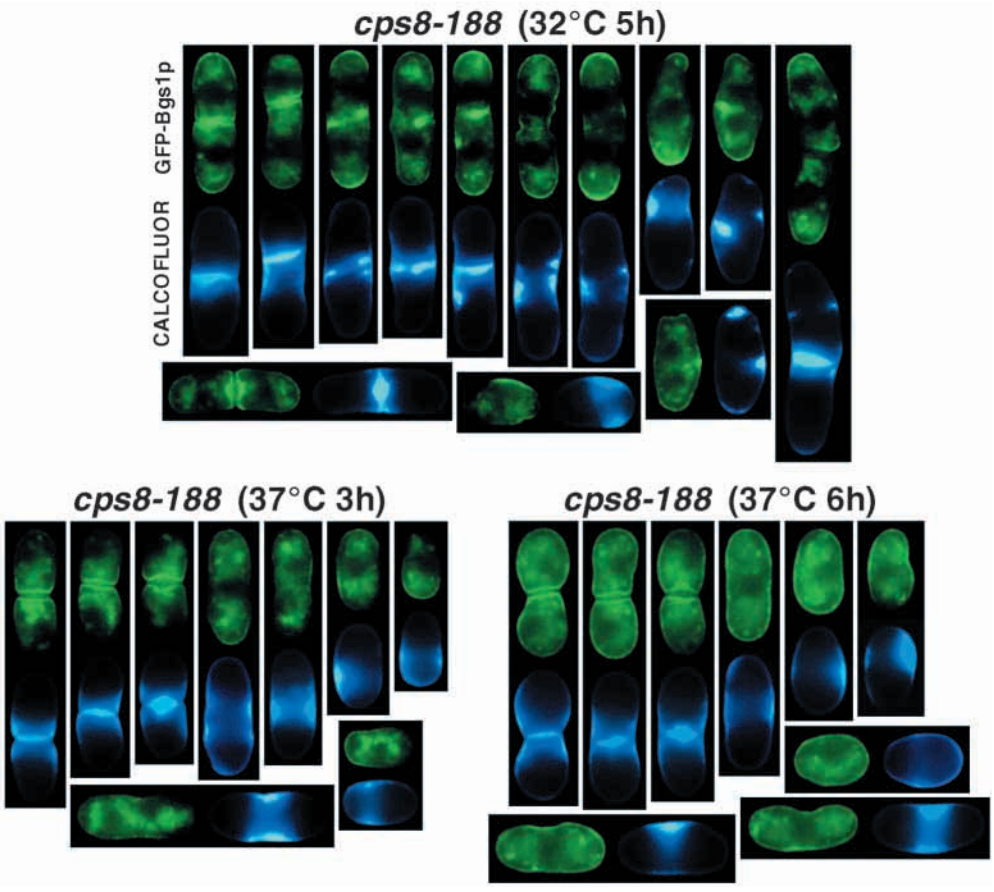


Fig. 5. Bgs1p localizes to all around the cell and septum and to regions of altered cell wall deposition in the actin mutant *cps8-188*. *GFP-bgs1*$^{+}$ *bgs1*Δ mutant cells were grown as in Fig. 2, shifted to 32°C for 5 hours or to 37°C for 3 or 6 hours, and examined for GFP and Calcofluor white staining. Cells representative of the different mutant phenotypes at each temperature and incubation time are shown.

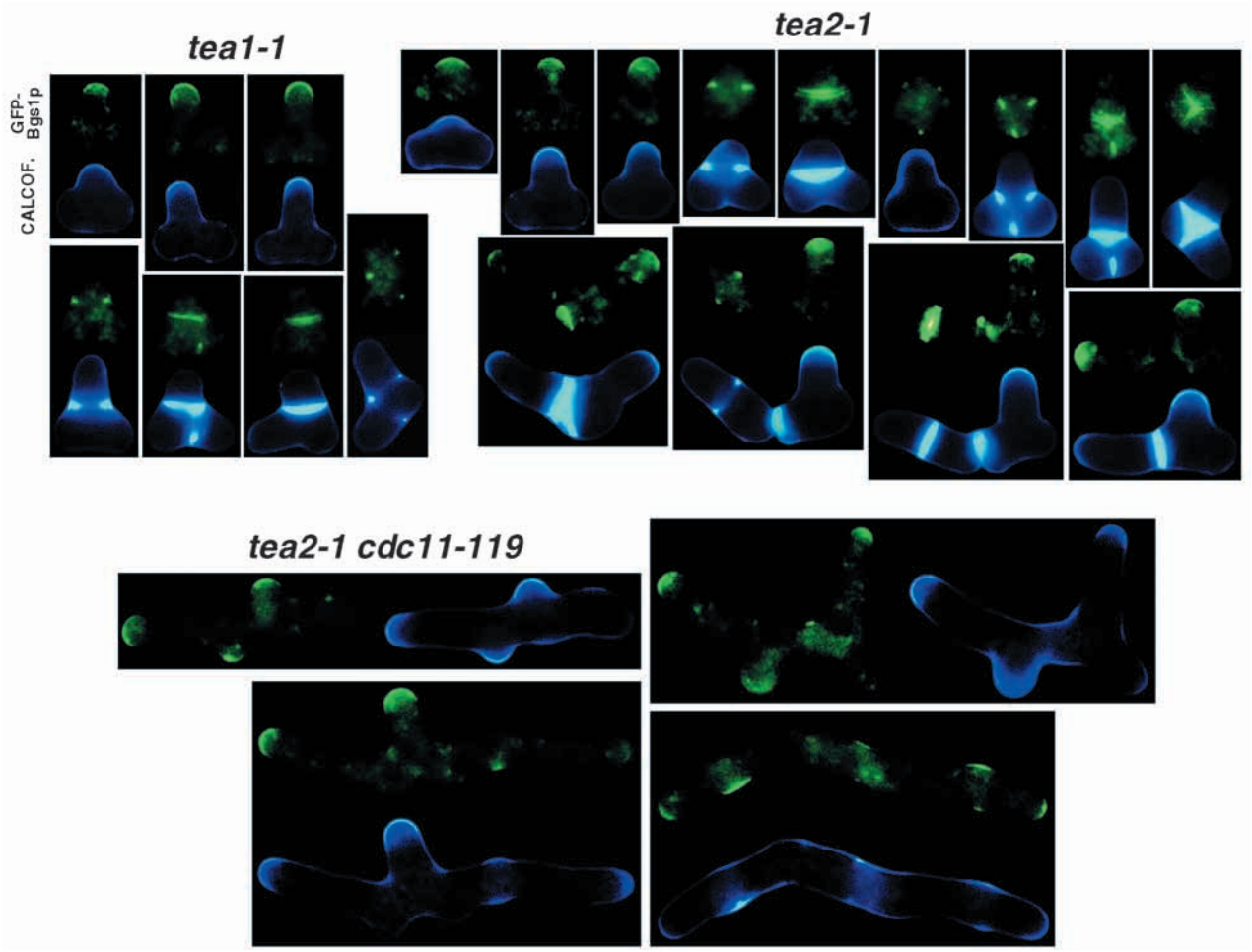


Fig. 6. Bgs1p localizes to the altered growing ends and septa in the end marker mutant strains *tea1-1*, *tea2-1* and *tea2-1 cdc11-119*. *GFP-bgs1*$^{+}$ *bgs1*Δ mutant cells were grown as in Fig. 2, shifted to 37°C for 6 hours (*tea2-1 cdc11-119*) or 8 hours (*tea1-1* and *tea2-1*), and examined for GFP and Calcofluor white staining. Cells representative of the different mutant phenotypes are shown.

cases, Bgs1p was polarized, appearing at the growing tips or in the altered septa (Fig. 6). In the *tea2-1 cdc11-119* mutant, Bgs1p localized to multiple new growing ends (Fig. 6).

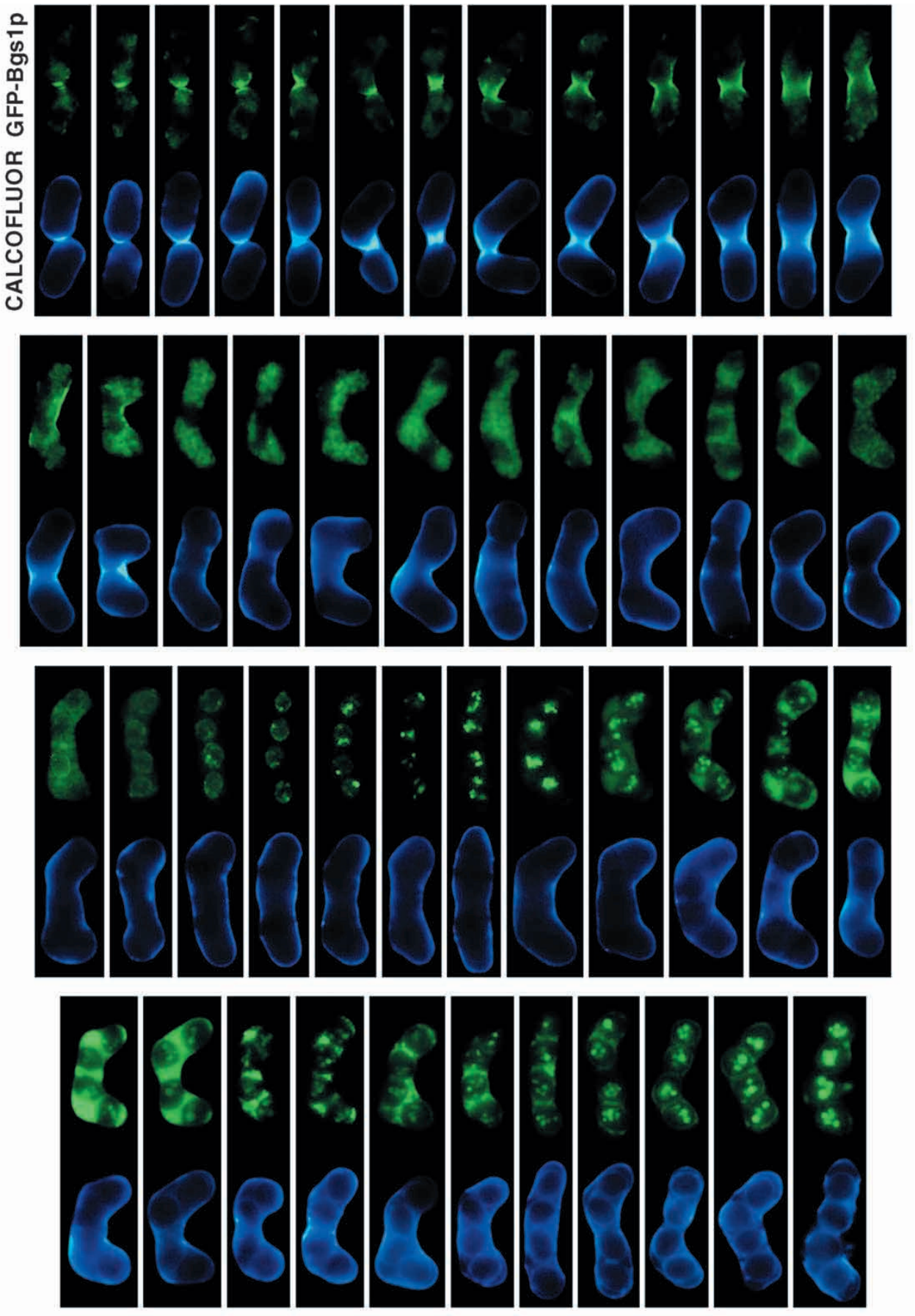
These results indicate that Bgs1p localization in these mutants remains polarized to the growing poles and septum, which are mislocalized due to the failure of the polarity system to establish and maintain the polarized growth along the long axis of the cell.

Bgs1p is also present and localized to the growing zones during mating, spore wall formation and spore germination

Bgs1p shows a localized pattern in all stages of vegetative growth. As it is present at every point where growth takes place, we wondered whether it might also be present at the cell wall growth sites during the sexual phase of the life cycle. Thus, Bgs1p localization was evaluated along the sporulation process in a homothallic *bgs1*Δ *GFP-bgs1*$^{+}$ h^{90} strain.

During the mating process, Bgs1p appears well localized to the mating projection of the cells (Fig. 7). Initially, Bgs1p is found in only one of both cells, even before any mating projection is observed. When the mating projection is present, Bgs1p is even more apparent. Then, the other cell begins to respond to the call of the first cell, and Bgs1p and the mating projection appears in the second cell. Then, the cell walls of both cells fuse and Bgs1p stays as a band in the septum that separates both cells. When the cells fuse and the septum disappears, Bgs1p appears as a wide band at both sides of the neck between both cells (Fig. 7); it then disappears first from one side of the neck-membrane and then from the other. At that time, Bgs1p is filling in all the cytoplasm, uniformly and with intense fluorescence, but is absent at the fused nucleus, which extends along the zygote and divides, giving rise to the four nuclei. While remaining in

Fig. 7. Bgs1p localization during the sexual phase of the life cycle suggests that it is involved in mating projection synthesis, cell fusion control and forespore and spore wall synthesis. Homothallic *GFP-bgs1*$^{+}$ *bgs1*Δ h^{90} cells grown at 28°C in EMM liquid medium until early-stationary phase were collected, transferred onto a SPA plate and incubated at 28°C. Samples were taken after 3, 5, 8, 24 and 48 hours, resuspended in EMM liquid medium containing 50 μg/ml Calcofluor white, and visualized for GFP and Calcofluor white staining. Cells and zygotes representative of each mating and sporulation step were selected and aligned to show a sexual phase progression.


CALCOFLUOR GFP-Bgs1p

the cytoplasm, Bgs1p begins to appear inside or associated with the membranes of the four well-defined oval or round shaped forespores (Fig. 7). Bgs1p disappears from the cytoplasm, remaining in the prospores and accumulating as very bright patches that will remain during the rest of spore maturation processes. Again, Bgs1p appears in the cytoplasm as uniform and extremely bright fluorescence. During all this process, Calcofluor white only shows the zygote shape, with no staining of prospore cell wall, although the prospores are perfectly distinguishable at phase contrast microscopy (data not shown). At this point, Calcofluor white begins to stain the spores inside the ascus, probably because the prospore is maturating its wall to become a spore (Fig. 7). Next, Bgs1p accumulates outside the spores as bright and random patches, usually close to the spore wall, and then, the general Bgs1p fluorescence all around the cytoplasm gradually disappears, until it only remains localized to the entire spore periphery and as bright patches inside the mature spores. At that time, the spores are completely mature and the envelope that forms the ascus will eventually lyse to release the spores (Fig. 7). All these observations suggest that Bgs1p function extends beyond the vegetative cycle. According to its specific localization pattern, Bgs1p might have a dual role during mating: first, helping the cell to grow and form the mating projection; and second, forming part of a security mechanism that compensates any excess of cell wall degradation during the cell fusion process. In addition, these observations also suggest that Bgs1p has multiple roles during sporulation, helping to synthesize both prospore and spore walls.

To analyze whether Bgs1p is also present during the spore germination process, germinating spores of homothallic *bgs1*Δ *GFP-bgs1*$^{+}$ h^{90} strain were examined (Fig. 8). In order to avoid any treatment with a cell wall hydrolytic enzyme complex that might alter the spore wall structure and perhaps modify Bgs1p localization, the spores were released by spontaneous lysis of the asci during a long incubation period in SPA solid medium. The spore analysis showed that Bgs1p is also involved in several germination steps (Fig. 8). Initially, the spores show an isotropic growth. Bgs1p reorganizes, causing the internal bright patches to disappear and become distributed uniformly in the spore periphery and weakly inside the spore (Fig. 8). Then, Calcofluor white staining shows the beginning of a polarized growth and Bgs1p increases its localization to that place. The spore elongates by a monopolar growth and Bgs1p is always present at this growth pole. When the spore stops growing, Bgs1p reorganizes to the middle of the cell, being first with the contractile ring and later in the spore septum. During cell division, Bgs1p remains between the spore and the new cell. Before division is completed, it localizes to the old end of the new cell, but remains in the septation pole of the spore, probably because the spore will reinitiate its growth at this pole, which resembles a vegetative cell.

Discussion

Cell wall synthesis and its regulation is a crucial morphogenetic process during cell growth and division in yeast and filamentous fungi (reviewed by Arellano et al., 1999; Cabib et al., 1998; Cabib et al., 2001). During the past years, a big effort has been made to understand how fission yeast establishes and regulates its polarized growth (Brunner and Nurse, 2000; Chang, 2001; Mata and Nurse, 1998; Sawin and Nurse, 1998; Verde, 1998), and the mechanism by which cytokinesis is regulated and coupled to the cell cycle (reviewed by Balasubramanian et al., 2000; Gould and Simanis, 1997; Guertin et al., 2002; Le Goff et al., 1999a; McCollum and Gould, 2001; Sawin, 2000). However, the knowledge about how fission yeast performs and regulates the synthesis of its main cell wall components during polarized growth and cytokinesis has scarcely evolved.

In this work, we present a series of genetic and localization results, which suggest that Bgs1p function is implicated in septum synthesis. We show that the *cps1-12* mutant interacts genetically with some *cdc* septation mutants. The double mutants displayed several aggravated phenotypes. Interestingly, some septation mutants did not show negative interaction with *cps1-12*, measured as increased sensitivity to Novozym 234. Perhaps the former class of septation mutants affects the cell wall integrity in a different way compared with the *cps1-12* mutant, whereas the latter class might be involved

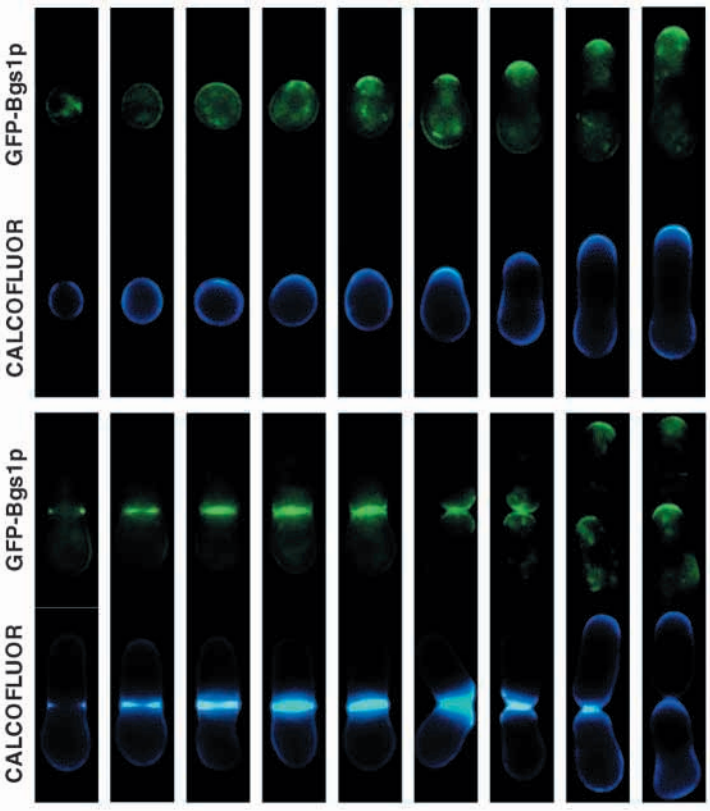


Fig. 8. Bgs1p localizes around the spore during isotropic growth, to the growing pole during spore germination, and to the contractile ring and septum during cytokinesis of the first cell. Homothallic *GFP-bgs1*$^{+}$ *bgs1*Δ h^{90} strain was grown and sporulated as in Fig. 7. Cells were incubated on SPA plates at 28°C for 7 days to allow most of the asci to spontaneously lyse and liberate the spores. The culture was checked to confirm that most of the spores were released. The spores were collected, incubated in YES liquid medium at 28°C, and samples were taken at 5 and 9 hours. Calcofluor white was added (50 μg/ml final concentration) and the spores were examined for GFP and Calcofluor white staining. Spores representative of each germination step were selected and aligned to show an ordered germination process.

in the Bgs1p pathway itself. However, additional experiments are needed to support this hypothesis. Another reason why some septation mutants did not show negative interaction with *cps1-12* could be the specificity of the *bgs1* mutant allele. In fact, similar results have been described for other *bgs1* mutant alleles and some *cdc* septation mutants, including *cdc* mutants that in our case show negative interaction and vice versa (Le Goff et al., 1999b; Liu et al., 1999). We also describe epistatic interactions between *cps1-12* and the *cdc* septation mutants. Similar epistatic interactions have been described for other *bgs1* mutant alleles (Le Goff et al., 1999b; Liu et al., 1999) but in these cases all the analyzed septation mutants were epistatic to the *bgs1* mutants and, therefore, Bgs1p function was not well localized as it could act after septum synthesis. In our case, we showed that *cdc16-116* is epistatic to *cps1-12*, which suggested that Bgs1p function is located in the period between the beginning and end of septum synthesis at cytokinesis. This result is consistent with the proposed function of Bgs1p as one of the *S. pombe* GS catalytic subunits, and in that case, Bgs1p would be responsible for septum cell-wall (1,3)β-D-glucan synthesis.

We were interested in studying Bgs1p localization along the cell cycle. For this purpose, we constructed a *bgs1*$^+$/*bgs1*Δ mutant strain that had the complete *bgs1*$^+$ ORF removed. Sporulation of this strain showed *bgs1*$^+$ is essential at least for spore germination. A similar result was obtained for a mutant with a partial disruption of the *bgs1*$^+$ gene (Liu et al., 1999). Germination of *bgs1*Δ spores results in spherical and enlarged cells that eventually will lyse (Liu et al., 1999). This phenotype points to the importance of Bgs1p localization for polarized cell growth, at least during spore germination. Although it is likely that *bgs1*$^+$ is also essential for vegetative cells, it has not been proven yet. In fact, repression of the *bgs1*$^+$ gene by the 81X version of the thiamine-repressible *nmt1*$^+$ promoter (pJG25) is not lethal in a *bgs1*Δ strain (see Materials and Methods). Nevertheless, we cannot discard the possibility of a residual *bgs1*$^+$ expression, high enough to maintain cell viability.

In order to avoid any artifact in Bgs1p localization studies, we tried to mimic the physiological state for Bgs1p. An integrated single copy of *GFP-bgs1*$^+$ gene, regulated by its own *bgs1*$^+$ promoter, made the *bgs1*Δ mutant revert to the wild-type. Therefore, GFP-Bgs1p is functional and expressed at physiological levels. During septum synthesis, Bgs1p localization is coincident with that of the contractile ring, appearing as bright fluorescence signals ahead of the newly synthesized septum cell wall. These results suggest that Bgs1p might be responsible for the synthesis of a specific lineal (1,3)β-D-glucan that constitutes the fission yeast primary septum (Humbel et al., 2001) just as CalS1p is responsible for the formation of the cell plate in plant cells, made of callose (Verma and Hong, 2001), in a process that mimics the synthesis of the primary septum in *S. cerevisiae*, made of chitin, by Chs2p (Schmidt et al., 2002). Nonetheless, *S. cerevisiae* might present some differences, as ring contraction and primary septum formation depend on each other (Schmidt et al., 2002). Initially, our results of Bgs1p localization indicate that its requirements are coincident with those of *S. cerevisiae*: Bgs1p localization depends on the presence of an actomyosin ring and on its contraction, promoted by the septation initiation network (SIN) (McCollum and Gould, 2001; Sawin, 2000). However, *cps1-12* mutant may apparently form normal septa when grown under certain conditions, although the cells are unable to separate. If the *cps1-12* mutant phenotype were due to the absence of Bgs1p function and if its function were the primary septum synthesis, this would mean that the medial ring can contract and a septum can be made, even in the absence of primary septum.

After submitting this manuscript for publication, another work describing Bgs1p localization appeared (Liu et al., 2002). The described localization data and conclusions are coincident with part of the results presented in our work. The authors reach the same conclusions about Bgs1p localization to the contractile ring and its dependence on F-actin and SIN proteins for its localization. Furthermore, it is stated that Bgs1p is not required for cell elongation and is visualized only in cells undergoing mitosis and cytokinesis. It is not detected in disc-like structures following assembly of the primary septum. In our case, Bgs1p is clearly detected in every process of cell wall growth. We do not know the exact reason why it was not detected by Liu et al., but it is probably due to one of the following possibilities. First, Bgs1p localization is very unstable and extremely sensitive to cell wall stress, as has been reported for the *S. cerevisiae* homologue Fks1p (Delley and Hall, 1999). In the studies by Liu et al., it is likely that the cells lost Bgs1p from the poles or septum during manipulation. In our experiments, despite careful handling of the cells, the GFP sometimes disappeared from the cell poles and accumulated inside the cell after two or three pictures or even at the beginning. Moreover, we noticed different degrees of Bgs1p stability: very unstable at the poles, more stable along the septum and very stable at the contractile ring. However, after ethanol fixation, Bgs1p localization seemed to be stable throughout the cell. Second, the GFP-Bgs1p fusion protein used by Liu et al. might display a different behavior. They did not describe whether the gene replacement was done in haploid or diploid strains. If the fusion protein is not completely functional, the gene replacement in haploid strains might promote gene duplication. In that case, wild-type Bgs1p could be more stable or have more affinity for growth poles than the fusion protein. Finally, this specific fusion protein might be less stable, making its detection outside the contractile ring more difficult.

Bgs1p localization in *tea* mutants suggests that it depends on the microtubule-dependent growth establishment. In addition, it has been demonstrated that actin is another key component directing Bgs1p localization, as it is for Ags1p, the putative (1,3)α-D-glucan synthase (Katayama et al., 1999). Disappearance of Bgs1p fluorescence from the poles at the time of cytokinesis and vice versa, favors the idea of a mechanism for a drastic and rapid Bgs1p recruitment to the medial ring zone at the time of septum formation and to the poles at the time of cell growth, coincident with the location of actin patches and cytoskeleton components at the same region of active cell-wall synthesis. In fact, it has recently been shown that a GFP fusion of the *S. cerevisiae* homologue Fks1p moves on the cell surface to the sites of cell wall synthesis. This Fks1p mobility is dependent on the movement of cortical actin patches and is necessary for correct cell wall synthesis (Utsugi et al., 2002).

It is likely that Bgs1p is an integral constituent of the plasma membrane. Therefore, its recycling or degradation is probably

channeled through endocytosis. Our observation of intermediate stages of cells with Bgs1p localized between cell poles and septum, and of growing cells with internalized Bgs1p, revealed the presence of lumpy and granular Bgs1p structures in the cytoplasm. This suggests that Bgs1p is processed by endocytosis rather than diffusing freely. If this were the case, it would be especially interesting to search for the final destination of Bgs1p. This processing might lead to complete degradation in the vacuole or lysosome, or to an alternative route of Bgs1p recycling, as has been described for *S. cerevisiae* chitin synthases Chs2p and Chs3p, respectively (Chuang and Schekman, 1996).

The presence of Bgs1p in the growing sites during the mitotic cycle is coincident with the localization of Rho1p GTPase, the GS regulatory subunit (Arellano et al., 1997). This colocalization of both proteins is consistent with the idea that Bgs1p is probably a GS catalytic subunit or forms part of a GS catalytic complex. However, it is still not known whether Rho1p has a function and/or localizes to the same sites during mating, sporulation and spore germination.

All this work suggests that the Bgs1p role is widely extended along the life cycle, probably involved in the synthesis of all or part of the cell wall (1,3)β-D-glucan. Alternatively, it might also have a cell wall repair or protection function during cell growth or zygote formation. This is consistent with the idea there are three Bgs1p homologues, which might have specific and separate functions during cell wall synthesis, as is the case for chitin synthesis in *S. cerevisiae* (Shaw et al., 1991). Supporting this idea, the Bgs2p homologue has been found to be essential for sporulation but not for vegetative growth (Liu et al., 2000a; Martin et al., 2000). Furthermore, *bgs3*$^{+}$ and *bgs4*$^{+}$ have already been cloned and both are essential genes (M. V. Martin, B. Garcia, E. Carnero, A. D. et al., unpublished; J. C. G. C., J. I., A. D. et al., unpublished). Moreover, *bgs4*$^{+}$ complements all *cwg1-1* mutant phenotypes (Ribas et al., 1991), which display reduced GS activity and cell wall (1,3)β-D-glucan (J. C. G. C., I. P. Martins, E. Carnero et al., unpublished). An appealing idea is that Bgs3p and Bgs4p would be responsible for other essential (1,3)β-D-glucans that form the cell wall and secondary septum, either by synthesizing different types of glucan or by displaying a differential localization, either spatial or temporal, along the cell cycle. This would also be the case for Bgs1p during sporulation. Bgs2p has been found to be essential for sporulation, but it does not imply that all the (1,3)β-D-glucan made in the spore wall is due to Bgs2p. In fact, a further Bgs1p study will be necessary to ascertain its specific role for spore wall formation.

S. pombe is the only described model system with four different essential Bgs proteins to apparently synthesize the same (1,3)β-D-glucan. What is the function and regulation of any of them? It is believed that they form part of the GS catalytic subunit but none contains the proposed UDP-glucose binding consensus R/K-X-G-G of glycogen synthases (Farkas et al., 1990; Furukawa et al., 1990). Instead, they all contain the proposed consensus RXTG (Inoue et al., 1996; Ishiguro et al., 1997), but more experimental work will be needed to prove their function as GS catalytic subunits as well as to ascertain the specific role of each Bgs subunit in the fission yeast life cycle.

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