deletion: 104, 5′-AGCAGACGAGTCGACAGCTTCCATTAGCTTCACGGTTTCG-3′; 105, 5′-GACCTGCAGACGCGTCCTCTGACCCTACGAGTTCCACC-3′; 106, 5′-GTCAGAGGACGCGTCTGCAGGTCGACTCTAGAGGATCC-3′; 107, 5′-GACATCAGAGTCGACTGATGGCCTCCACGCACGTTGTG-3′; H deletion #2: 162, 5′-AGCAGACGAGTCGACGCTGGGGCCAAGGAGTCAG-3′; 161, 5′-GTTGTAAAACGACGGATCCCAGACTCACACATGGTCCCTG-3′; 160, 5′-AGTCTGGGATCCGTCGTTTTACAACGTCGTGACTGGG-3′; 107, 5′-GACATCAGAGTCGACTGATGGCCTCCACGCACGTTGTG-3′; H deletion #3: 184, 5′-AGCAGACGAGTCGACAGCTGTGGTGATAGTATGAAGTATGAC-3′; 183, 5′-GTTGTAAAACGACGTGTAGTTATGTAACAATCGAACG-3′; 182, 5′-ACATAACTACACGTCGTTTTACAACGTCGTGACTGGG-3′; 107, 5′-GACATCAGAGTCGACTGATGGCCTCCACGCACGTTGTG-3′; R1 deletion: 142, 5′-GGCAGTTATTGGTGCCCTTAAACG-3′; 132, 5′-CAAATAGTACTATGCGTGTAAATGAAGCCAATGCTAAGTGG-3′; 124, 5′-GCTTCATTTACACGCAATGTACTATTTGATTTGAGGACC-3′; 154, 5′-GACATCAGAGTCGACAACTCGAAGTAACAGGTCAGAAAGC-3′; R1YFP: 5′sal/r1, 5′-AGCAGACGAGTCGACACAACCAATCTCCCGGAAGAATGC-3′; 3′r1/yfp, 5′-CTTGCTCACCATGGTTTCTAAGCTACCTGGGAACAATG-3′; 5′r1/yfp, 5′-GCTTAGCCACCATGGTGAGCAAGGGCGAGC-3′; 3′yfp/r1, 5′-AAGGAGGCAGCTTACTTGTACAGCTCGTCCATGCC-3′; 5′yfp/r1, 5′-GCTGTACAAGTAAGCTGCCTCCTTGCCGTCTACCC-3′; 3′r1/sal, 5′-GACATCAGAGTCGACGGTGTGTGGGGGTGCCACTC-3′; R2 deletion: 129, 5′-AGCAGACGAGTCGACGGCAAGGTCACATAGCATTACTTGG-3′; 130, 5′-GATATGAAATAGTGACTATAGGAATTACCAGCAAAATGTTG-3′; 133, 5′-TGATATCCTTAATGGTCGTTTTACAACGTCGTGACTGGG-3′; 107, 5′-GACATCAGAGTCGACTGATGGCCTCCACGCACGTTGTG-3′; intergenic region deletion: 152, 5′-GCAGAGTCGACGAGCTAAGGTCTCACTATTTTCACAG-3′; 113, 5′-GTGACCTTGCCAGAGACCCATTGGTTAGCCCAATG-3′; 114 5′-AATGGGTCTCTGGCAAGGTCACATAGCATTACTTGG-3′; and 151, 5′-GACATCAGAGTCGACTGACTCCTGCCAAGAAATCCTTCC-3′. The construction of the R2GFP shuttle vector was as described (*12*).

27. We thank N. Heintz and W. Yang for help with BAC technology, F. Isdell and M. Genova for help with flow cytometry, D. Dorsett for helpful discussions, and E. Besmer and members of the Nussenzweig lab for suggestions. Supported by NIH Medical Scientist Training Program grant GM07739, the Surdna Foundation, and the William Randolph Hearst Foundation (W.Y); NIH (M.C.N. and R.R.H.); and the Howard Hughes Medical Institute (M.C.N.).

5 May 1999; accepted 14 July 1999

Control of the Terminal Step of Intracellular Membrane Fusion by Protein Phosphatase 1

C. Peters,[1] P. D. Andrews,[2] M. J. R. Stark,[2] S. Cesaro-Tadic,[1] A. Glatz,[1] A. Podtelejnikov,[3] M. Mann,[3] A. Mayer[1]*

Intracellular membrane fusion is crucial for the biogenesis and maintenance of cellular compartments, for vesicular traffic between them, and for exo- and endocytosis. Parts of the molecular machinery underlying this process have been identified, but most of these components operate in mutual recognition of the membranes. Here it is shown that protein phosphatase 1 (PP1) is essential for bilayer mixing, the last step of membrane fusion. PP1 was also identified in a complex that contained calmodulin, the second known factor implicated in the regulation of bilayer mixing. The PP1-calmodulin complex was required at multiple sites of intracellular trafficking; hence, PP1 may be a general factor controlling membrane bilayer mixing.

Intracellular membrane fusion can be divided into distinct subreactions: priming, tethering and docking of the membranes, and subsequent mixing of the bilayers and contents (*1*). Most components identified so far, such as NSF (NEM-sensitive fusion protein), α-SNAP (soluble NSF attachment protein), SNAREs (SNAP receptors), Rab-like guanosine triphosphatases (GTPases) and their cofactors, and the LMA1 complex (low molecular weight activity), act in the early steps of intracellular membrane fusion, mediating recognition and association of the appropriate membranes. In contrast, there is little information about the transition from docking to bilayer mixing.

In the yeast *Saccharomyces cerevisiae*, the fusion of vacuoles to each other involves reactions that are identical to those that mediate fusion of intracellular membranes in other eukaryotic cells (*1*). Vacuole fusion also requires an efflux of calcium from the vacuolar lumen (*2*), which is controlled by priming and docking of the vacuoles. The release of calcium into the cytosol results in a transient association of the cytosolic calcium-binding protein calmodulin with the vacuole membrane, which triggers the final events of bilayer and contents mixing. The final stage is also characterized by its sensitivity to certain low molecular weight compounds such as the serine-threonine phosphatase inhibitor microcystin LR, mastoparan, and guanosine 5′-*O*-(3′-thiotriphosphate) (*3*).

The target of microcystin LR is membrane-associated because microcystin LR inhibited vacuole fusion in the absence of cytosol (*4*). To identify the target of microcystin LR on yeast vacuolar membranes, we fractionated purified vacuole membranes by affinity chromatography with microcystin LR that was immobilized on Sepharose beads (*5*). In contrast to free microcystin LR, which forms covalent adducts with protein phosphatases (*6*), the immobilized form can associate with these enzymes noncovalently. Purified vacuolar membranes (*7*) were solubilized in detergent and passed over microcystin LR–Sepharose (*5*). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that only one protein of ~35 kD could be specifically eluted from the Sepharose with free microcystin LR (Fig. 1A). Incubation of the solubilized membrane with free microcystin LR inhibited binding of the 35-kD protein to microcystin LR–Sepharose. Association of the other proteins was not completely blocked by free microcystin LR, and we regarded them as nonspecific. An excised region of the gel containing the 35-kD protein was degraded by trypsin (*8*), and the resulting peptide mixture was analyzed by high-accuracy matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (*9*).

Eight peptide masses matched the calculated tryptic peptide masses of the yeast open reading frame YER133w (*10*) and identified the protein as protein phosphatase 1 (PP1). The involvement of PP1 in vacuole fusion was supported by the fact that affinity-purified antibodies to PP1 inhibited vacuole fusion in vitro (*11*) (Fig. 1B). Furthermore, purified yeast inhibitor 2 (*12*), a highly specific negative regulator of PP1 (*13*), inhibited vacuole fusion (Fig. 1C). Addition of purified recombinant PP1 (*14*) rescued the block to fusion caused by microcystin LR (Fig. 1D).

PP1 is an essential protein (*15*). To further examine the role of PP1 in fusion, we generated temperature-sensitive (ts) PP1 mutants (*16*). Vacuoles prepared from mutant strains that were grown at the permissive temperature (25°C) were fusion competent at 25°C (Fig. 2A). In contrast, short incubation of the vacuoles at the nonpermissive temperature (37°C) inhibited fusion. Vacuoles from wild-type yeast retained their fusion competence at 37°C. Inactivation of PP1 in the mutant strains by a temperature shift also disrupted vacuolar structure in vivo. At the nonpermissive temperature the vacuole disintegrated into small vesicles that aggregated (Fig. 2B). Under the same conditions, wild-type cells maintained a single large vacuole, as did the mutant cells at the permissive temperature. Overexpression of yeast inhibitor 2 in wild-type cells also caused vacuole fragmentation

[1]Friedrich-Miescher-Laboratorium, Spemannstrasse 37-39, 72076 Tübingen, Germany. [2]Department of Biochemistry, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, Scotland. [3]Protein Interaction Laboratory, Department of Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark.

*To whom correspondence should be addressed. E-mail: andreas.mayer@tuebingen.mpg.de

(*4*). Hence, PP1 was essential for vacuole fusion in vivo and in vitro.

Once a vacuole completes a step of the membrane fusion reaction, it becomes resistant to inhibitors of that particular step (*3*). This sensitivity to inhibitors can reveal the sequence of events in vacuole fusion. To determine if PP1 is required for an early or late stage of membrane fusion, we supplemented an ongoing in vitro fusion reaction with various inhibitors at different times and incubated the preparations until the end of the fusion period (70 min). At each given time point a sample was set on ice to stop fusion to monitor progression of the fusion reaction. All inhibitors prevented vacuole fusion when added at the start of the reaction (Fig. 3A). The vacuoles became resistant to the inhibitor Gdi1p after 30 min, indicating that the docking phase had been completed (*7*). Full resistance to anti-PP1 or to microcystin LR did not occur (*17*); therefore, PP1 functions close to the final step of the reaction, that is, during bilayer mixing. It remains to be determined whether this function is related to observations on eosinophil exocytosis where fusion pore expansion is controlled by phosphorylation (*18*).

Microcystin LR also affects the release of LMA1 from the membrane (*19*). LMA1 is functionally connected to factors acting early in intracellular membrane fusion: it stimulates Sec18p (NSF) adenosine triphosphatase activity and binds to and stabilizes activated SNAREs (*19*). Finally, it is released from the membrane. It is unclear if LMA1 release per se is of functional importance or rather a side effect of the reaction, because release occurs also in the absence of priming (Sec18p activity) and hence does not depend on overall fusion activity (*19*). We could not resolve whether PP1 has two targets: LMA1 and another, late-acting factor. Alternatively, LMA1 release itself could be connected to a late event that is regulated by PP1. In light of the functional relation of LMA1 to NSF and SNAREs, we consider this second possibility to be less likely.

Components that are essential for intracellular fusion reactions operate at many different transport steps in the cell (*1*). We examined the role of PP1 in regulating vesicle transport from the endoplasmic reticulum (ER) to the Golgi apparatus and also during endocytosis. The PP1 ts mutants were incubated at the nonpermissive temperature before they were metabolically labeled (*20*) with [^{35}S]methione and [^{35}S]cysteine for 5 min. Cells were then incubated in nonradioactive medium (chase) for different lengths of time before they were lysed. Carboxypeptidase Y (CPY) was immunoprecipitated from the lysates (Fig. 3B). CPY is a vacuolar protease that is translocated into the ER as a proenzyme (p1 form) and travels to the Golgi by vesicular transport (*21*). There it becomes glycosylated (p2 form). CPY is further transported through the prevacuolar-endosomal compartment and delivered to the vacuole where the propeptide is cleaved off, resulting in the active vacuolar form of the enzyme (m form). In wild-type cells, CPY was rapidly transported from the ER (0 min) to the Golgi (2.5 min) and finally to the vacuole (20 min). In contrast, in the ts mutant cells, CPY accumulated in the ER. At the permissive temperature, transport of CPY was similar in the mutant and wild-type cells (*4*). Thus, rapid inactivation of PP1 by a temperature shift blocked ER-to-Golgi transport.

To assess the role of PP1 in endocytic transport, we used the fluorescent styryl dye FM4-64 to pulse-label the plasma membrane (*22*). After incubation of the cells with FM4-

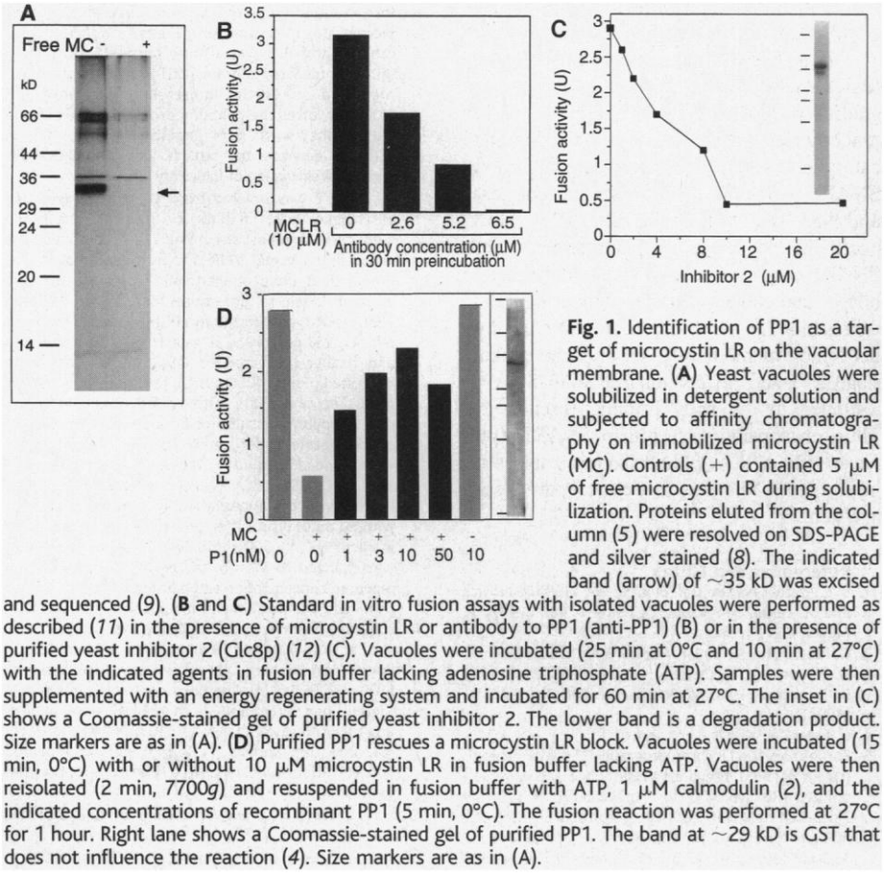

Fig. 1. Identification of PP1 as a target of microcystin LR on the vacuolar membrane. (**A**) Yeast vacuoles were solubilized in detergent solution and subjected to affinity chromatography on immobilized microcystin LR (MC). Controls (+) contained 5 μM of free microcystin LR during solubilization. Proteins eluted from the column (*5*) were resolved on SDS-PAGE and silver stained (*8*). The indicated band (arrow) of ~35 kD was excised and sequenced (*9*). (**B** and **C**) Standard in vitro fusion assays with isolated vacuoles were performed as described (*11*) in the presence of microcystin LR or antibody to PP1 (anti-PP1) (B) or in the presence of purified yeast inhibitor 2 (Glc8p) (*12*) (C). Vacuoles were incubated (25 min at 0°C and 10 min at 27°C) with the indicated agents in fusion buffer lacking adenosine triphosphate (ATP). Samples were then supplemented with an energy regenerating system and incubated for 60 min at 27°C. The inset in (C) shows a Coomassie-stained gel of purified yeast inhibitor 2. The lower band is a degradation product. Size markers are as in (A). (**D**) Purified PP1 rescues a microcystin LR block. Vacuoles were incubated (15 min, 0°C) with or without 10 μM microcystin LR in fusion buffer lacking ATP. Vacuoles were then reisolated (2 min, 7700*g*) and resuspended in fusion buffer with ATP, 1 μM calmodulin (*2*), and the indicated concentrations of recombinant PP1 (5 min, 0°C). The fusion reaction was performed at 27°C for 1 hour. Right lane shows a Coomassie-stained gel of purified PP1. The band at ~29 kD is GST that does not influence the reaction (*4*). Size markers are as in (A).

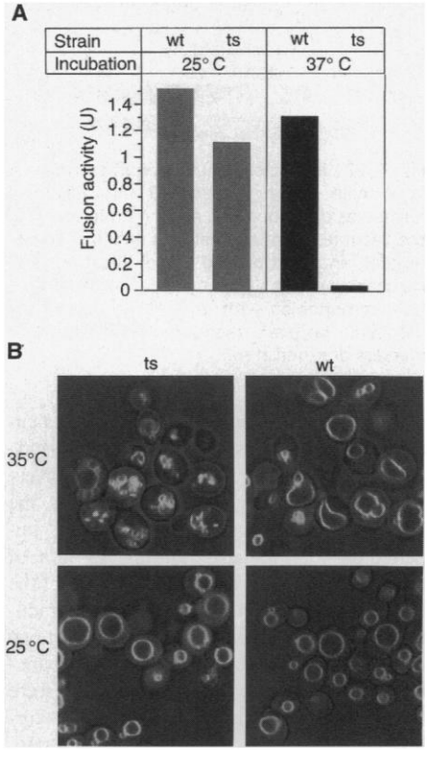

Fig. 2. Vacuole fusion in PP1 ts mutants in vitro and in vivo. (**A**) Vacuoles were isolated from PP1 ts mutants (PAY810-3Aα and PAY 821-2Bα) and from the corresponding wild-type (PAY813-2Cα and PAY820-1Dα) strains (*16*) and incubated in fusion buffer that lacked ATP for 5 min at 25° or 37°C. Vacuoles were chilled to 25°C before addition of ATP and initiation of the fusion reaction. (**B**) Ts (PAY700-4) and wild-type (PAY704-1) cells were grown (10 hours, 25°C) and then stained with the fluorescent dye FM4-64 (*2*). Cells were then incubated in medium without stain at 25° or 35°C for 5 hours and analyzed by confocal microscopy. The transmission and fluorescence channel of the microscope were overlaid. Image width for all panels is 25 μm.

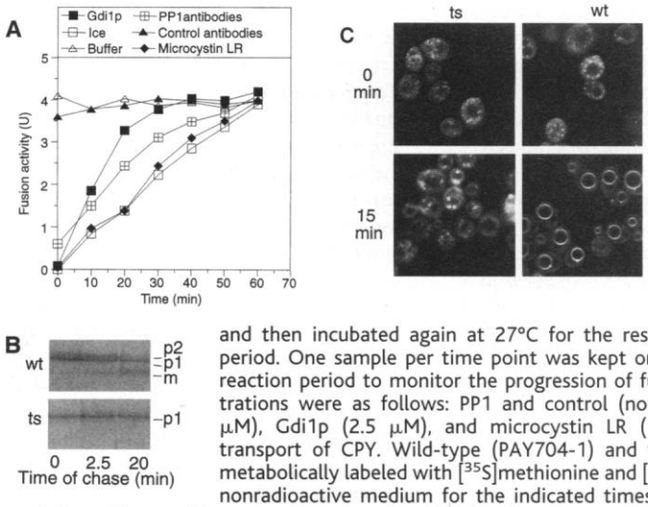

Fig. 3. PP1 acts after the docking phase and at multiple steps of membrane trafficking. (**A**) Fusion reactions (six times the standard volume) were incubated at 27°C. At the indicated times they were divided into 30-μl standard fusion reactions, supplemented with the indicated agent or buffer only, kept on ice for 10 min, and then incubated again at 27°C for the rest of the 70-min reaction period. One sample per time point was kept on ice until the end of the reaction period to monitor the progression of fusion. Antagonist concentrations were as follows: PP1 and control (nonimmune) antibodies (13 μM), Gdi1p (2.5 μM), and microcystin LR (10 μM). (**B**) ER-to-Golgi transport of CPY. Wild-type (PAY704-1) and ts (PAY702-4) cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and chased in nonradioactive medium for the indicated times (*20*). CPY was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and autoradiography. The forms of CPY are indicated. (**C**) Endocytic uptake of FM4-64. The same cells as in (A) were grown (12 hours, 25°C) to OD_{600} = 2 and transferred to 37°C for 30 min. They were pulse-labeled with FM4-64 (*23*), chased for the indicated times, and analyzed by confocal microscopy as in Fig. 2.

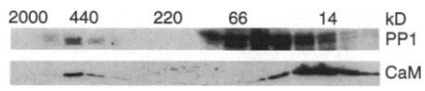

Fig. 4. PP1 is part of a multisubunit complex. A large-scale fusion reaction (3 ml) was performed as described (*7*). After 45 min at 27°C, the vacuoles were reisolated, solubilized in detergent, and fractionated by gel filtration (*24*). Fractions were analyzed by SDS-PAGE and protein immunoblot with anti-PP1 and anti-calmodulin. The peak fractions of molecular size markers are indicated.

64 for 2 min (*23*), small intracellular vesicular structures were stained in both wild-type and PP1 ts cells (Fig. 3C). Many of these vesicles were mobile inside the cells. Thus, the formation of endocytic vesicles is not impaired in PP1 mutants. During 15 min of chase in medium without dye, staining of the small vesicles in wild-type cells was gradually lost and the vacuolar membrane became increasingly fluorescent. Slightly larger punctate structures (three to five per cell) were also stained, but transiently, which might correspond to endosomes. After 15 min, all stain had been transferred to the vacuoles. PP1 ts cells, however, failed to transfer the stain to the vacuoles, indicating a block of the endocytic pathway. Under permissive conditions, endocytic transport in the ts mutant was similar to that in wild-type cells (*4*). PP1 activity appeared essential not only for vacuolar fusion, but also for ER-to-Golgi and endocytic vesicular trafficking.

To test if PP1 action might be related to that of calmodulin, another late-acting component in vacuole fusion (*2*), we solubilized purified vacuolar membranes in detergent and analyzed the extract by gel filtration (*24*) (Fig. 4). A fraction of PP1, which is a 35-kD protein, was recovered from a high molecular weight complex of about 500 kD. Calmodulin was also found in the complex. Preliminary attempts to isolate the PP1 complex indicated that, besides PP1 and calmodulin, it contained several other components (*4*) that could be target proteins of PP1. It is possible that Ca^{2+} and calmodulin signal the completion of docking. Calcium efflux from the lumen of the vacuoles may activate the PP1 complex to catalyze the final events in fusion.

On the basis of reconstitution of liposome fusion with purified proteins, two recent studies proposed different minimal fusion machineries as necessary and sufficient to catalyze intracellular membrane fusion: v-SNARE–t-SNARE complexes in one case and NSF–α-SNAP in the other (*25*). However, in physiological membrane systems, SNARE complexes can be disassembled before bilayer and contents mixing without inhibiting the reaction (*26*). In addition, anti-SNARE does not inhibit membrane fusion after the docking stage (*27*), and the function of NSF–α-SNAP is restricted to the early priming reaction (*3*). Thus, an apparatus other than SNARE complexes and NSF–α-SNAP likely catalyzes bilayer mixing. PP1 may be a critical part of such a system.

References and Notes

1. S. R. Pfeffer, *Nature Cell Biol.* **1**, 17 (1999); P. Novick and M. Zerial, *Curr. Opin. Cell Biol.* **9**, 496 (1997); A. Mayer, *ibid.*, in press.
2. C. Peters and A. Mayer, *Nature* **396**, 575 (1998).
3. A. Mayer, W. Wickner, A. Haas, *Cell* **85**, 83 (1996); B. Conradt, A. Haas, W. Wickner, *J. Cell Biol.* **126**, 99 (1994).
4. C. Peters *et al.*, data not shown.
5. For microcystin LR chromatography, 2 mg of vacuoles (strain BJ3505), prepared as described (*7*), were solubilized (30 min, 4°C) in 5 ml of buffer A (10 mM Pipes-KOH pH 6.8, 200 mM sorbitol, 150 mM KCl, 500 μM $MnCl_2$, 40 mM CHAPS, 75 μM pefablock SC, 150 nM leupeptin, 37.5 μM *o*-phenanthroline, 500 nM pepstatin A) and centrifuged (100,000*g*, 30 min, 4°C). The supernatant was passed over 100 μl of microcystin LR–Sepharose (*6*) (200 μl/min). The matrix was washed with 25 ml of buffer A and eluted with 1 ml of buffer A containing 50 μM microcystin LR. Eluted proteins were concentrated by ultrafiltration in microcon-10 (Amicon).
6. G. Moorhead, R. W. Mackintosh, N. Morrice, T. Gallagher, C. Mackintosh, *FEBS Lett.* **356**, 46 (1994); R. W. MacKintosh *et al.*, *ibid.* **371**, 236 (1995).
7. A. Mayer and W. Wickner, *J. Cell Biol.* **136**, 307 (1997).
8. A. Shevchenko, M. Wilm, O. Vorm, M. Mann, *Anal. Chem.* **68**, 850 (1996).
9. A. Shevchenko *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14440 (1996); O. N. Jensen, A. Podtelejnikov, M. Mann, *Rapid Commun. Mass Spectrom.* **10**, 1371 (1996).
10. YER133w is the *GLC7* gene. The peptides matched within 20 parts per million and covered 33% of the Glc7p sequence. For mass spectrometry, protein bands were excised from the gels and digested by trypsin (*8*). We analyzed 2% of the supernatant by MALDI mass spectrometry on a Bruker REFLEX instrument (Bruker Daltonics, Bremen, Germany) and searched the resulting peptide mass maps in a nonredundant sequence database using PeptideSearch software developed by P. Mortensen and M. Mann. Hits were verified with second-pass searches taking account of chemical modifications.
11. Standard fusion yeast strains were BJ3505 and DKY6281 (*3*). One unit of fusion activity is defined as 1 μmol of *p*-nitrophenol developed per minute and microgram of Δpep4 vacuoles. Vacuole isolation, cytosol preparation, and fusion were performed and assayed as described (*7*); however, the cells for the cytosol preparation were not lysed in a bead beater but by freezing the cell suspension and blending it in liquid nitrogen. The cytosol concentration in fusion assays was only 0.6 mg/ml. Anti-PP1 was raised by blotting PP1 from an SDS gel onto nitrocellulose, dissolving the nitrocellulose in dimethyl sulfoxide, and subcutaneously injecting a 1:1 mixture with Freund's adjuvant into rabbits. Anti-PP1 was affinity purified with a PP1–glutathione *S*-transferase (GST) fusion protein immobilized on CH-Sepharose 4B (Pharmacia) (*3*). However, after the coupling procedure the column was washed with 6 M guanidinium-HCl, 10 mM EDTA to denature the protein. This was critical for successful extraction of inhibitory antibodies.
12. Inhibitor 2 was isolated from yeast overexpressing His-tagged yeast inhibitor 2 (YMR311c) from the *GAL* promoter. Yeast strain PAY 704-1 containing the expression plasmid YMR311 (from Invitrogen) was grown in 8 liters of Hartwell's complete medium without uracil to an optical density at 600 nm (OD_{600}) of 1, transferred to YPGal medium (1% yeast extract, 2% peptone, 2% galactose, 0.1% raffinose), and further incubated to OD_{600} = 6. Cells were harvested (5 min, 4000*g*, 2°C); resuspended in 50 mM potassium phosphate buffer pH 8, 300 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM imidazole; and frozen in liquid N_2. Frozen pellets were blended in liquid N_2, thawed, and centrifuged (1 hour, 150,000*g*, 2°C). Nickel-Sepharose (1 ml) was loaded with the supernatant (0.5 ml/min), washed with 50 ml of lysis buffer, and eluted by an imidazole gradient (50 to 500 mM). The buffer of the eluate was changed to 50 mM tris pH 8, 50 mM NaCl by repeated concentration and dilution in a centricon-10 (Amicon). The protein was loaded onto a MonoQ^{FPLC} column (Pharmacia; 0.2 ml/min). The column was washed with 10 ml of 50 mM tris pH 8, 50 mM NaCl. Bound proteins were eluted with an NaCl gradient (100 mM to 1.5 M). Fractions containing inhibitor 2 (determined by protein immunoblot) were pooled, and the buffer was changed to 10 mM Pipes-KOH pH 6.8, 200 mM sorbitol, 150 mM KCl by repeated concentration and dilution in a centricon-10. Samples were stored at −20°C.
13. H. Y. L. Tung, W. Wang, W. Chan, *Mol. Cell. Biol.* **15**, 6064 (1995).
14. For purification of PP1, *Escherichia coli* strain PCC605

(*13*) (4 liters) was grown to OD_{600} = 0.5, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, incubated (25°C, 18 hours), and harvested (10 min, 8000*g*, 2°C). Cells were resuspended in lysis buffer (50 mM tris-HCl pH 7.4, 150 mM KCl, 1 mM $MnCl_2$, 1 mM PMSF) and lysed by repeated freeze-thaw cycles and sonication. The lysate was centrifuged (200,000*g*, 30 min, 2°C). A glutathione column (5 ml, Pharmacia) was loaded with the supernatant and washed with 300 mM KCl, 50 mM tris-HCl pH 7.4, 1 mM CHAPS, 1 mM $MnCl_2$, and the fusion protein was cleaved on the column by incubating with factor Xa (Biolabs, 0.01 mg/ml in lysis buffer with 2 mM $CaCl_2$) overnight at 4°C and eluted with 10 ml of Q buffer (50 mM KCl, 1 mM $MnCl_2$, 50 mM tris-HCl pH 8). Cleavage was inefficient (<10%). The eluate (1.5 mg) was loaded onto a Mono Q (FPLC) column (Pharmacia; 0.2 ml/min). Bound proteins were eluted with a gradient of 100 to 750 mM NaCl in Q-buffer and screened by protein immunoblotting. Fractions containing PP1 were pooled, tested for factor Xa activity (with Boehringer Chromzyme A, according to the manufacturer's instructions), and found to be free from Xa-cleavage activity. PP1 was transferred to 10 mM Pipes-KOH pH 6.8, 200 mM sorbitol, 150 mM KCl, 1 mM $MnCl_2$, 50% glycerol by repeated concentration and dilution in a centricon-10 and stored at −80°C.

15. M. J. R. Stark, *Yeast* **12**, 1647 (1996).
16. All these strains are based on W303 (*ade2-1 his3-11,15 leu2-3 trp1-1 ura3-1 can1-100 ssd1-d2*). Temperature-sensitive PP1(*glc7*) alleles were generated as described (*28*). Strains PAY704-1 (*MAT***a** glc7::LEU2 *trp1*::GLC7::*TRP1*), PAY700-4 (*MAT***a** *glc7*::LEU2 *trp1*::*glc7*-10::*TRP1*), and PAY702-4 (*MAT***a** *glc7*::LEU2 *trp1*::*glc7*-13), in which wild-type or mutant *GLC7* alleles integrated at *trp1* substitute for the copy of *GLC7* on chromosome IV, will be described elsewhere (*29*). A null allele of *PHO8* was generated in the W303 background by one-step polymerase chain reaction (PCR)–mediated gene disruption with the *Schizosaccharomyces pombe* $His5^+$ gene (*30*), and the correct gene replacement verified by PCR. Strains PAY810-3Aα (*MAT*α *glc7::LEU2 trp1::glc7-10::TRP1 pho8Δ::his5*$^+$) and PAY813-2Cα (*MAT*α *glc7::LEU2 trp1::GLC7::TRP1 pho8Δ::his5*$^+$) were generated by crossing this strain with PAY700-4 and PAY704-1, respectively, then selecting haploid segregants with the appropriate combination of genetic markers after tetrad analysis. PAY 821-2Bα (*MAT*α *glc7::LEU2 trp1::glc7-10::TRP1 pep4Δ::URA3*) and PAY820-1Dα (*MAT*α *glc7::LEU2 trp1::GLC7::TRP1 pep4Δ::URA3*) were similarly generated after crossing WZY333 (*MAT*α*pep4Δ::URA3*) with PAY700-4 and PAY704-1 and additionally verified by using a standard plate assay to confirm the lack of CPY activity after replating several times (*31*).
17. Gdi1p extracts the Rab-like GTPase Ypt7p from the membrane and prevents docking (*7*). Anti-PP1 was not as effective in this type of experiment as in Fig. 1B because the antibody binds slowly to PP1 on the membrane (*4*). Because the kinetic analysis in Fig. 3 did not allow a preincubation of vacuoles with the antibodies as in Fig. 1B, the inhibitory effect of the antibodies was not complete, shifting the inhibition curve to the left. Microcystin LR binds covalently to PP1, resulting in rapid inactivation.
18. S. Scepek, J. R. Coorssen, M. Lindau, *EMBO J.* **17**, 4340 (1998).
19. Z. Xu, A. Mayer, E. Muller, W. Wickner, *J. Cell Biol.* **136**, 299 (1997); Z. Xu, K. Sato and W. Wickner, *Cell* **93**, 1125 (1998).
20. For pulse-labeling with ^{35}S, cells were grown overnight at 25°C in CMM [complete minimal medium (*32*) minus methionine and cysteine] to OD_{600} = 1 and transferred to 37°C for 1 hour. Cells from 15 ml of culture were harvested (2 min, 3000*g*, 25°C), incubated in 30 mM tris pH 8.9, 10 mM dithiothreitol (37°C, 5 min), reisolated, and incubated for 15 min at 37°C in CMM containing 1.3 M sorbitol, 20 mM tris pH 7.4, 40 mM ammonium sulfate, and oxalyticase (1000 U/ml). The spheroplasts were pelleted (2 min, 3000*g*) and resuspended in CMM without ammoniumsulfate, but with 1.3 M sorbitol and Tran-^{35}S-label (300 μCi/ml; ICN). After 5 min at 37°C, the spheroplasts were re-isolated and resuspended in CMM containing 40 mM ammonium sulfate, 10 mM cysteine, 10 mM methionine, and 1.3 M sorbitol. After incubation for the indicated times (37°C), the cells were pelleted (2 min, 3000*g*, 4°C) and lysed in 2% SDS, 2% Tween 80, 50 mM tris-HCl pH 7.4, 10 mM NaN_3, and 1 mM PMSF for 5 min at 95°C. The SDS concentration was adjusted to 0.2% by dilution with IP buffer (50 mM tris pH 7.4, 0.2% Tween 20, 150 mM KCl, 100 μM EDTA, 1 mM PMSF). CPY was immunoprecipitated by adding affinity-purified antibodies (2 μg/ml) and 50 μl of a 1:1 slurry of protein A–Sepharose to 1 ml of lysate. After 2 hours of shaking at 4°C, the beads were pelleted and washed three times with IP buffer and once with phosphate-buffered saline. Proteins were eluted with SDS sample buffer.
21. J. H. Stack, B. Horazdovsky, S. D. Emr, *Annu. Rev. Cell Dev. Biol.* **11**, 1 (1995).
22. This dye rapidly associates with the plasma membrane, becomes endocytosed, and migrates by vesicular transport to the vacuole where it accumulates [T. Vida and S. D. Emr, *J. Cell Biol.* **128**, 779 (1995)].
23. For pulse-labeling with FM4-64, cells were grown in YPAD medium (1% yeast extract, 2% peptone, 0.004% adenine sulfate, 2% dextrose) (12 hours, 25°C), labeled with 200 μM FM4-64 (2 min, 25°C), reisolated (30 s, 5000*g*, 25°C), washed with YPAD, and reisolated as before. The cells were resuspended in YPAD at OD_{600} = 1 and chased at 37°C for various times. The cells were reisolated (30 s, 5000*g*, 2°C) and resuspended in the supernatant at OD_{600} = 10. A 7-μl sample of the suspension was mixed with 7 μl of 0.4% Seaplaque agarose in 10 mM Pipes-KOH pH 6.8, 200 mM sorbitol (kept liquid at 35°C), transferred to a microscopy slide, and chilled at 4°C for 5 min to immobilize the cells. Cells were quickly investigated with a confocal microscope (Leica TCS) under minimal excitation.
24. For gel filtration, an upscaled fusion reaction was performed (3-ml volume, no cytosol, 45 min). The vacuoles were sedimented (2 min, 20000*g*, 4°C), resuspended in 0.5 ml of buffer S [10 mM Pipes-KOH pH 6.8, 150 mM KCl, 200 mM sorbitol, 0.5 mM MnCl, 10 μM $CaCl_2$, 750 μM pefablock SC, leupeptin (750 ng/ml), 375 μM o-phenanthroline, pepstatin A (3.75 μg/ml)], and solubilized by addition of CHAPS to a final concentration of 40 mM (10 min, 4°C, mild shaking). The lysate was centrifuged (15 min, 100,000*g*, 4°C) and the supernatant applied to a $Superose12^{FPLC}$ column (Pharmacia) equilibrated in buffer S with 4 mM CHAPS. The column was run at 0.5 ml/min (4°C). We collected 1-ml fractions.
25. T. Weber *et al.*, *Cell* **92**, 759 (1998); M. Otter-Nilsson, R. Hendriks, E.-I. Pecheur-Huet, D. Hoekstra, T. Nilsson, *EMBO J.* **18**, 2074 (1999).
26. C. Ungermann, K. Sato, W. Wickner, *Nature* **396**, 543 (1998); J. R. Coorssen, P. S. Blank, M. Tahara, J. Zimmerberg, *J. Cell Biol.* **143**, 1845 (1998).
27. B. J. Nichols, C. Ungermann, H. R. B. Pelham, W. T. Wickner, A. Haas, *Nature* **387**, 199 (1997).
28. S. H. MacKelvie, P. D. Andrews, M. J. R. Stark, *Mol. Cell. Biol.* **15**, 3777 (1995).
29. P. D. Andrews and M. J. R. Stark, in preparation.
30. M. S. Longtine *et al.*, *Yeast* **14**, 953 (1998).
31. E. W. Jones, *Genetics* **85**, **23** (1977).
32. F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1997).
33. We thank C. MacKintosh for helpful hints in the initial phase of the project, K. Nasmyth for strain WZY333, M. Bayer and O. Müller for discussions, and E. Schlegel for help in preparing the manuscript. Supported by the Wellcome Trust (project grants 046956 and 049778, M.J.R.S.), the Deutsche Forschungsgemeinschaft (grant SFB446, A.M.), and the Boehringer Ingelheim Stiftung (A.M.).

4 May 1999; accepted 8 July 1999

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