Bem1p, a scaffold signaling protein, mediates cyclin-dependent control of vacuolar homeostasis in *Saccharomyces cerevisiae*

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How proliferating cells maintain the copy number and overall size of their organelles is not clear. We had previously reported that in the budding yeast *Saccharomyces cerevisiae* the G1 cyclin Cln3p is required for vacuolar (lysosomal) homotypic fusion and loss of Cln3p leads to vacuolar fragmentation. The Cdc42p GTPase is also required for vacuole fusion. Here we show that the scaffold protein Bem1p, a critical regulator of Cdc42p activity, is a downstream effector of Cln3p and the cyclin-dependent kinase (Cdk) Cdc28p. Our results suggest that Bem1p is phosphorylated in a Cdk-dependent manner to promote vacuole fusion. Replacing Ser72 with Asp, to mimic phosphorylation at an optimal Cdk-consensus site located in the first SH3 domain of Bem1p, suppressed vacuolar fragmentation in cells lacking Cln3p. Using in vivo and in vitro assays, we found that Cln3p was unable to promote vacuole fusion in the absence of Bem1p or in the presence of a nonphosphorylatable Bem1p-Ser72Ala mutant. Furthermore, activation of Cdc42p also suppressed vacuolar fragmentation in the absence of Cln3p. Our results provide a mechanism that links cyclin-dependent kinase activity with vacuole fusion through Bem1p and the Cdc42p GTPase cycle.

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scribed [Spellman et al. 1998]. Cln3p/Cdc28p phosphorylates Whi5p, a repressor of the G1/S transcription factor SBF, thereby releasing Whi5p from SBF and activating START transcription [Costanzo et al. 2004; de Bruin et al. 2004; Schaefer and Breeden 2004]. In addition, our earlier findings provided evidence for a novel function of Cln3p in vacuolar homotypic fusion, separate from its role in G1/S transcription and not shared by other G1 cyclins [Han et al. 2003].

A central polarity-establishment factor in a variety of organisms [from yeast to humans] is Cdc42p, a Rho-type small GTPase that orchestrates numerous processes necessary for polarization, such as septin and actin organization and membrane trafficking, in response to cell cycle transitions and environmental cues [Etienne-Manneville 2004; Irazoqui and Lew 2004]. Cdc42p membrane localization is not restricted to the plasma membrane but includes internal membranes, notably vacuolar membranes [Richman et al. 2004]. Furthermore, Cdc42p is one of several GTPases required for vacuolar homotypic fusion [Etzen et al. 2001; Muller et al. 2001]. Reorganization of vacuole-bound actin is needed for vacuolar homotypic fusion, and proteins of the Cdc42p-dependent processes necessary for actin remodeling are enriched on vacuolar membranes [Etzen et al. 2002]. Among numerous Cdc42p effectors and interacting proteins, the scaffold protein Bem1p is critical for proper Cdc42p activation [Irazoqui et al. 2003]. After the Cln3p/Cdc28p-mediated initiation of the G1/S transcription program, the burst of late G1-phase Cdk activity involving Cln1,2p/Cdc28p and Pcl1,2p/Pho85p (another cyclin/Cdk complex) triggers a pathway that leads to phosphorylation of Cdc24p. Cdc24p is a Cdc42p guanine nucleotide exchange factor (GEF). Once at the bud site Cdc24p binds Bem1p, and Cdc24p-dependent actin reorganization necessary for bud emergence takes place [Gulli et al. 2000; Bose et al. 2001; Moffat and Andrews 2004].

In this report we show that Bem1p is required for vacuolar homotypic fusion and that the overall vacuolar compartment in bem1Δ cells is enlarged but fragmented, similar to cln3Δ cells. Furthermore, Bem1p is phosphorylated in a Cln3p-dependent manner at Ser72. A single S72D substitution in Bem1p (which mimics phosphorylation) or overexpression of CDC42 or CDC24 suppresses the vacuolar fragmentation of cln3Δ cells. Conversely, substituting Ser72 in Bem1p with Ala blocks the ability of Cln3p to promote vacuole fusion, in vivo and in vitro. The results we report here suggest that Cln3p impacts on vacuolar homeostasis through Bem1p and Cdc42p.

Results

Cln3p is required for an early step of vacuolar homotypic fusion

We had previously shown that Cln3p is required for vacuolar homotypic fusion [Han et al. 2003]. Vacuolar homotypic fusion is composed of several distinct sequential steps: priming, docking, and fusion (Wickner and Haas 2000). To study the kinetics of the reaction, order of addition experiments can be done, whereby the fusion reaction progressively becomes resistant to the addition of various inhibitors, once the inhibitor-sensitive step has been completed. For example, placement on ice blocks all steps and thus results in a “late-stage” inhibition profile, which is also similar to the GTP-γ-S inhibition profile. There are several GTPases involved in more than one step, including the last fusion step, which leads to a late-stage inhibition profile by GTP-γ-S [Etzen et al. 2000, 2001]. On the other hand, the ionophore FCCP blocks the docking step and it does not significantly inhibit the overall reaction if added late.

Using this approach, we decided to determine the stage of the overall reaction that requires Cln3p for its completion, with cytosol from cells expressing HA-tagged Cln3p. We then added an anti-HA antibody as an inhibitor at various time points during the reaction and measured the overall fusion activity at the end of the 90-min incubation period (Fig. 1). This experiment was

Figure 1. Cln3p is required for an early step during vacuole fusion. (A) A schematic of the experimental strategy is shown. (B) Fusion reactions were incubated at 27°C with cytosol from a CLN3-HA strain. At the indicated times of addition (t1) shown on the X-axis, aliquots were removed and added to tubes containing anti-HA Ab, FCCP, GTP-γ-S, or buffer and incubated at 27°C. The activity from the buffer-containing tube at 27°C was set as 100% fusion activity. Another buffer-containing tube was incubated on ice and its activity was set as 0% fusion activity. As a control, aliquots of reactions with cytosol from the untagged HA tagged isogenic strain were also added at various time points to tubes containing the anti-HA Ab or buffer, and treated in the same manner as above. Reactions were incubated for a total of 90 min and then assayed for alkaline phosphatase, the relative values of which are shown on the Y-axis. (C) Fusion reactions were performed as described in Materials and Methods, using cytosol from cln3Δ cells and recombinant proteins from bacteria as indicated. Fusion was evaluated colorimetrically and the average and standard deviation of the relative fusion activities from at least three independent experiments is shown.
Bem1p impacts on vacuolar biogenesis, downstream of Cln3p

To understand Cln3p’s vacuolar function, we reasoned that one of the numerous known regulators of homotypic fusion might be targeted by Cln3p. Possible downstream effectors of Cln3p/Cdc28p in vacuolar homotypic fusion may display the following properties: Loss-of-function mutations should lead to vacuolar and overall cell size enlargement, loss-of-function mutations should lead to vacuolar fragmentation, and the putative effectors should be Cdk targets. Recent genome-wide studies focused on these properties, namely, altered cell size [Jorgensen et al. 2002, Zhang et al. 2002], fragmented vacuolar morphology [Seeley et al. 2002], and proteins that are phosphorylated by Cdc28p in whole-cell extracts [Ubersax et al. 2003]. There are >100 gene products in each data set (Fig. 2A). For example, there were 181 proteins that were significantly phosphorylated by Clb2p/Cdc28p (with a P-score ≥ 2, which is the logarithm of the amount of phosphate incorporated per nanogram of protein). Yet, only Cln3p itself and Bem1p were present in all three data sets (Fig. 2A).

Using the in vitro fusion assay, we found that reactions with cytosol from BEM1-TAP cells in the presence of inhibitory rabbit IgG (which targets the TAP domain) had significantly reduced fusion activity (Fig. 2B). Loss of Bem1p or Cln3p also leads to vacuolar fragmentation in ∼40–60% of the cells [see Fig. 3A,B; Seeley et al. 2002, Han et al. 2003]. Furthermore, cells lacking BEM1 were larger overall (∼40%) [Figs. 2, 3] than wild-type cells and, importantly, their vacuolar compartment was also disproportionately enlarged (about twofold) [Figs. 2, 3]. Finally, Cdc28p readily phosphorylated Bem1p [P-score = 3.4] in the phosphorylation assays by Ubersax et al. [2003]. Consequently, we decided to evaluate Bem1p as a putative Cln3p/Cdc28p effector in vacuolar biogenesis.

To test whether Cln3p might mediate its effects in vacuolar biogenesis through Bem1p, we measured the overall cell size and vacuolar size of CLN3 and BEM1 mutant combinations and also microscopically examined their vacuolar morphology. Loss of Cln3p or Bem1p leads to cellular and vacuolar enlargement and vacuolar fragmentation (Fig. 3). Conversely, cells expressing a stabilized form of Cln3p from the dominant gain-of-function CLN3-2D allele are smaller overall [Cross 1988], and their vacuolar compartment is also smaller [see Fig. 3A, Han et al. 2003]. However, the CLN3-2D allele was unable to reduce cell and vacuole size in the absence of Bem1p [Fig. 3A]. Furthermore, combined loss of Bem1p and Cln3p did not lead to an additive cellular or vacuolar enlargement or vacuolar fragmentation [Fig. 3B]. These results are consistent with the idea that Bem1p is required for Cln3p’s effects on cell and vacuole size, and that Bem1p and Cln3p do not affect vacuolar biogenesis through separate independent pathways.
Bem1p is phosphorylated in a Cdk-dependent manner

It has been previously mentioned in an earlier report that Bem1p might be a phosphoprotein, because phosphatase treatment leads to faster migration of Bem1p during SDS-PAGE [Leeuw et al. 1995]. Indeed, immunoprecipitated TAP-tagged Bem1p migrated faster upon phosphatase treatment [Fig. 4A]. Bem1p has four potential Cdk phosphorylation sites: two [ST]-P-X-[KR] sites at Thr51 and Ser72 and two [ST]-P sites at Thr26 and Ser60, respectively. As a first step toward establishing possible Cdk-mediated phosphorylation events, we asked whether an antibody directed against phosphorylated [ST]-P recognized Bem1p immunoprecipitated from cell extracts. From these cell extracts we sequentially immunoprecipitated Bem1p-TAP using IgG and calmodulin beads [Fig. 4B]. The immunoprecipitated protein was readily recognized by the PAP reagent, via the protein A domain [Fig. 4B]. To use the anti-phospho-[ST]-P antibody, after immunoprecipitations with IgG beads, we cleaved the immunoprecipitated product with TEV protease to remove the protein A part of the TAP epitope. Immunoblotting with the anti-phospho-[ST]-P antibody recognized the cleaved Bem1p-CBP [which now lacks the protein A part of the TAP tag] [Fig. 4B]. Recognition by the anti-phospho-[ST]-P antibody was phosphorylation dependent, because the signal was significantly reduced after treatment with phosphatase [Fig. 4C]. When normalized for Bem1p levels using an antibody that recognizes the remaining part of the TAP tag after TEV cleavage, treatment with phosphatase removed >70% of the signal [Fig. 4C, bottom].

To further evaluate whether Cdc28p activity contributes to Bem1p phosphorylation, we used cdc28-as1 cells, which express an engineered version of Cdc28p that is inhibited by the ATP analog 1NM-PP1 [Bishop et al. 2000]. These cells were then transformed with a low-copy centromeric plasmid carrying cMyc-tagged Bem1p [Irazoqui et al. 2003]. Upon treatment with the inhibitory drug, the mobility of Myc-tagged Bem1p increased on SDS-PAGE, consistent with dephosphorylation [Fig. 4D]. No such effects were seen upon treatment with DMSO or in the isogenic strain that does not carry the engineered cdc28-as1 allele [Fig. 4D]. We had previously reported that shifting temperature-sensitive cdc28-1 cells to their nonpermissive temperature led to vacuolar fragmentation [Han et al. 2003]. To further confirm that Cdc28p-associated kinase activity is required for vacuole homotypic fusion, we performed the in vitro fusion reaction using cytosol from cdc28-as1 cells, and we found that upon treatment with the inhibitory drug, fusion activity was reduced to ∼40% of the activity when DMSO alone was added [Fig. 4E]. Together, all of the above results support the hypothesis that Bem1p is phosphorylated in a Cdc28p-dependent manner in vivo.

Ser72 is critical for Bem1p’s role in vacuolar homeostasis

We then examined whether the Bem1p potential Cdk phosphorylation sites are conserved in other Saccharomyces species [Clifton et al. 2003; Kellis et al. 2003] and in a couple of Candida species for which genome infor-
Figure 4. Bem1p might be phosphorylated in a Cdk-dependent manner. [A] Schematic of the TAP-tagged Bem1p. Bem1p-TAP was isolated from cells carrying a chromosomal TAP-tagged BEM1 copy (BEM1-TAP, strain 7499374) [see Table 1] by immunoprecipitation using IgG and treated with calf intestinal phosphatase (CIP) as indicated. The samples were then processed for SDS-PAGE and immunoblotting with the PAP reagent. (B) Bem1p-TAP was isolated by tandem immunoprecipitations using IgG and calmodulin beads. The IgG-immunoprecipitated material was treated with TEV protease as indicated, before immunoprecipitations with calmodulin beads. Immunoblotting was done using the indicated antibodies. [C] The immunoprecipitated samples from Bem1p-TAP cell extracts were treated as in B and also with λ-phosphatase (λ-PPase) and phosphatase inhibitor (PPase-[I]) as indicated. The antibodies used for immunoblotting are shown on the right, and the relative ratios of the signal intensities are shown at the bottom. (D) Extracts from CDC28+/cdc28-as1 cells carrying cMyc-tagged Bem1p on a low-copy plasmid [plasmid pDLB2226] [see Table 2] were prepared using a urea extraction buffer and analyzed by SDS PAGE and immunoblotting with an anti-Myc antibody. The cells were treated with DMSO alone or 1 NM-PP1 (at 5 µM) for the indicated times. [E] Vacuole fusion activity using cytosol from cdc28-as1 cells. DMSO alone or 1 NM-PP1 (at 5 µM for 15 min) was added as indicated. Fusion was evaluated colorimetrically and the average and standard deviation of the relative fusion activities from at least three independent experiments is shown.

mation is available [Fig. 5A]. Not surprisingly, all the sites are conserved in the sensu stricto Saccharomyces species [S. mikatae, S. kudriavzevii, S. paradoxus, and S. bayanus]. S. castellii is a more distantly related sensu lato Saccharomyces species, and alignment of Bem1p to its ortholog in this species is likely to be more useful [Cliften et al. 2003]. In S. castellii only positions 26 and 72 are conserved, while in the two Candida species positions 51 and 72 are conserved [Fig. 5A]. The extreme N terminus of the C. albicans Bem1p does not show significant conservation with the S. cerevisiae Bem1p, and the highlighted position may not correspond to Ser51. Overall, the only conserved site in all of these cases is Ser72 [Fig. 5A], which is a preferred [ST]-P-X-[KR] Cdk consensus site. Ser72 also represents the first amino acid residue of the SH3-1 domain, which spans from position 72 to 132, based on PROSITE software predictions [Gattiker et al. 2002].

To test whether position 72 might be targeted for Cdk-mediated phosphorylation, we replaced Ser72 with Ala [to abolish phosphorylation] or Asp [to mimic phosphorylation] and evaluated the vacuolar morphology of bem1Δ or cln3α cells expressing these Bem1p mutants from low-copy plasmids as C-terminal cMyc fusions [Fig. 5B]. The S72A and S72D mutants were expressed at levels similar to the wild-type cMyc-tagged Bem1p, and their subcellular localization was indistinguishable from wild type [data not shown]. We noticed that introduction of the otherwise wild-type cMyc-tagged Bem1p on a low-copy centromeric plasmid only partially suppressed the vacuolar fragmentation of bem1Δ cells [Fig. 5B]. The plasmid is clearly functional because it does suppress the bud emergence defect of bem1Δ cells (see Fig. 7, below; Irazoqui et al. 2003). The lack of strong suppression of the vacuolar fragmentation might be due to the relative underexpression of cMyc-tagged Bem1p from this plasmid compared with endogenous levels [Irazoqui et al. 2003]. Consistent with this interpretation, cMyc-tagged Bem1p expressed from a high-copy (2µ) plasmid at levels no more than twofold higher compared with endogenous Bem1p [Irazoqui et al. 2003] showed strong suppression of the vacuolar fragmentation of bem1Δ cells [Fig. 5D, below]. Nonetheless, it is important to note that the fact that wild-type cMyc-tagged Bem1p from a low-copy plasmid only partially suppressed vacuolar fragmentation conveniently allows one to evaluate the effects of the S72D substitution even in bem1Δ [but CLN3+] cells. It was clear that S72D Bem1p suppressed vacuolar fragmentation better than wild-type Bem1p, and, conversely, the S72A Bem1p mutant did not suppress at all the vacuolar fragmentation of bem1Δ cells [Fig. 5B,C]. Because the S72A mutant is functional in bud emergence (see below, Fig. 7), the lack of suppression in vacuolar fragmentation is unlikely to be due to the production of a nonfunctional unfolded protein. Finally, introduction of the S72D, but not the S72A, Bem1p mutant in cln3α cells also significantly suppressed their vacuolar fragmentation [Fig. 5B,C].

To further test the role of Ser72, we overexpressed
blocks the ability of Cln3p to promote vacuole fusion in vivo. We then performed in vitro fusion reactions using cytosol from cells lacking both Cln3p and Bem1p, supplemented with various combinations of recombinant Cln3p, Bem1p, Bem1p-S72A, and Bem1p-S72D [Fig. 5E]. The highest activity was observed upon addition of Bem1p-S72D with or without Cln3p, and the lowest upon addition of Bem1p-S72A. Also, addition of Cln3p alone or with Bem1p-S72A did not increase fusion activity [Fig. 5E]. Therefore, these in vitro results completely support our in vivo evidence that Cln3p requires Bem1p to promote fusion. Furthermore, the S72A substitution in Bem1p blocks fusion, while the S72D mutation promotes it. Interestingly, addition of wild-type Bem1p also increased fusion activity in the absence of Cln3p, albeit not to the same level as Bem1p-S72D. Thus, it is possible that in the absence of Cln3p, when large amounts of exogenous Bem1p are added, Bem1p can still be modified in these extracts, perhaps by other cyclin/Cdk complexes. Taken together, our results from the in vitro fusion assays are in very good agreement with our in vivo evidence, and suggest that Cln3p and Bem1p play direct roles in vacuole fusion, with Bem1p acting downstream of Cln3p.

Overall, the evidence supports the notion that the conserved Ser at position 72 in Bem1p might be targeted for Cdk-mediated phosphorylation, and that the phosphorylated form promotes vacuolar homotypic fusion. It is possible that the other sites may also be phosphorylated. However, because the S72D mutant significantly suppressed the vacuolar fragmentation of cln3Δ and bem1Δ cells in vivo and in vitro, while the S72A mutant did not, it is reasonable to conclude that even if additional sites are phosphorylated, phosphorylation of Ser72 has the most significant biological consequences for vacuolar biogenesis.

We then used 2D gel electrophoresis to better resolve putative Bem1p isoforms [Fig. 6A]. Indeed, an acidic isoform present in wild-type Bem1p [Fig. 6A, top panel] was absent in cells lacking Cln3p [Fig. 6A, middle panel] or in cells carrying Bem1p-S72A [Fig. 6A, bottom panel]. We also noticed that Bem1p-S72A migrated slightly faster than wild-type Bem1p in standard one-dimensional SDS-PAGE, similar to wild-type Bem1p in cln3Δ cells [Fig. 6B]. Together, these results strongly suggest that Bem1p is phosphorylated at Ser72 in a Cln3p-dependent manner in vivo.

Finally, we examined whether Bem1p expressed in bacteria could be phosphorylated in vitro. For this experiment we overexpressed CLN3 from a high-copy plasmid carrying epitope-tagged CLN3 under the control of a galactose-inducible promoter. It has been previously shown that overexpression of Cln3p allows for the recovery of detectable Cln3p-associated kinase activity against histone H1, and that most of that activity is Cdc28p-dependent [Tyers et al. 1992, 1993]. Indeed, the immunoprecipitated Cln3p had associated kinase activity against recombinant Bem1p, albeit to significantly lower levels compared with histone H1 [Fig. 6C]. We also performed similar reactions using commercially avail-
Opposing role of Ser72 in bud emergence

Since Bem1p was originally identified for its role in bud emergence (Bender and Pringle 1991), we next examined whether our Bem1p Ser72 mutants have phenotypes associated with bud emergence. To quantify the bud emergence defect we measured the frequency of unbudded cells containing spindles. The S72A mutant was fully functional in bud emergence (Fig. 7), in contrast to its total lack of complementing activity in vacuole fusion (Fig. 5). Interestingly, the S72D substitution did not fully complement the bud emergence defect of bem1Δ cells (Fig. 7). Thus, while phosphorylation of Ser72 promotes vacuole fusion, it is not required for and it might even negatively affect bud emergence.

Overexpression of Cdc42p or Cdc24p suppresses the vacuolar fragmentation of cln3Δ cells

We next examined whether Cln3p’s requirement for vacuolar homotypic fusion might reflect perturbations of Cdc42p activity, since Bem1p is a well-established regulator of Cdc42p, and Cdc42p is also required for the docking step of vacuolar homotypic fusion (Eitzen et al. 2001; Muller et al. 2001). To perhaps bypass the requirement for Cln3p, we activated the Cdc42p GTPase cycle by overexpressing wild-type CDC42, or its exchange factor CDC24, in cln3Δ cells and evaluated their vacuolar morphology. Remarkably, overexpression of CDC42 or CDC24 completely suppressed the fragmented vacuolar morphology of cln3Δ cells (Fig. 8A,B). On the other hand, overexpression of CDC42 did not significantly suppress the vacuolar fragmentation of bem1Δ cells (Fig. 8B). CDC24 overexpression weakly suppressed the vacuolar fragmentation in bem1Δ cells (Fig. 8B). Overall, overexpression of Cdc42p, in the presence of Bem1p, is sufficient to bypass the requirement for Cln3p in vacuole fusion.

Furthermore, in the in vitro fusion assay, addition of Cdc42p did not rescue the fusion defect of bem1Δ cytosolic extracts (Fig. 8C), suggesting again that Bem1p is required for Cdc42p to promote fusion. Interestingly, when the extracts were supplemented with Bem1p-S72A together with Cdc42p there was an increase in fusion activity (~60%) [Fig. 8C], but not to the same extent as when the extracts were supplemented with Bem1p-S72D (see Fig. 5E).

We next examined several other Bem1p amino acid substitutions at positions that are important for Bem1p’s biological roles (Irazoqui et al. 2003) for their ability to suppress vacuolar fragmentation of bem1Δ cells [Fig. 8D]. For example, a P208L substitution affects interactions with various effectors and scaffolds, P355A inter-
indicated vacuolar morphology is shown. The percentage of cells with the examined microscopically. The number of cells examined is medium, and allowed to grow for another 4 h before they were medium containing FM4-64 for 1 h, washed, resuspended in fresh growing cells of the indicated genotype were transferred in me-

Figure 8. CDC42 or CDC24 overexpression suppresses the vacuolar fragmentation of cln3Δ cells. [A] Diploid cln3Δ cells [BY4743 background] were transformed with high-copy plasmids as indicated. The transformants were then stained with FM4-64 to observe their vacuolar morphology and photographed through phase optics [top panels] and by fluorescence [bottom panels]. [B] The same analysis as in A was also done for bem1Δ and wild-type (WT) cells, and the percent of cells with vacuolar fragmentation and the number of cells scored in each case [n] is shown. [C] Fusion reactions were performed as described in Ma-
terials and Methods, using cytosol from bem1Δ cells and recombinant proteins from bacteria as indicated. Fusion was evaluated colorimetrically and the average and standard deviation of the relative fusion activities from at least three independent experiments is shown. [D] Homozygous diploid bem1Δ cells [BY4743 background] were transformed with the empty high-copy vector or carrying wild-type or mutant cMyc-tagged Bem1p as indicated. The transformants were then stained with FM4-64 to observe their vacuolar morphology by fluorescence microscopy. The percent of cells with vacuolar fragmentation and the number of cells scored in each case [n] is shown. [E] Cells lacking Bem1p are not defective in vacuolar segregation. Exponentially growing cells of the indicated genotype were transferred in me-
dium containing FM4-64 for 1 h, washed, resuspended in fresh medium, and allowed to grow for another 4 h before they were examined microscopically. The number of cells examined is shown in parentheses. The scored cells had a bud diameter ≤0.4 of the mother cell's diameter. The percentage of cells with the indicated vacuolar morphology is shown.

typic fusion. To varying degrees, the other Bem1p mut-
tants also significantly suppressed vacuolar fragmentation of bem1Δ cells [Fig. 8D]. Notably, the weakest suppression, albeit still significant, was observed with the P208L substitution [Fig. 8D], which affects protein–

Discussion

In this study we present experiments that link the G1 cyclin Cln3p with vacuole fusion, through Bem1p and the Cdc42p GTPase. We discuss our findings in the context of the known roles of these proteins and how they might affect vacuolar homeostasis.

We were initially led to Bem1p because bem1Δ cells are large and their vacuolar compartment is also enlarged and fragmented, similar to the situation in cln3Δ cells [Fig. 2; Han et al. 2003]. The experiments we report here suggest that Bem1p is phosphorylated in a Cdk-dependent manner. The biological significance of Ser72 phosphorylation is underscored by the fact that a substitution to Asp that probably mimics phosphorylation suppresses vacuolar fragmentation, while an Ala substitution does not [Fig. 5E]. Our data strongly point to the link between Cln3p and modification of Ser72 [Fig. 6]. In vitro, however, it appears that multiple Cdk complexes can phosphorylate Bem1p. Addition of Bem1p from bacteria can still promote vacuole fusion in vitro, in the absence of Cln3p [Fig. 5E], suggesting that other cyclin/Cdk com-

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cell cycle and we have previously reported that loss of Cln3p leads to mild defects in vacuolar segregation [Fig. 8E, Han et al. 2003], but there are no such defects in bem1Δ cells [Fig. 8E]. Thus, although the mechanism we describe here linking Cln3p with Bem1p certainly impacts on the “steady-state” overall vacuolar homeostasis, it may not necessarily impart an oscillatory feature to it.

On the other hand, Cln3p’s vacuolar roles strongly affect the cell size phenotypes associated with CLN3 mutations (see Fig. 3, Han et al. 2003). As we have discussed previously [Han et al. 2003], this does not extend to all cell size mutants. Vacuolar enlargement is usually accompanied by cellular enlargement [Efe et al. 2005] but the converse is not necessarily true and not all large cell size mutants have large vacuoles. This is exemplified in double mutant cln1ΔΔ cells, which are large overall although they have small vacuoles [Han et al. 2003]. A general correlation between vacuolar fragmentation and cell size also does not seem to exist [Fig. 2A]. All these observations suggest that Cln3p’s role in vacuole fusion is separate from and probably does not impact on the other function of Cln3p in accelerating initiation of DNA replication.

How might cyclin-dependent phosphorylation of Bem1p impact on vacuole fusion? Cdc42p is involved in actin dynamics [Etienne-Manneville 2004; Irazoqui and Lew 2004] and vacuole fusion [Eitzen et al. 2001; Muller et al. 2001]. Importantly, the temporal requirement of Cln3p or Cdc42p for fusion appears to be similar. Loss of either Cln3p or Cdc42p leads to vacuolar fragmentation, but once the docking step of fusion is completed neither Cln3p [Fig. 1] nor Cdc42p [Eitzen et al. 2001; Muller et al. 2001] are required anymore for fusion. While it is clear that noncytoskeletal actin is found on the surface of vacuoles and remodeling of this actin pool is required for vacuole fusion [Eitzen et al. 2002], it is not known how it contributes to fusion. This requirement is distinct from the actin cytoskeleton-dependent processes of vacuole segregation [Weisman 2003; Pruyne et al. 2004]. Since we find no evidence for a defect in vacuolar segregation in bem1Δ cells [Fig. 8D], there is no reason to think that the cyclin-dependent phosphorylation of Bem1p we describe here affects this process. Consequently, our data placing Bem1p downstream of Cln3p [Figs. 3, 5] and the ability of Cdc42p to suppress vacuole fragmentation in cln3ΔΔ cells [Fig. 8] probably reflect the noncytoskeletal role of actin for vacuole fusion that has been proposed by the Wickner and Mayer laboratories [Eitzen et al. 2001, 2002; Muller et al. 2001].

Organelle-bound actin could also act as a fusion barrier, which must transiently disassemble for vesicles to dock, while later promoting actin assembly is thought to somehow help the docked vesicles to finally fuse [Eitzen 2003]. Based on chemical inhibition experiments, it appears that the actin depolymerizing drug latrunculin B inhibits the last fusion step of vacuole homotypic fusion [Eitzen et al. 2001]. Interestingly, the F-actin binding and stabilizing drug jasplakinolide inhibited the docking step of fusion only, and not the last latrunculin B-sensitive step [Eitzen et al. 2001]. Given that Cln3p [Fig. 1] and Cdc42p [Eitzen et al. 2001; Muller et al. 2001] are not required after the docking step of fusion, the fact that Ser72 of Bem1p has distinct and even opposing roles in vacuole fusion versus bud emergence [Figs. 5, 7], and the fact that other Bem1p mutants that affect bud emergence do not affect vacuole fusion [Fig. 8], it is possible that the effects we are observing reflect the “actin as a barrier” model of fusion [Eitzen 2003].

Does cyclin-dependent phosphorylation of Bem1p affect other Bem1p-regulated processes? The S72A substitution is incapable of suppressing vacuolar fragmentation in bem1Δ cells, but it is still fully active in bud emergence [Figs. 5, 7]. On the other hand, the S72D substitution fully suppresses vacuole fragmentation, but it only partially suppresses bud emergence [Figs. 5, 7]. These results suggest that Bem1p has distinct roles in bud emergence and vacuole fusion. While presumably locking Ser72 to the phosphorylated state [in the S72D mutant] suffices to suppress vacuolar fragmentation [Fig. 5], it might adversely affect bud emergence [Fig. 7].

The notion that Bem1p’s roles in vacuole fusion and bud emergence may be distinct is also suggested by the fact that other Bem1p mutants [for example, the K482A mutant] that were shown to be unable to suppress bud emergence defects [Irazoqui et al. 2003] retain full complementing ability in vacuole fusion [Fig. 8D]. Even the P208L mutant, which cannot complement bud emergence [Irazoqui et al. 2003], partially suppressed vacuolar fragmentation [Fig. 8D]. The P208L mutant affects interactions between the SH3-2 domain of Bem1p with various effectors, including the Cla4p kinase. Interestingly, loss of Cla4p leads to vacuolar fragmentation [Secley et al. 2002], and it is possible that interactions mediated by the SH3-2 domain of Bem1p might be important for vacuole fusion, explaining the incomplete suppression of vacuolar fragmentation we got from the P208L mutant [Fig. 8D].

In any case, since the actin remodeling necessary for vacuole fusion is not the same as the rearrangement of the actin cytoskeleton that takes place during bud emergence, probably identical mechanisms/effectors do not operate in both processes. It should also be noted that while Cln1p and Cln2p have established roles in bud emergence [Gulli et al. 2000; Bose et al. 2001; Moffat and Andrews 2004], they do not affect vacuole fusion [Han et al. 2003]. Finally, although there are no known protein–protein interactions mediated by the SH3-1 domain of Bem1p, it is possible that Ser72 might impact on vacuole fusion through interactions with effectors that have specific roles in fusion. Given the scaffold role of Bem1p and the fact that addition of Cdc42p in the presence of the Bem1p-S72A mutant did not promote fusion as efficiently as the Bem1p-S72D mutant alone [cf. Figs. 8C and 5E], it is possible that phosphorylation of Ser72 is important for some kind of protein–protein interactions that promote vacuole fusion. Future experiments need to address in full detail the mechanisms that account for the differences between Bem1p’s roles in vacuole fusion and bud emergence.
Materials and methods

Strains and DNAs

Cell cultivation, media, and yeast molecular biology techniques were performed as described by Kaiser et al. (1994), unless otherwise indicated. The strains and plasmids used in this study are listed in Tables 1 and 2, respectively. To generate the bem1Δ cln3Δ strain (SMY01) we disrupted one CLN3 copy in diploid bem1Δ cells as described previously [PolyMenis and Schmidt 1997]. The resulting heterozygote was sporulated, and the segregants were obtained by random spore analysis (Kaiser et al. 1994). All the single cyclin deletions and the bem1Δ strain in the BY4741 background were obtained by sporulation of the corresponding homozygous diploid deletion strains distributed from Research Genetics. The phenotypes reported for each strain were obtained after examining several independent transformants or segregants for the strain in question.

The putative phosphorylation site amino acid substitutions were introduced in the BEM1-12MYC low-copy centromeric plasmid pDLB2226 [Irazoqui et al. 2003]. We first PCR amplified BEM1 sequences from pDLB2226 using forward primers that encoded the desired mutation [BEM1-S72A-FWD, 5′-CCGAAACGAGTTATAAAAGCCAAATAC-3′, BEM1-S72D-FWD, 5′-CCAAGACATAATTCTAAAGATATTACTGCTCCAGAGA-3′] and a reverse primer corresponding to sequences up to position +574 of the BEM1 ORF [BEM1+(+574)-REV, 5′-GGTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCA-3′]. The PCR products were purified by agarose gel electrophoresis and used as primers in a second PCR reaction with pDLB2226 as template and a forward primer corresponding to BEM1 sequences up to position −186 [BEM1−(−186)-FWD, 5′-ATTACCCCTAAAGGGCAAAATG-3′]. The PCR product of this reaction was also purified by agarose gel electrophoresis and cotransformed into yeast cells together with plasmid pDLB2226, which was previously linearized with SmaI and HindIII digestion (cutting at positions −166 to +330 of the BEM1 ORF, respectively). The gap-repaired plasmid derivatives were then recovered from yeast transformants by standard methods [Kaiser et al. 1994].

To construct plasmids for bacterial expression, we digested the BG1805-based plasmids [purchased from Open Biosystems; see Table 2] with AgeI, which cuts once in the GAL1 promoter all the BG1805 plasmids used here. The digested plasmids were then transformed into a Ura− yeast strain for gap repair together with an oligonucleotide that encodes the arabinoose PRED promoter and ribosome-binding site [Guzman et al. 1995] [GAL1-PBAD-FWD, 5′-CGGGAACGGATTAAGGCGGGCGGCGGTCGAGCTCCTTTATCGCAAAATAC-3′], GAL1-PBAD-FWD at its 5′ end carries sequences complementary to GAL1 sequences upstream of the AgeI site, and at its 3′ end sequences complementary immediately upstream of the ATG start codon of the ORF in the BG1805 plasmids, which correspond to the bacteria phase λ attR1 site.

The mutant plasmid derivatives were sequenced to verify the introduced mutation and the absence of any other mutations at the Genome Technologies Laboratory of Texas A&M University.

Vacuolar morphology and size

For microscopic examination of vacuolar membranes the cells were stained with N-(3-triethylammoniumpropyl)-4-(6-[diethyloxycarbonyl]hexatrienyl)pyridinium dibromide, FM4-64, from Molecular Probes, as described by Han et al. (2003), and then examined microscopically with a Nikon Eclipse TS100 inverted fluorescence microscope.

Vacuolar size was evaluated by flow cytometry after staining with the vital vacuolar stain 5′-[6′-carboxy-2′,7′-dichlorofluorescein diacetate, CDCFDA, from Molecular Probes as described previously [Han et al. 2003].

The mean cell volume of live unfixed samples was measured using a Beckman Coulter Z2 Channelizer. The data were analyzed using the manufacturer’s AccuComp software. The geometric mean is indicated in each case.

In vitro vacuole fusion

Cytosol and vacuoles were prepared and used as described previously [Mayer et al. 1996; Han et al. 2003]. Briefly, to measure fusion activity the reaction mixture was equally split, and to each aliquot the same volume of buffer with inhibitor [or antibody] was added. Buffer-added aliquots were incubated at 27°C or on ice, the fusion activities of which were measured after 90 min and set as 100% or 0% fusion activity, respectively. The inhibitor-added aliquot was incubated at 27°C for the same time and its fusion activity was measured to evaluate the relative fusion activity.

Protein analysis

Unless otherwise indicated, the gels for SDS-PAGE [Laemmli 1970] contained 8% of a 29:1 acrylamide/bis-acrylamide solution. Immunoprecipitations and TEV cleavage of TAP-tagged Bem1p were performed according to the protocols and reagents recommended by Rigaut et al. [1999]. Briefly, 5 × 10^6 cells of

Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
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<td>Res. Genetics</td>
</tr>
<tr>
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<td>MATa hisΔ3 leu2Δ met15Δ ura3Δ</td>
<td>Res. Genetics</td>
</tr>
<tr>
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<td>BY4741/BY4742</td>
<td>Res. Genetics</td>
</tr>
<tr>
<td>W303a</td>
<td>MATa ade2 trp1 leu2 his3 ura3 can1</td>
<td>B. Futcher</td>
</tr>
<tr>
<td>GT108</td>
<td>CLN3-3HA::URA3 (W303a otherwise)</td>
<td>Open Biosystems</td>
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<tr>
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<td>SMY01</td>
<td>bem1Δ::KanMX cln3Δ::URA3 [BY4741 otherwise]</td>
<td>This study</td>
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<td>DOM30</td>
<td>cdc28::cdc28-as1 [DOM90 otherwise]</td>
<td>D. Morgan</td>
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</table>
untagged or TAP-tagged BEM1 cells from the exponential phase of growth were used. Cell extracts were prepared in the presence of protease and phosphatase inhibitor cocktails (Sigma). After the first immunoprecipitation with IgG-agarose beads, the beads were washed twice with RIPA buffer and once with TEV reaction buffer. Eighty units of TEV for 30 min at 30°C were used to cleave the protein A domain. The phosphatase experiment shown in Figure 4A was done as described previously (Liakopoulos et al. 2003). For the phosphatase experiments in Figure 4C, Bem1p-CBP on calmodulin beads was washed three times with RIPA buffer and once with lambda phosphatase reaction buffer. Then 500 U of lambda phosphatase and 10 mM PPi were incubated at 30°C for 30 min in the presence or absence of 100 μg/ml phosphatase inhibitor cocktail.

For protein surveillance, cell extracts were prepared using a urea extraction buffer as described by Ubersax et al. (2003). The differences in Bem1p’s electrophoretic mobility in 1D SDS-PAGE are better resolved if the samples are desalted prior to electrophoresis (using a kit from Pierce, according to their instructions) and electrophoresis is performed at a constant voltage of 70 V (instead of the typical 200 V applied to minigels).

Two-dimensional gel electrophoresis was performed using carrier ampholytes (pH 4–6, GE Healthcare) in acrylamide tubes according to the method of O’Farrell (1975). Seven-centimeter-long tube gels containing 0.5% ampholytes were focused for ∼1000 volt hours without prefocusing. The tube gels were extruded and soaked, sequentially, in SDS sample buffer containing dithiothreitol or iodoacetamide. The tube gels containing the reduced and alkylated proteins were subsequently placed on top of 8% polyacrylamide slab minigels and subjected to SDS-PAGE (Laemmli 1970). Prior to immunostaining, the proteins were electroblotted onto PVDF membranes (Immobilon P®, Millipore) at 100–200 mA overnight using 10 mM CAPS, 10% methanol (pH 11).

For immunostaining, protein A fusion proteins were detected with the peroxidase-anti-peroxidase (PAP) soluble complex reagent from Sigma. The anti-Pgk1p antibody was from Molecular Probes. The anti-phospho-[ST]p, anti-CBP, anti-HA, and anti-Myc antibodies were from Abcam. The horseradish peroxidase-conjugated secondary antibodies used for immunoblotting were also from Abcam. All antibodies were used at the dilutions recommended by the manufacturers. The blots were processed with reagents from Pierce.

Bacterial expression

The pBAD-based plasmids were transformed into Escherichia coli (strain XL1-Blue). For protein expression we followed previously published procedures (Guzman et al. 1995). From 50–100-mL cultures we obtained soluble protein from all the constructs we describe here. The exception was Cln3p, which required larger cultures (0.5L–1 L) because the majority of the recombinant protein appeared to be insoluble. We purified the recombinant proteins through their 6×His epitope, using TALON Co²⁺ affinity beads (BD Biosciences), according to the manufacturer’s instructions.

Kinase assays

Human Cdc2/cyclin B and Cdk2/cyclin A were from New England Biolabs, and used at 5 U per reaction. Cln3p-associated activity was obtained from yeast cells (strain W303a) carrying plasmid BG1805-CLN3 (see Table 2), using TALON Co²⁺ affinity beads (BD Biosciences), according to the manufacturer’s instructions. The Cln3p-associated activity was from ∼10¹⁰ cells initially grown in raffinose, but 4 h prior to harvesting, the culture was induced with 2% galactose. Histone H1 was from Sigma and used as a substrate at 5 μg per reaction. Bem1p was obtained from bacteria as described above. The reactions also contained 5 μCi [γ-³²P]ATP, 100 μM ATP, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, and 0.01% Brij 35. All the reactions (30 μL total volume) were performed at room temperature for 20 min.

Immunofluorescence microscopy

Unless otherwise indicated, we followed the protocols of the Botstein laboratory as described at http://genome-www.
Bem1p and vacuolar homeostasis


Bem1p, a scaffold signaling protein, mediates cyclin-dependent control of vacuolar homeostasis in *Saccharomyces cerevisiae*

Bong-Kwan Han, Lydia M. Bogomolnaya, James M. Totten, et al.

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Access the most recent version at doi:10.1101/gad.1361505

References

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