Docked vacuoles are believed to undergo rapid lipid mixing during hemifusion, then a slow, rate-limiting completion of fusion and mixing of luminal contents. Previous genomic analysis has suggested that Bem1p, a scaffold protein critical for cell polarity, may support vacuole fusion. We now report that bem1Δ strains have fragmented vacuoles (vps class B and C). During in vitro fusion reactions, vacuoles from bem1Δ strains showed a strong reduction in the rate of lipid mixing when compared to vacuoles from the BEM1 parent. The reduction in the overall rate of fusion with bem1Δ vacuoles was modest, consistent with lipid mixing as a non-rate limiting step in the pathway. Though the fusion of either BEM1 (wild-type) or bem1Δ vacuoles is stimulated by recombinant Bem1p, the lipid mixing of docked bem1Δ vacuoles is highly dependent on rBem1p under certain reaction conditions. Bem1p-stimulated lipid mixing is blocked by well characterized fusion inhibitors including lipid ligands and antibodies to Ypt7p, Vps33p and Vam3p. Although full-length Bem1p is required for maximal stimulation, a truncation mutant comprising the SH3 domains and the PX domain retains modest stimulatory activity. In contrast to an earlier report, we do not find phosphorylation of Bem1p at Ser 72 to be required for Bem1p-stimulated fusion. Taken together, Bem1p is a positive regulator of lipid mixing during vacuole hemifusion and fusion.

Membrane fusion in eukaryotic cells is highly regulated. At the core of the fusion machinery are Rab GTPases and their effectors (1), SNAREs (SNAP receptors) (2) and regulatory lipids (3-5). Additional fusion regulators include Sec18p/NSF (N-ethyl melamide sensitive fusion factor) (6,7), Sec17p/αSNAP (soluble NSF attachment protein) (8,9), SM proteins (10), calmodulin (11), synaptotagmin (12,13), kinases (Gerst 2003) and phosphatases (14-16), Rho GTPases and actin (17), V0 (the integral membrane domain of the vacuolar H+-ATPase) (18,19) and others (20).
mixing followed by a slower, rate-limiting content mixing (Jun and Wickner, manuscript in preparation).

To identify the proteins which catalyze each step of the fusion cascade, we used a genomic approach to identify genes which regulate vacuole size and copy number in growing cells (32). One candidate is Bem1p, a multi-domain protein required for cell polarization during budding and mating. Among its many binding partners (33,34), Bem1p interacts with GTP-Cdc42p via its N-terminal SH3 domains (35) and Cdc24p (a GDP-GTP exchange factor for Cdc42p) via its C-terminal PB1 domain (36,37). The central PX domain of Bem1p may interact with PI(3)P (38,39) and with specific proteins (40). In addition, Bem1p can associate with actin (41). Since Cdc42p (42,43), actin (44) and PI(3)P (5) are implicated in vacuole fusion, we decide to examine whether Bem1p regulates fusion directly.

Besides its reported presence at the bud site and bud tip, we find that Bem1p is associated with vacuoles. Vacuoles from \textit{bem1} \textit{/} strains show a dramatic reduction in the rate of lipid mixing. Though the overall fusion of either \textit{BEM1} (wild-type) or \textit{bem1} \textit{Δ} vacuoles is stimulated by \textit{rBem1p}, the subreaction of lipid mixing of docked \textit{bem1} \textit{Δ} vacuoles is most highly dependent on Bem1p and requires added \textit{rBem1p} under certain reaction conditions. We propose that Bem1p regulates lipid mixing during homotypic vacuole fusion.

**EXPERIMENTAL PROCEDURES**

**Strain Construction - Saccharomyces cerevisiae** strains \textit{BY4742}, \textit{BY4742 bem1Δ::kanMX6}, \textit{BY4742 pep4Δ::kanMX6} and \textit{BY4742 pho8Δ::kanMX6} were from Research Genetics. \textit{PEP4} or \textit{PHO8} was deleted from \textit{BY4742 bem1Δ::kanMX6} by transformation with a PCR product amplified from \textit{pRS405} (45) to generate \textit{BY4742 bem1Δ::kanMX6 pep4Δ::LEU2} or \textit{BY4742 bem1Δ::kanMX6 pho8Δ::LEU2}. \textit{BEM1} was deleted from \textit{BJ3505} and \textit{DKY6281} (21) by transformation with a PCR product amplified from \textit{pUG72} (46) to generate \textit{BJ3505 bem1Δ::URA3} and \textit{DKY6281 bem1Δ::URA3}.

\textit{BEM1} cDNA was amplified from plasmid pDLB1185 (35) using 5'-CGCAGATCTGCTGATGTTGGAATATCGTGAA CGGAAATTTTC. The cDNA was inserted into pMBP-Parallel1 (47) between the BamHI and EcoRI sites to generate pMBP-Bem1-his \textsubscript{6}, which was sequenced and transformed into \textit{E. coli} Rosetta (DE3) (Novagen).

\textit{BEM1} truncation mutants were generated in a similar fashion except that 5'-CGCAGATCTGCTGATGTTGGAATATCGTGAA CGGAAATTTTC were used for “1-277”, 5'-CGCAGATCTGCTGATGTTGGAATATCGTGAA CGGAAATTTTC for “1-407”, 5'-CGCAGATCTGCTGATGTTGGAATATCGTGAA CGGAAATTTTC for “408-551”. \textit{BEM1} mutants S72A and S72D were created using quick change mutagenesis (48) with overnight Dpn1 digestion. Oligonucleotides (Invitrogen) corresponding to the mutation sites were 5'-GACATAATTCTAAAGATATTACTGCTCAGAGAAAGTTATAAAAGCC and 5'-GGCTTTTATAACTTTCTCTGGATCAGTAATATCTTTAGAATTATGTC for S72A and 5'-GACATAATTCTAAAGATATTACTGCTCAGAGAAAGTTATAAAAGCC and 5'-GGCTTTTATAACTTTCTCTGGATCAGTAATATCTTTAGAATTATGTC for S72D. pMBP-Bem1-his \textsubscript{6} was the template for initial primer extension.

For expression in yeast, the \textit{BEM1} gene was amplified from \textit{S. cerevisiae} S288C genomic DNA (Invitrogen) using 5'-CGCAGATCTAATGTCGTTATATTTTCAATC and 5'-CGGAATTCTCGAGTTAAATCTTTCATATAATTC and then subcloned into centromeric plasmid \textit{pRS413} (between BamHI and EcoI sites) (45).

\textit{Proteins and antibodies - MBP-Bem1-his \textsubscript{6}} and mutant Bem1 proteins were purified using their C-terminal his \textsubscript{6} tag. The MBP tag is important to maintain protein solubility. Rosetta (DE3) /pMBP-Bem1-his \textsubscript{6} cultures (1L) in Terrific Broth (49) at 37 °C were induced at \textit{A}_{600} = 2.5 with 0.5 mM IPTG at 25 °C for 6h. Cells were collected by centrifugation (5,000 x g, 23 °C, 5 min), suspended in 25 mL of PBS, pH 7.4 (49), 5mM...
Following 1 h at 4°C, the agarose beads were washed twice with 20 mL of wash buffer (2 M DTT, 0.25 μg/mL pepstatin A, 0.25 μg/mL leupeptin, 20 mM imidazole in 2 x PBS). Recombinant Bem1p was eluted by 5 mL of 250 mM NaCl, and 3 times with 20 mL of wash buffer plus an additional 250 mM mercaptoethanol, 250 mM NaCl), twice with 20 mL of wash buffer and added to a nitrocellulose strip (Bio-Rad) in 2 x PBS, 10 mM imidazole, 8 mM DTT, 200 μg/mL pefabloc-SC) and 250 mM NaCl, and 3 times with 20 mL of wash buffer. Recombinant Bem1p was eluted by 5 mL of 250 mM imidazole in 2 x PBS.

MBP-Fapp1(PH)N-K, defective for PI(4)P binding (50), is a gift from C. Stroupe.

Antibodies to GST-Bem1p (a gift from A. Merz) were purified from rabbit serum. Serum (5 mL) was mixed at 4°C for 4 h with 50 μL of 0.5 M of EDTA, 50 μL of proteinase inhibitor cocktail (62 μg/mL leupeptin, 0.4 mg/mL pepstatin A, and 2.44 mg/mL pefabloc-SC) and 250 μL of 20 x PBS, and added to a nitrocellulose strip (Bio-Rad) bearing 200 μg of MBP-Bem1 for overnight incubation at 4°C. Following 5 washes (10 mL) with PBS, the blot was incubated with 800 μL of 0.2 M glycine (pH 2.3) for 3 min at RT and the eluate was neutralized with 200 μL of 1 M TrisCl, pH 8.0. Affinity-purified anti-Bem1p (0.2 μg/mL) was used in immuno-blot analysis.

Other antibodies for immuno-blot analysis were described previously (42,44) except anti-Vps1p IgG (purified by protein A sepharose; 10 μg/mL), anti-CoI1p (affinity-purified from rCoI1p cross-linked to Affi-Gel 15 agarose; 1μg/mL) and anti-Pfy1p (affinity-purified from rPfy1p cross-linked to Affi-Gel 15 agarose; 1μg/mL).

Fusion inhibitors were used at the following concentrations: anti-Sec17p IgG (51), 57 μg/mL; anti-Sec18p IgG (51), 70 μg/mL; anti-Ypt17p peptide antibody (42), 33 μg/mL; anti-Vps33p IgG (27), 42 μg/mL; anti-Vam3p IgG (52), 67 μg/mL; MED, 10 μM (25).

**Lipid mixing assay** - Vacuoles were isolated (21) with 20 mM PIPES-KOH, pH 6.8. Purified vacuoles (160 μg) were incubated with 0.12 mM octadecyl rhodamine B chloride (R18; Molecular Probes) on ice for 15 min. Labeled vacuoles were separated from the unbound dye and used to measure lipid mixing as described (Jun and Wickner, manuscript in preparation). Standard (+ ATP) fusion assays (30 μL) contained 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 6 mM MgCl₂, 1 mM ATP, 1 mg/mL creatine kinase, 29 mM creatine phosphate, 10 mM coenzyme A, 2.9 μg/mL IB₂, 0.67 μg R18-labeled vacuoles and 5.33 μg unlabeled vacuoles. Bypass fusion assays (- ATP) contained 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl₂, 20 mM glucose, 0.61 mg/mL hexokinase (1 unit; Sigma), 13 μg/mL rVam7p (prepared according to A. Merz (personal communication), 10 μg coenzyme A, 2.9 μg/mL IB₂ and vacuoles. Aliquots (25 μL) of the reaction mixture were transferred to a 384-well plate (round bottom, non-binding surface; Corning, NY) and fluorescent signals were measured every minute and processed as described (Jun and Wickner, manuscript in preparation).

**ALP maturation assay** - Vacuoles from pep4Δ and pho8Δ strains (3 μg each) were mixed in 30 μL under either the standard condition (+ ATP) or the bypass conditions (- ATP) as above. Following incubation on ice or at 27°C for 80 min, alkaline phosphatase activity was assayed at 30°C for 5 min (21).

**Docking assay** - Docking reactions with 6 μg DKY6281 vacuoles were performed in a volume of 30 μL of 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 100 mM KCl, 0.5 mM MgCl₂, 0.3 mM ATP, 0.07 mg/mL creatine kinase, 13.3 mM creatine phosphate, 0.7 μg/mL Sec18p, 20 μM coenzyme A, 14.3 μg/mL IB₂ and 0.13 μM Cy3-rBem1p. After 30 min at 27°C, reactions were transferred to ice, mixed with 1 μL of 16 μM MDY-64 and 50 μL of 4% agarose and mounted on slides.

MBP-Bem1-his₆ was labeled with Cy3 maleimide (Amersham) according to the manufacturer’s instruction. Unlabeled dye was quenched by addition of 10 mM mercaptoethanol and 10 mM cysteine and separated from the
labeled protein using a PD-10 column (Amersham).

Microscopy - Fluorescence microscopy methods were as described (26). To visualize FM4-64 or Cy3, Cy5, or MDY4-64 fluorophores, we used TRITC/Dil, Cy5, or Endow GFP filter sets (Chroma Technologies).

RESULTS

Yeast vacuoles undergo constant fusion and fission during cell growth. An imbalance between the fusion rate and the fission rate will cause abnormal vacuole morphology (vam, fragmented vacuole morphology) (32,53). Deletion of the BEM1 gene from the BY4742 chromosome causes type B vacuole fragmentation (Fig. 1B), as reported (32). Transforming BY4742 (Fig. 1A) or BY4742 bem1Δ (Fig. 1B) cells with vector alone does not change their vacuole morphologies. However, transforming BY4742 bem1Δ with pRS413-BEM1 restored normal vacuole morphology (compare Fig. 1D to 1B). To determine whether the vam phenotype is strain specific, we deleted BEM1 from DKY6281 and BJ3505. DKY6281 bem1Δ vacuoles exhibit a type B fragmentation phenotype (Fig. 1F) whereas BJ3505 bem1Δ vacuoles exhibit a more severe fragmentation (type C; Fig. 1H). This is supported by quantification of the number of vacuole lobes in random fields of yeast cells. For example, of 97 DKY6281 cells, 91.8% had less than 5 vacuole lobes, 7.2% had more than 5 vacuole lobes and 1% had highly fragmented vacuoles. Of 96 DKY6281 bem1Δ cells, only 38.5% had less than 5 vacuole lobes, whereas 46.9% had more than 5 vacuole lobes and 14.6% had highly fragmented vacuoles. Thus BEM1 is an authentic VAM gene.

Bem1p does not affect the vacuolar distribution of known fusion catalysts - Bem1p must associate with the vacuoles to directly regulate fusion. We therefore assayed BEM1 and bem1Δ vacuoles for their content of Bem1p by immunoblot with affinity-purified anti-Bem1p antibody (Fig. 2A). The antibody recognized a doublet close to the predicted M, of Bem1p (62 KDa) from WT vacuoles but not from bem1Δ vacuoles. The protein band with slower mobility may represent the phosphorylated form of Bem1p (54). Compared to proteins such as Pho8p and Vam3p (Fig. 2B), which are predominantly localized to the vacuole, Bem1p is not as highly enriched in the vacuole preparation (Fig. 2B). This is not surprising, since Bem1p cooperates with Cdc42p at the cell surface to regulate polarized growth (36).

To determine whether BEM1 deletion causes trafficking defects that affect fusion, we compared the vacuolar level of known fusion catalysts on BEM1 and bem1Δ vacuoles by immunoblot (Fig. 2A). Both BEM1 and bem1Δ vacuoles have comparable amounts of SNAREs, Ypt7p, HOPS, most other reported fusion regulators and Pho8p (alkaline phosphatase), suggesting that Bem1p does not critically regulate trafficking to the vacuole. Vacuoles from bem1Δ strains have more Cdc42p than normal (Fig. 2A), probably due to the up-regulation of Cdc42p in bem1Δ cells (Fig. 2B).

To examine whether Bem1p has a direct affinity for docked vacuoles, we labeled recombinant Bem1p with Cy3 and added the fluorescent protein to a standard docking assay. rBem1p (Fig. 2D, E) associates with vacuoles at punctate structures.

Bem1p stimulates lipid and content mixing in vitro - Since vacuoles from a bem1Δ strain have normal levels of most proteins, they are suitable for study of the roles of Bem1p in fusion. We isolated BEM1 and bem1Δ vacuoles and examined their fusion activities through the content-mixing assay (Materials and Methods). We performed the assay under either the standard reaction condition (with ATP) or the bypass condition with no ATP but with added rVamp7 (25). Vacuoles from BEM1 and bem1Δ strains exhibit comparable fusion activities under either condition (Fig. 3A,B). Since vacuoles from BEM1 and bem1Δ strains have similar amounts of maturable pro-ALP (data not shown), Bem1p is not essential for fusion in vitro. However, when recombinant Bem1p is added to the reaction, it stimulates the fusion of vacuoles from either BEM1 or bem1Δ strains (Fig. 3A,B). A slightly more potent stimulation is observed for vacuoles from a bem1Δ strain.

To understand why deletion of the BEM1 gene causes striking vacuole fragmentation in vivo but little or no fusion defect in vitro, we postulated that Bem1p might regulate a stage of the fusion pathway that is not rate-limiting in vitro but which
may be rate-limiting \textit{in vivo}. Recent studies (Jun and Wickner, manuscript in preparation) indicate that vacuole fusion proceeds from a very rapid lipid mixing step to a slow and rate-limiting content mixing step. We therefore examined the effect of Bem1p on lipid mixing. We monitor the rate of lipid mixing by an R18 dequenching assay. In this assay, rhodamine with an 18-carbon acyl chain (R18) is bound to vacuoles at a level which self-quenches the rhodamine fluorescence. The mixing of the lipid phase of these vacuoles with that of a docked neighbor vacuole, which is Rab, HOPS, and SNARE regulated and thus on the authentic fusion pathway (ibid), dilutes the self-quenched R18 and hence causes a large increase in its fluorescence. \textit{BEM1} and \textit{bem1}/.notdef. vacuoles labeled with R18 were re-isolated and mixed with unlabeled vacuoles. Lipid mixing assays were performed under standard or bypass conditions and were supplemented with rBem1p and anti-Vam3p where indicated. The fluorescent signal was recorded by a plate reader every minute (Fig. 3C to 3F). The initial dequenching rate was calculated (Materials and Methods) and is presented in Fig. 3G and 3H. There is a remarkable difference in the Vam3p-dependent dequenching rates between vacuoles from either \textit{BEM1} or \textit{bem1}/.notdef. strains, whether under regular or bypass fusion conditions. This contrasts with the results from the content mixing assay, where little difference was seen for these same vacuoles. The rate of lipid mixing of vacuoles from a \textit{bem1}/.notdef. strain is strongly dependent on added Bem1p under the bypass condition.

\textbf{Bem1p-dependent lipid mixing is sensitive to established fusion inhibitors} - Under the bypass fusion condition, the Bem1p-dependent R18 dequenching rate is sensitive to anti-Ypt7p, anti-Vps33p (Vps33p is a subunit of the HOPS complex), anti-Vam3p and MARCKS effector domain (MED; a ligand for PI(4,5)P2) (Fig. 4), showing that Bem1p stimulates the Rab, HOPS, and SNARE-dependent fusion pathway. The signal is not sensitive to priming inhibitors such as anti-Sec17p and anti-Sec18p (Fig. 4), consistent with our observation that added rVam7p bypasses the need for ATP-dependent priming (25). MBP-Fapp1(PH)N-K, a control for the effects of the MBP tag on MBP-Bem1p, did not affect the rate of lipid dequenching.

\textit{Full-length Bem1p is required for maximal activity} - Bem1p is composed of 2 N-terminal SH3 domains, a central PX domain and a C-terminal PB1 domain. To examine the importance of each domain, we generated deletion mutants and purified the encoded proteins (Fig. 5A). In the dequenching assay, 1.75 \(\mu\)M of each Bem1-derived protein was used for comparison (Fig. 5B). The full-length Bem1p exhibits maximum stimulatory effect. A protein (277-551) lacking the 2 SH3 domains has no stimulatory activity whereas a protein (1-407) with only the PB1 domain removed maintains modest activity. A protein consisting of only the PB1 domain is inert in the dequenching assay, but the SH3 domains alone show very weak activity. The results from the dequenching assay are mirrored in the content-mixing assay (Fig. 5C). Bem1p or related derivatives (up to 1.75 \(\mu\)M) were added to bypass fusion reactions. Only full-length Bem1p and 1-407 exhibited stimulatory effects. Taken together, the full-length Bem1p and its SH2 and PX domains are crucial for Bem1p activity in vitro. Of course, we cannot rule out the possibility that the mutants could be mis-folded. Bem1p did not interfere with the reporting system, since it has no effect when added at the end of a fusion reaction (Fig. 5C, compare lanes 1 & 2).

\textit{Bem1p activity does not require S72 phosphorylation} - It has been reported that Cln3p-dependent phosphorylation of Bem1p at S72 is required to promote fusion (54). In this study, the replacement of this serine by aspartic acid, which mimics phosphorylation, supported fusion whereas the S72A mutant, which could not undergo phosphorylation, had no fusion activity. Because the fusion activities in this report required cytosol, it is not clear whether these Bem1p mutant proteins act directly on the vacuolar fusion machinery or through recruitment of cytosolic factors. We purified these two Bem1p mutant proteins (Fig. 5A) and compared their activity with the WT Bem1p in a cytosol-free vacuole fusion reaction (Fig. 6). Under the bypass fusion condition, which eliminates the possibility of Bem1p phosphorylation during the assay, wild-type Bem1p and its S72D and S72A derivatives stimulate fusion in a comparable fashion. Similar results were seen for the standard (+ ATP) fusion condition. Interestingly, supplementing the
reaction with cytosol did not change the behavior of Bem1p derivatives (data not shown). Thus, phosphorylation at S72 does not appear to be required for Bem1p activity in our assay.

DISCUSSION

We find that Bem1p stimulates the lipid mixing step of vacuole fusion. Lipid mixing takes place upon merger of the two apposed leaflets of the docked organelles (hemifusion), which can occur well before the merger of distal leaflets and aqueous content mixing. SNARE-dependent hemifusion structures have been implicated in proteoliposome fusion (55,56), engineered cell fusion (57), and vacuole fusion (Jun and Wickner, manuscript in preparation). For vacuole fusion, the rapid hemifusion step is followed by a slow rate-limiting step of content mixing. This notion is supported by our finding that bem1Δ vacuoles exhibit a significant reduction of the rate of lipid mixing but not of fusion when compared to BEM1 vacuoles. “Bypass” reaction conditions permit rapid, Bem1p-dependent lipid mixing, indicating that Bem1p can act at, or prior to, the lipid mixing step. Because no homologs for Bem1p have been discovered in higher organisms, it is possible that the role of Bem1p in fusion is unique to the yeast vacuole.

How might Bem1p regulate lipid mixing during vacuole fusion? As a multidomain protein, Bem1p has the capacity to bind several proteins and lipids. The small GTPase Cdc42p and actin are two Bem1p binding partners that have already been implicated in fusion. Although it is unclear whether these interactions take place on the vacuole or how they might regulate lipid mixing, there is more Cdc42p on bem1Δ vacuoles, suggesting that the functional relationship between Bem1p and Cdc42p may extend to vacuole. Moreover, deletion of the Cdc42p binding domain of Bem1p abolishes Bem1p activity (Fig. 5B,C). Bem1p may also act on proteins which are critical for lipid mixing via unidentified interactions. The PX domain of Vam7p allows Vam7p interaction with the HOPS complex (26); both these proteins regulate important steps which are required for lipid mixing. It is conceivable that the PX domain of Bem1p might regulate lipid mixing by regulating SNARE-HOPS interaction. It is also possible that Bem1p regulates lipid mixing via direct interaction with PI(3)P, an important regulatory lipid in the fusion reaction. Addressing these possibilities may require generating Bem1p derivatives with point mutations that abolish individual inter-molecule interactions.

Recent studies of p47phox suggest that the function of its PX domain can be masked by an intramolecular interaction with its SH3 domain (58). Phosphorylation of the SH3 domain exposes the PX domain by causing a transition from a close to an open conformation. Interestingly, Bem1p also appears to be activated by phosphorylation at S72 in the first SH3 domain (54). However, in our hands, S72D, which mimics the phosphorylated form, exhibits similar in vitro activity to S72A or the wild-type. This discrepancy may arise from the fact that fusion is cytosol-dependent in the studies of Han et al. but not in our assays. Bem1p may stimulate fusion via different pathways under these two conditions.

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**FOOTNOTES**

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**FIGURE LEGENDS**

**Figure 1.** Vacuoles in *bem1Δ* strains are fragmented. BY4742 transformed with vector pRS413 (A) or pRS413-BEM1 (C), BY4742 *bem1Δ* transformed with pRS413 (B) or pRS413-BEM1 (D), DKY6281 (E), DKY6281 *bem1Δ* (F), BJ3505 (G), and BJ3505 *bem1Δ* (H) were grown at 30 °C to stationary phase and stained with 3 μM FM4-64 for 1h before microscopy. Scale bar in (G), 10 μM.

**Figure 2.** Proteins on vacuoles from *BEM1* and *bem1Δ* strains. BY4742 *pep4Δ* and BY4742 *pep4Δ bem1Δ* strains were grown at 30 °C to log phase in YPD medium. Spheroplasts and vacuoles were prepared as described in Materials and Methods. Vacuolar proteins (10 μg, or 3 μg for Cdc42p and Rho1p); or spheroplast proteins (50 μg, or 15 μg for Cdc42p) were subject to SDS-PAGE and immunoblot (A and B). A routine docking assay with 6 μg DKY6281 vacuoles and 0.13 μM Cy3-rBem1p in 30 μL was performed at 27 °C for 30 min. Vacuoles were stained with 5 μM MDY-64 at the end of the reaction. Images were acquired using Endow GFP (C) and TRITC/Dil filter sets (D). (E) Merged image of (C) and (D). Scale bar in (E), 5 μM.

**Figure 3.** Bem1p stimulates lipid mixing and content mixing. ALP maturation assays were performed for 80 min by mixing BY4742 *pep4Δ* and BY4742 *pep4Δ bem1Δ* vacuoles were grown at 30 °C to log phase in YPD medium. Spheroplasts and vacuoles were prepared as described in Materials and Methods. Vacuolar proteins (10 μg, or 3 μg for Cdc42p and Rho1p); or spheroplast proteins (50 μg, or 15 μg for Cdc42p) were subject to SDS-PAGE and immunoblot (A and B). A routine docking assay with 6 μg DKY6281 vacuoles and 0.13 μM Cy3-rBem1p in 30 μL was performed at 27 °C for 30 min. Vacuoles were stained with 5 μM MDY-64 at the end of the reaction. Images were acquired using Endow GFP (C) and TRITC/Dil filter sets (D). (E) Merged image of (C) and (D). Scale bar in (E), 5 μM.
100%, the values for other conditions were adjusted relative to the “no inhibitor” value. Standard deviations were calculated using Excel. To measure the rate of lipid mixing, BY4742 and BY4742 bem1Δ vacuoles were labeled with R18 on ice for 15 min and re-isolated by step-gradient flotation. Dequenching assays were performed by mixing 1 part of labeled vacuoles with 8 parts of unlabeled vacuoles (6 μg total). Fluorescent signals were recorded every minute using a Gemini XPS microplate reader. The “lipid mixing” signal at each time point was standardized by subtracting the signal at 0 min, then dividing by the maximum dequenching signal seen upon Triton X-100 addition and is expressed as a per cent of this maximum (C to F). The initial rates of dequenching are presented in (G) and (H). The mean dequenching rate for the “no inhibitor” condition was averaged from 4 independent experiments. The dequenching rates for other conditions were derived as above.

Figure 4. Bem1p-dependent dequenching is blocked by fusion inhibitors. Vacuoles from bem1Δ cells were labeled with R18 and mixed with unlabeled vacuoles under the bypass condition (no ATP plus Vam7p). Dequenching rates and standard deviations were calculated from three independent experiments as described in Fig. 3.

Figure 5. Full length recombinant Bem1p is required maximal activity. MBP- Bem1-his6 and mutant Bem1 proteins were purified from Ni2+-agarose beads. Eluted proteins (3 μg) were subjected to SDS-PAGE followed by Coomassie blue staining (A). Lipid mixing assays had 1.75 μM of Bem1 proteins (B). Fusion assays used BY4742 pep4Δ bem1Δ and BY4742 pho8Δ bem1Δ vacuoles under the bypass condition (C). Data from three independent experiments were processed as in Fig. 3.

Figure 6. Phosphorylation at Ser 72 is not essential for Bem1p activity. Mutations at S72 were created using PCR based quick-change mutagenesis (Materials and Methods). MBP-S72A-his6 and MBP-S72D-his6 were purified by the same method as WT MBP-Bem1-his6. The bypass fusion assay was performed using BY4742 pep4Δ bem1Δ and BY4742 pho8Δ bem1Δ vacuoles.
### Fig 2

**A.**

| Vacuoles | 1 | 2 |
|----------|---|---|
| BEMI     | Bem1p | Vam3p |
| bentΔ    |     | Vam7p |
|          | Vac8p | Vti1p |
|          | Vps1p | Nyv1p |
|          | Act1p | Ykt6p |
|          | Las17p | Sec18p |
|          | Cof1p | Sec17p |
|          | Pfy1p | Ypt7p |
|          | Rho1p | Vps16p |
|          | Cdc42p | Vps33p |
|          | Pho8p | Vps39p |

**B.**

| Vacuoles | Cells |
|----------|-------|
| BEMI     | Pho8p |
| bentΔ    | Vam3p |
|          | Bem1p |
|          | Cdc42p |

**C.**

**D.**

**E.**
Fig 3

A. Standard fusion (+ATP)

B. Bypass fusion (-ATP, +rVam7p)

C. Standard fusion (+ATP)

D. Standard fusion (+ATP)

E. Bypass fusion (-ATP, +rVam7p)

F. Bypass fusion (-ATP, +rVam7p)

G. Standard fusion (+ATP)

H. Bypass fusion (-ATP, +rVam7p)
Dequenching rate (K value)

- no inhibitor
- + anti-Vam3
- + MBP-Fapp1(PH)N-K
- + MBP-Bem1-his6
- + anti-Sec17
- + anti-Sec18
- + anti-Ypt7
- + anti-Vps33
- + anti-Vam3
- + MED
Fig 5

A.

B.

C.
Fusion (units)

- ice
- no Bem1p or inhibitor
- αVam3
- 0.19 μM
- 0.58 μM
- 1.75 μM
- 1.75 μM + αV3
- 0.19 μM
- 0.58 μM
- 1.75 μM
- 1.75 μM + αV3
- 0.19 μM
- 0.58 μM
- 1.75 μM
- 1.75 μM + αV3

WT
S72A
S72D

Bem1A bypass
BEM1 bypass

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Hao Xu and William Wickner

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