Determination of Four Biochemically Distinct, Sequential Stages during Vacuole Inheritance In Vitro

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Abstract. Vacuole inheritance in *Saccharomyces cerevisiae* can be reconstituted in vitro using isolated organelles, cytosol, and ATP. Using the requirements of the reaction and its susceptibility to inhibitors, we have divided the in vitro reaction into four biochemically distinct, sequential subreactions. Stage I requires exposure of vacuoles to solutions of moderate ionic strength. Stage II requires "stage I" vacuoles and cytosol. In stage III, stage II vacuoles react with ATP. Finally, during stage IV, stage III vacuoles at a certain, minimal concentration complete the fusion reaction without further requirement for any soluble components. Reagents that inhibit the overall vacuole inheritance reaction block distinct stages. Stage III of the reaction is sensitive to the proton ionophore CCCP, to inhibitors of the vacuolar ATPase such as bafilomycin A_1, and to the ATP-hydrolyzing enzyme apyrase, suggesting that an electrochemical potential across the vacuolar membrane is required during this stage. Inhibition studies with the amphiphilic peptide mastoparan and GTPγS suggest that GTP-hydrolyzing proteins might also be involved during this stage. Microcystin-LR, a specific inhibitor of protein phosphatases of type 1 and 2A, inhibits stage IV of the inheritance reaction, indicating that a protein dephosphorylation event is necessary for fusion. The definition of these four stages may allow the development of specific assays for the factors which catalyze each of the consecutive steps of the in vitro reaction.

CYTOPLASMIC organelles are not synthesized de novo after cell division but arise from the growth and duplication of preexisting organelles. Specific, cell cycle–coordinated processes are required to ensure that low- and single-copy number organelles are properly divided and partitioned between mother and daughter cells (Birkey, 1983; Warren, 1993). Cytological studies, mainly performed in mammalian cells, support the involvement of organelle-specific, cell-cycle–regulated processes in the inheritance of cytoplasmic organelles (Warren, 1993). However, little is known about the molecular mechanisms underlying these important events.

The budding yeast *Saccharomyces cerevisiae* has developed specific mechanisms to ensure the correct inheritance of its organelles from one generation to the next (Yaffe, 1991). *S. cerevisiae* undergoes a "closed" mitosis during which the nucleus does not break down. Instead, it elongates into the growing daughter cell and the two nuclei are formed by septation before cytokinesis (Byers, 1981). The Golgi apparatus in *S. cerevisiae* is comprised of single cisternae distributed throughout the cellular cytoplasm (Preuss et al., 1992). Soon after bud emergence, several Golgi cisternae cluster near the neck of the bud prior to entry (Redding et al., 1991; Preuss et al., 1992). Most of the endoplasmic reticulum in *S. cerevisiae* is associated with the plasma membrane and the nuclear envelope and enlarges and elongates along with these organelles (Preuss et al., 1991). Mitochondria occur at a varying copy number in *S. cerevisiae* (Stevens, 1981). Nevertheless, they enter the newly emerging bud by a specific mechanism which is defective in a number of *mdm* mutants (McConnell et al., 1990; Steward and Yaffe, 1991; McConnell and Yaffe, 1992, 1993). The *S. cerevisiae* vacuole, an acidic, lysosomal-like organelle, is present at a low copy number (Weisman et al., 1987). This organelle can be visualized using either endogenous or exogenous fluorophores (Weisman et al., 1987). *S. cerevisiae* vacuoles can be readily isolated and several soluble and membrane-bound, vacuolar marker enzymes have been characterized (Roberts et al., 1991). These tools make the yeast vacuole not only amenable to cytological and genetic studies, but also to biochemical analyses. We have therefore begun to investigate the process of vacuole inheritance in *S. cerevisiae*.

Cytological studies have confirmed that vacuole inheritance is coordinated with the cell division cycle. It is also spatially regulated, as it is restricted to a specific path between the mother and daughter cell (Weisman and Wickner, 1988; Gomes de Mesquita et al., 1991). During this complex process, the yeast vacuole undergoes distinct morphological

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changes. The mother cell organelle forms a membranous, tubulovesicular structure, termed a segregation structure. This structure elongates into the emerging bud and delivers vacuolar material to establish the daughter cell vacuole. This step is followed by the assembly of this vacuolar material to form the new daughter cell organelle, a process which involves the fusion of vacuolar material and leads to mixing of vacuolar contents (Weisman and Wickner, 1988). A number of *vac* mutants have been isolated which are defective for the proper partitioning of vacuolar material in vivo (Weisman et al., 1990; Shaw and Wickner, 1991; Weisman and Wickner, 1992). These mutants define several complementation groups. The fact that *vac* mutations are specific for the vacuole and do not affect the inheritance of other organelles in *S. cerevisiae*, along with the observation that partitioning of various organelles occurs at different times during the cell cycle (Yaffe, 1991), indicates that a specific mechanism is involved in the inheritance of each organelle.

We have previously shown that the process of vacuole inheritance can be reconstituted in vitro using isolated vacuoles (Conradt et al., 1992). When incubated at physiological temperatures in the presence of salt, cytosol, and ATP, vacuoles isolated from wild-type cells form tubular and vesicular structures and assemble through vacuole-to-vacuole fusion. Vacuoles isolated from *vac* mutants, in contrast, fail to undergo these reactions and, instead, fragment during the in vitro incubation (Conradt et al., 1992; Haas et al., 1994). In the accompanying paper we describe a quantitative assay for the reaction of vacuole-to-vacuole fusion. We now show that the in vitro reaction can occur in four successive subreactions with distinct requirements and inhibitor sensitivities. These subreactions provide specific assays for activities required in the overall reaction.

Materials and Methods

Yeast Strains and Media

Vacuoles required for the in vitro reactions of vacuole-to-vacuole fusion were isolated from *S. cerevisiae* strains BJ3505 [*Matα pep4::HIS3 prbl-Δ1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can1*] (Moehle et al., 1986) and DKY6281 [*Matα ura3-52 leu2-3 leu2-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Δpho8::TRP1*]. DKY6281 was kindly provided by Dr. D. Klionsky (University of California, Davis, CA). Cytosol was prepared from the *S. cerevisiae* strain K91-1A [*Matα ura3 pho8::pAL134 pho13::pPH13 lys1*] which was provided by Dr. Y. Kaneko (Institute for Fermentation, Osaka, Japan). All strains were grown in rich medium (YEPD) at 30°C.

Materials

Oxalyticase was purchased from Enzogenetics (Corvallis, OR), DEAE-dextran and Ficoll 400 from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) and creatine phosphokinase, creatine phosphate, and guanosine 5′-*o*-(3-thiotriphosphate) (GTPγS)[1] from Boehringer Mannheim Corp. (Indianapolis, IN). ATP, apyrase (grade VIII), carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), and *para*-nitrophenyl phosphate (*p*NPP) were obtained from Sigma Chemical Company (St. Louis, MO). Bafilomycin A_1 was kindly provided by Dr. K. Altendorf (Universität Osnabrück, Osnabrück, FRG). Mastoparan7 was purchased from Peninsula Laboratories, Inc. (Belmont, CA) and microcystin-LR from LC Laboratories (Woburn, MA). Protein concentrations were measured using Bio-Rad protein assay reagents from Bio-Rad Laboratories (Richmond, CA).

1. *Abbreviations used in this paper*: CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; GTPγS, 5′-*o*-(3-thiotriphosphate); *p*NP, *p*-nitrophenol; *p*NPP, *para*-nitrophenyl phosphate.

In Vitro Reactions of Vacuole-to-Vacuole Fusion

Vacuoles were isolated from the strains BJ3505 and DKY6281 as described by Conradt et al. (1992), except that $MgCl_2$ was omitted from the vacuole isolation buffers. Cytosol was prepared according to Conradt et al. (1992) using the strain K91-1A as a source of cytosol (S100).

In vitro reactions contained 10 μl of each of the two types of vacuoles (BJ3505 and DKY6281 vacuoles) at a concentration of 0.25 mg/ml each. To these, salt, cytosol, ATP, and an ATP-regenerating system were added to final concentrations of 0.2 M sorbitol, 20 mM Pipes-KOH, pH 6.8, 100 mM KCl, 50 mM K(OAc), 5 mM $MgCl_2$, 2–3 mg/ml cytosol (S100), 1 mM ATP, 40 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase. The volume of the reactions was adjusted to 30 μl with vacuole isolation buffer (0.2 M sorbitol, 10 mM Pipes-KOH, pH 6.8). In vitro reactions were incubated for 2 h at 25°C, and then transferred to ice.

Alkaline Phosphatase (PHO8) Activity Assay

Vacuole inheritance in vitro was measured using the alkaline phosphatase maturation assay as described by Haas et al. (1994). Alkaline phosphatase activity was determined according to Mitchell et al. (1981) with the modifications described by Haas et al. (1994), except that 500 μl of the PHO8-substrate solution (250 mM TrisCl, pH 8.0, 0.4% Triton X-100, 10 mM $MgCl_2$, 1 mM *p*NPP) was added to each sample on ice. Reactions were transferred to 30°C and incubated for 3–10 min, and then stopped by the addition of 500 μl of 1 M glycine-KOH, pH 11.5. The amount of *p*-nitrophenol (*p*NP) was determined by measuring the OD_{400}. 1 U of alkaline phosphatase activity (1 U PHO8 activity) corresponds to 1 μmole of *p*NP released at 30°C per min and μg BF3505 vacuolar protein.

Results

Using two-stage incubations and order-of-addition experiments as described below, we can separate the in vitro reaction of vacuole inheritance into a sequence of four biochemically distinct subreactions (Fig. 1). The four stages have different requirements and are sensitive to different reagents. Moderate ionic strength (100 mM KCl, 50 mM K[OAc], 5 mM $MgCl_2$) is only needed during stage I, cytosol is required during stage II, and ATP during stage III. The stage IV reaction entails incubation of stage III vacuoles at a certain, minimal concentration and at physiological temperatures but requires no additional soluble components.

The Components of the In Vitro Reaction Are Required during Different Phases of a Standard Incubation

When measured using the alkaline phosphatase maturation assay (Haas et al., 1994), the in vitro reaction of vacuole inheritance requires vacuoles, cytosol, and ATP (Conradt et al., 1992; Haas et al., 1994). If complete reactions are prepared in buffer only without the regular mixture of salts (100 mM KCl, 50 mM K[OAc], 5 mM $MgCl_2$) and incubated

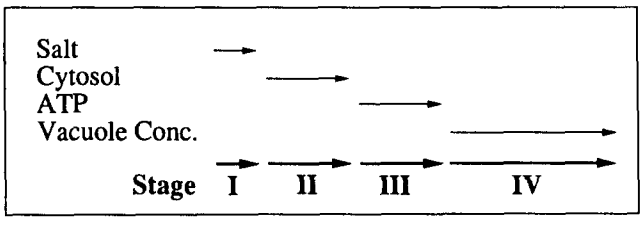
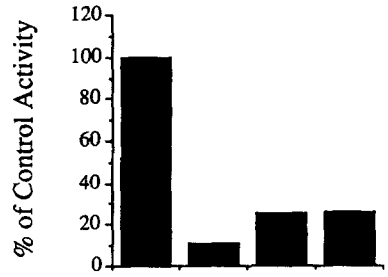


Figure 1. Working model for the requirements of the subreactions of vacuole inheritance in vitro. The in vitro reaction can be broken down into four biochemically distinct, sequential stages. See text for details.



Vacuoles [mg/ml]	0.17	0.17	0.17	0.017
Salt	+	+	-	+
Cytosol	+	+	+	+
ATP	+	+	+	+
Temperature [°C]	25	Ice	25	25

Figure 2. Requirements of the vacuole-to-vacuole fusion reaction as determined using the alkaline phosphatase maturation assay. In vitro reactions were prepared as described in Materials and Methods. Reactions were prepared in the standard volume of 30 μl with all components (vacuoles, [0.17 mg/ml], salt, cytosol, and ATP), without salt, or in 10-fold the standard volume (300 μl) with all components at the standard concentration except with 1/10 the concentration of vacuoles (0.017 mg/ml). The PHO8 activity of the complete reaction in the standard volume (0.98 U) was set to 100% activity.

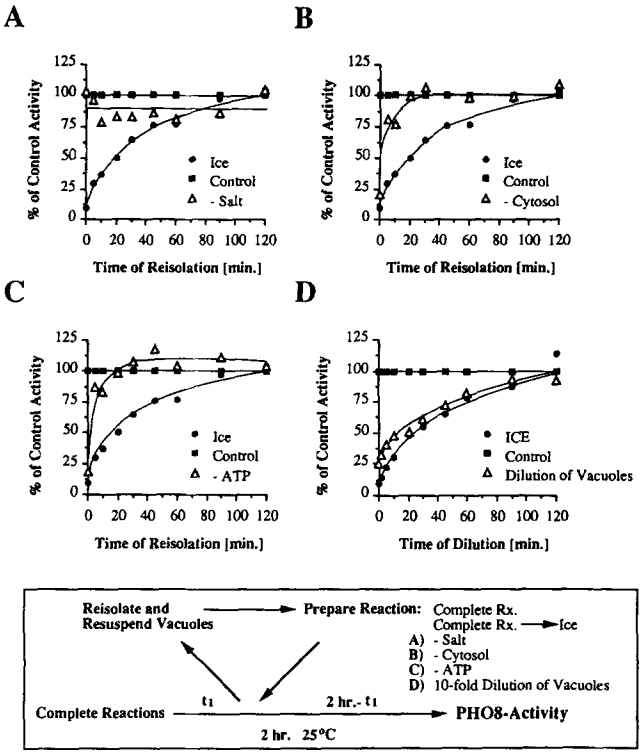


Figure 3. The components of the reaction are required during different phases of a standard incubation. Complete reactions were prepared as described in Materials and Methods. (*A–C*) A complete reaction corresponding to 50 reaction aliquots was prepared and incubated at 25°C for 2 h. At the time points indicated, an aliquot corresponding to five reactions was removed, vacuoles were sedimented using a microcentrifuge (10,000 *g*, 1 min, 4°C), resuspended in 100 μl of isolation buffer (0.2 M sorbitol, 10 mM Pipes-KOH, pH 6.8), and split into five aliquots of 20 μl. Two of these aliquots were made into complete reactions by adding salt, cytosol, and ATP. One of these was incubated at 25°C for the remainder of the 2-h incubation, the other was transferred to ice. All reaction components were added to the other three vacuole aliquots, except that either salt (*A*), cytosol (*B*), or ATP (*C*) was left out for the rest of the incubation at 25°C. Reactions were stopped by transferring them to ice. A complete reaction left on ice for 2 h was used as background. The units of PHO8 activity obtained from this reaction (0.092 U) was subtracted from the values obtained for the other samples. The alkaline phosphatase activity obtained for the vacuoles which were incubated in complete reactions at 25°C after reisolation (1.46 U ± 0.46 U; SD) was set to 100% activity. (*D*) A complete reaction corresponding to 30 standard reactions was prepared and incubated at 25°C for 2 h. At various times, three aliquots were removed from the main reaction and either put back to 25°C, transferred to ice, or diluted 10-fold (all components except vacuoles were kept at the same concentration) and incubated at 25°C for the remainder of the 2 h. Reactions were stopped by transferring them to ice. Before the addition of the alkaline phosphatase substrate solution, the vacuoles of the diluted reactions were sedimented (microcentrifuge, 5 min, 14,000 *g*, 4°C) and resuspended in 30 μl of isolation buffer. The values obtained for the undiluted reactions which were transferred back to 25°C (3.97 U ± 0.13 U; SD) were set to 100% activity. No background was subtracted.

under standard conditions (2 h, 25°C), or if a complete reaction is incubated on ice, little mature alkaline phosphatase is formed, indicating that little vacuole-to-vacuole fusion has occurred (Fig. 2). In addition, the concentration of vacuoles is critical for the in vitro reaction, since a standard amount of vacuoles incubated in 10 times the normal reaction volume gave only 22% the control units of alkaline phosphatase activity (Fig. 2).

To divide the in vitro reaction into biochemically distinct steps, we performed two-stage incubation experiments as outlined in Fig. 3. In these experiments, the components of a complete reaction were either removed (salt, cytosol, and ATP) or diluted (vacuoles) at various times during a standard incubation of 2 h. The effect which this had on the overall reaction was then used as a measure of when each particular component is required (Davidson and Balch, 1992). To remove a component, the vacuoles were reisolated by centrifugation and subsequent resuspension in buffer. To assure that this reisolation procedure did not interfere with the ongoing reaction one aliquot of the reisolated vacuoles was introduced into a complete reaction (salt, cytosol, and ATP) and incubated at 25°C for the remainder of the 2-h period. This set of reactions serves as the 100% or "control" activity curves in Fig. 3 (*A–D, filled squares*). To evaluate the effect of removing or diluting a component, we assayed the signal already generated at each time point during the 2-h incubation. For this purpose, a second reaction, comprised of vacuoles reisolated at each time point and introduced into a complete reaction, was transferred to ice instead to stop the reaction (Fig. 3, *A–D, filled circles*). If a component is only required at an early step, the reaction would not be inhibited by its late removal. If a component is required during a late phase of the reaction, its removal might arrest the reaction as effectively as ice at any time during the incubation.

Vacuoles can be shifted to low ionic strength almost im-

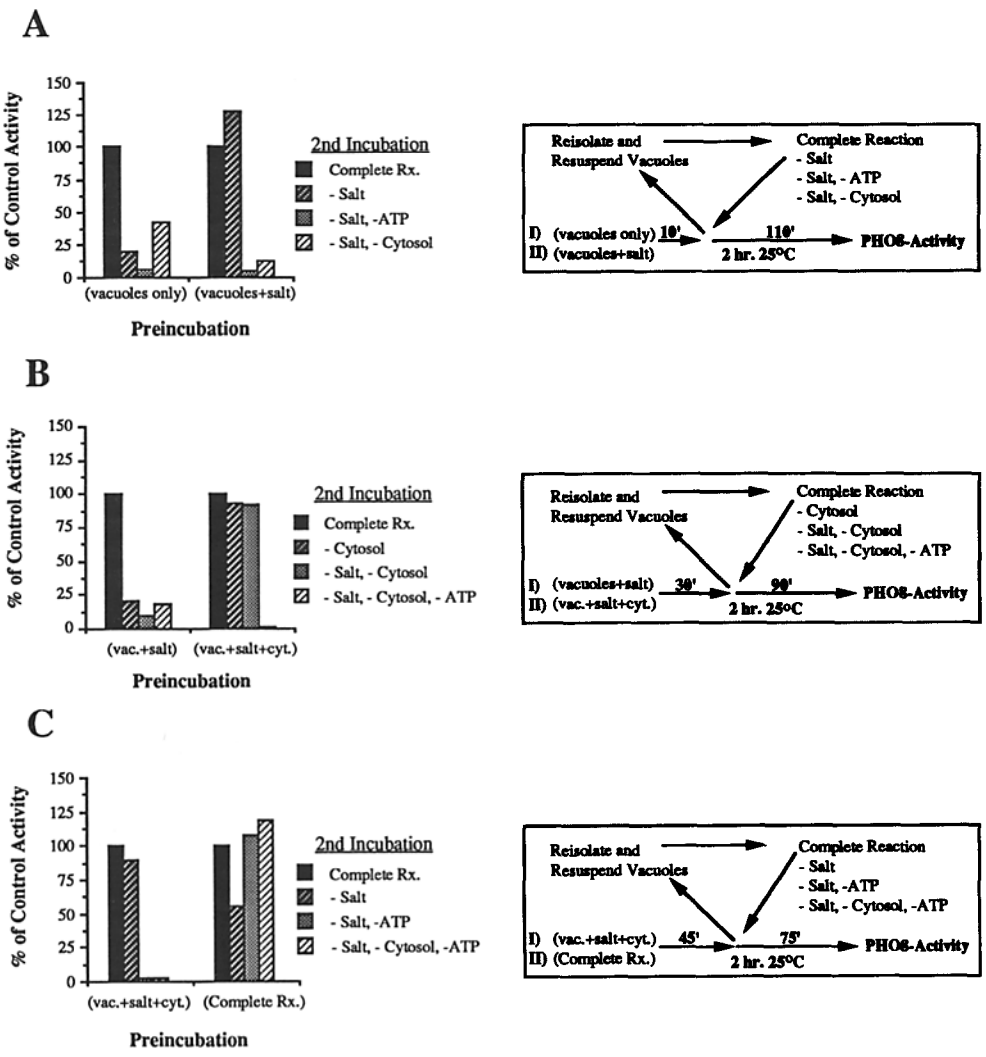


Figure 4. Requirements of each stage of the reaction. (*A*) A main reaction corresponding to eight reaction aliquots was prepared with either (*I*) vacuoles in isolation buffer only (0.2 M sorbitol, 10 mM Pipes-KOH, pH 6.8) or with (*II*) vacuoles and salt (0.2 M sorbitol, 20 mM Pipes-KOH, pH 6.8, 100 mM KCl, 50 mM KOAc, 5 mM $MgCl_2$). These were incubated for 10 min at 25°C and then transferred to ice. The vacuoles were sedimented in a microcentrifuge (10,000 *g*, 1 min, 4°C), and resuspended in isolation buffer. The vacuoles of reactions *I* and *II* were then divided into eight aliquots and made into complete reactions, or reactions in which either salt, salt and ATP, or salt and cytosol were omitted. One sample of each of these reactions was put on ice and one sample was transferred to 25°C and incubated for an additional 110 min. The reactions were stopped by chilling on ice. The PHO8 activity of the sample which was put on ice after the preincubation was used as background. The signal obtained for the complete reactions (*I*/0.653 U; *II*/1.661 U) was set to 100% activity. (*B*) Main reactions (eight reaction aliquots) were prepared containing either vacuoles and salt (*I*) or vacuoles, salt, and cytosol (*II*). These were incubated for 30 min at 25°C, transferred to ice, and vacuoles were collected by centrifugation. Vacuoles were resuspended and divided into eight aliquots each. Two samples of each of the following reactions were prepared: a complete reaction and reactions in which cytosol, salt and cytosol, or salt, cytosol, and ATP were omitted. One sample of each reaction was transferred to ice and served as background. The other sample was incubated at 25°C for an additional 90 min. The units of PHO8 activity measured for the complete reactions (*I*/0.837; *II*/1.081 U) were set to 100% activity. (*C*) Main reactions (eight reaction aliquots) containing either vacuoles, salt, and cytosol (*I*), or with all components present (*II*), were incubated for 45 min at 25°C, and then transferred to ice. Vacuoles were sedimented, resuspended and divided into eight aliquots. The following reactions were prepared in duplicate: a complete reaction, a reaction without salt, without salt or ATP, and without salt, ATP, or cytosol. One sample of each was transferred to ice and used as background. The other sample was incubated at 25°C for 75 min. The values of PHO8 activity obtained for the complete reactions (*I*/0.453 U; *II*/0.455 U) was set to 100%.

mediately after the start of the incubation at 25°C without affecting the ongoing reaction (Fig. 3 *A*, *open triangles*). Though the vacuoles must be exposed to solutions of moderate ionic strength (Fig. 2), this requirement is satisfied almost immediately during the reaction. Omission of either cytosol or ATP from the reaction after vacuole reisolation only caused a strong inhibition during the first few minutes (Fig. 3, *B* and *C*, *open triangles*). After 10–20 min, a time at which only 30–50% of the overall reaction has occurred, removal of cytosol or ATP has no effect. This indicates that cytosol and ATP are both required during early stages of the overall reaction. After a 20-min incubation at 25°C, the reaction has passed through these stages and has become cytosol and ATP independent.

To determine when the vacuole concentration is critical, we diluted the vacuoles 10-fold at various times throughout the 2-h incubation while all other components remained at the same concentration (Fig. 3 *D*, *open triangles*). Diluting the vacuoles is almost as effective as ice in inhibiting the reaction throughout the incubation. The concentration of vacuoles is therefore critical at a very late stage during the reaction; it may also be important at earlier stages.

Though individual reaction components may participate at more than one stage of the reaction, the data obtained from

these two-stage incubation experiments indicate that each component is last required at a defined step of the overall reaction. These studies suggest that there are at least three stages: a very early, brief stage which fulfills an ionic strength requirement, a second stage after which cytosol and ATP can be omitted, and a late stage which requires that the vacuoles be at least at some minimal concentration.

Stage I Requires Only Vacuoles and Moderate Ionic Strength

To determine which components are required for the stage I reaction, which yields vacuoles that can complete fusion at low ionic strength, we performed order-of-addition experiments as outlined in Fig. 4 *A*. Reactions containing either vacuoles only (I) or vacuoles and salt (II) were incubated for 10 min at 25°C. After this preincubation, the vacuoles were reisolated, resuspended, and divided into several aliquots. Reactions were prepared with either all components present or in which either salt, salt and cytosol, or salt and ATP were omitted. One sample of each reaction was transferred to ice as a control for any fusion that occurred during the 10-min preincubation period. The other reaction samples were returned to 25°C and incubated for an additional 110 min. The data in Fig. 4 *A* indicate that the vacuoles which were preincubated in isolation buffer only (10 mM Pipes-KOH, pH 6.8, 0.2 M sorbitol) require the presence of all components during the second incubation. When vacuoles were preincubated in the presence of salt, salt could be omitted during the second incubation without inhibiting the reaction. The reaction was still dependent on cytosol and ATP after the (vacuoles + salt) preincubation (Fig. 4 *A*). These results indicate that vacuoles and salt are sufficient for the stage I reaction, i.e., that cytosol and ATP are not required.

The Presence of Cytosol during Stage II Results in Cytosol Independence

To determine the requirements for stage II, either vacuoles and salt (I) or vacuoles, salt, and cytosol (II) were incubated for 30 min at 25°C (Fig. 4 *B*). After this preincubation, the vacuoles were reisolated, resuspended, and divided into aliquots. These were assayed either with all components, without cytosol, without salt and cytosol, or with neither salt, cytosol, nor ATP. The reactions were transferred to 25°C for an additional 90 min. One sample of each reaction was transferred to ice as a control. Preincubation of vacuoles with salt and cytosol fulfills the requirement for both salt and also for cytosol (Fig. 4 *B*). Though both salt and cytosol can be omitted in the second incubation, ATP is still required for the completion of the reaction. In contrast, if vacuoles and salt are preincubated, cytosol is still required during the second incubation. A (vacuoles + cytosol) preincubation also does not lead to cytosol independence (data not shown), indicating that stage I vacuoles are required for stage II to proceed. In addition, if vacuoles are preincubated with salt and ATP, no signal is obtained after the second incubation even in a complete reaction (data not shown). This suggests that this particular preincubation is unproductive and results in a loss of the ability of the vacuoles to fuse.

ATP Is Required during Stage III

If a complete reaction is incubated at 25°C for 45 min, the vacuoles can complete the fusion reaction in a second incubation in the absence of salt, cytosol, and ATP (Fig. 4 *C*). Stage IV of the reaction is therefore independent of these components and requires only stage III vacuoles. In contrast, if vacuoles are preincubated with salt and cytosol, ATP is still required during the second incubation (Fig. 4, *B* and *C*).

In summary, the two-stage incubation and order-of-addition experiments presented here suggest that there are four biochemically distinct stages occurring during vacuole inheritance in vitro: a very early stage which requires vacuoles and moderate ionic strength; a second stage which requires stage I vacuoles and cytosol; a third stage which requires stage II vacuoles and ATP, and a final stage which requires stage III vacuoles at a certain, minimal concentration.

Different Stages of the Reaction Are Differently Sensitive to Inhibitors

The in vitro reaction of vacuole inheritance is sensitive to bafilomycin A_1 and CCCP (Haas et al., 1994), compounds which interfere with the acidic pH in the vacuolar lumen and the electrochemical potential across the vacuolar membrane (Anraku et al., 1989; Bowman et al., 1989). The reaction is also sensitive to G protein ligands such as nonhydrolyzable GTP analogues and the amphiphilic peptide mastoparan (Haas et al., 1994) which affects the activity of certain heterotrimeric G proteins (Higashijima et al., 1988). In addition, the ATP-hydrolyzing enzyme apyrase and microcystin-LR, an inhibitor of protein phosphatases of type 1 and 2A (Honkanen et al., 1990), block the overall vacuole-to-vacuole fusion reaction (data not shown; Conradt et al., 1992).

To determine whether these reagents inhibit the reaction throughout a 90-min incubation or only during certain phases, two-stage incubation experiments were performed (Fig. 5). Complete reactions were incubated for 90 min at 25°C. At various times during the incubation, individual inhibitors were added to single reactions. To two reactions, buffer was added as a control and one was incubated at 25°C to serve as 100% control activity while the second was placed on ice, as shown in Fig. 3. According to their behavior in this two-stage incubation experiment, the various inhibitors can be divided into two groups. Apyrase, bafilomycin A_1, CCCP, GTPγS, and mastoparan appear to affect early or intermediate stages of the in vitro reaction. Despite the fact that there are consistent differences in the kinetics with which these reagents inhibit the reaction (with a $t_{1/2}$ of inhibition of 25 min for apyrase, 15–20 min for bafilomycin A_1 and CCCP, and 12 min for GTPγS and mastoparan) they all block a stage (or stages) which is completed after 30–60 min of incubation (Fig. 5). In contrast to these reagents, the addition of the protein phosphatase inhibitor microcystin-LR blocked any further increase in signal throughout the length of the experiment. This suggests that microcystin-LR probably blocks the last stage (though possibly also earlier stages) of the overall reaction, and that the other reagents tested affect stages that precede this last, microcystin-LR–sensitive stage. None of the inhibitors showed an effect when added at 90 min, indicating that their ability to inhibit was due to blocking the fusion reaction and was not caused by interfering with the alkaline phosphatase activity assay. These results suggest that the vacuole inheritance reaction is sensi-

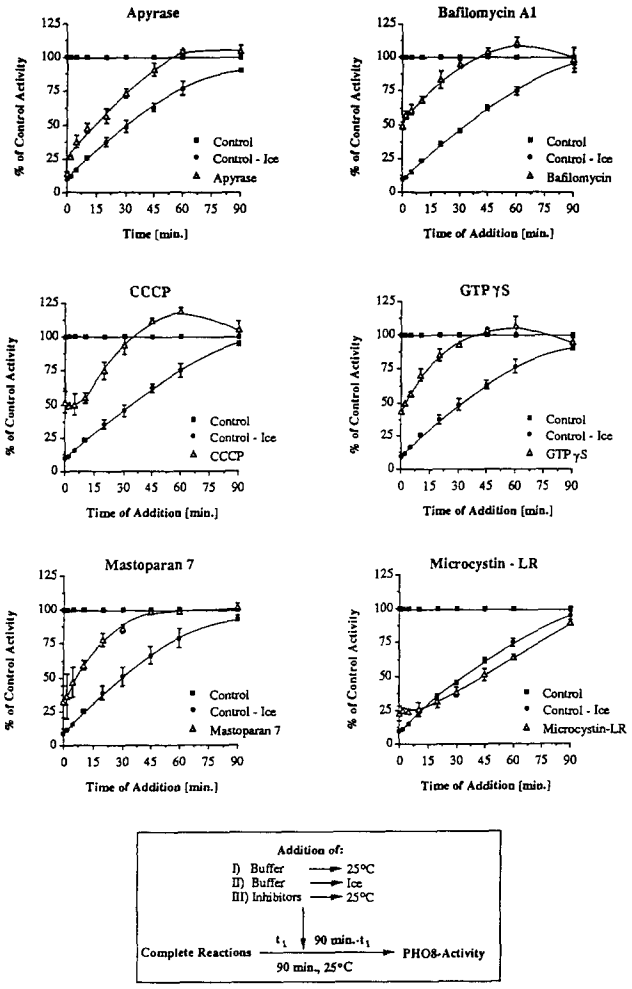


Figure 5. Inhibitors block different phases of the in vitro reaction. A complete reaction corresponding to 80 standard reactions was prepared as described in Materials and Methods and was incubated at 25°C for 90 min. At the indicated time points, eight reaction aliquots were removed. To two of these, buffer was added (*I* and *II*). One was transferred to 25°C (*I*) while the other was transferred to ice (*II*). To other reaction aliquots (*III*), individual inhibitors were added at the following concentrations: 16.7 U/ml apyrase, 10 μM bafilomycin A_1, 20 μM CCCP, 1 mM GTPγS, 15 μM Mas7, and 15 μM microcystin-LR. The reactions were incubated at 25°C for the remainder of the 90 min and stopped by transferring to ice. The PHO8 activity obtained for reaction I at a given time point (which was 2.54 U ± 0.19 U, 2.23 U ± 0.14 U, 4.37 U ± 0.61 U, and 1.86 U ± 0.20 U in four independent experiments) was set to 100% control activity. The data shown is expressed as percent of control activity and represents average values and error of two independent experiments.

tive to the inhibitors tested in this study during different phases of the overall reaction.

To determine which of the specific stages I-IV of the reaction might be sensitive to a particular inhibitor, we performed the reaction in a staged manner as outlined in Fig. 6. This experiment allowed us to determine the specific stage after which the reaction becomes insensitive to a particular agent. The effect of the inhibitors was assayed after each of the four stages of the reaction. As a control for this "staged" experiment, we measured the effect of the inhibitors on the overall reaction by adding them to complete reactions at the beginning of a 2-h incubation (Fig. 6, reactions *I-IV* in each panel). A stage I reaction, containing vacuoles and salt, was prepared and incubated for 10 min at 25°C. After this first incubation, an aliquot was removed and made complete by adding cytosol and ATP. This complete reaction was divided into eight reaction aliquots of standard volume. To six reaction aliquots, individual inhibitors were added. These reactions were then returned to 25°C for an additional 110 min to allow stage II-IV to proceed (Fig. 6, reactions *II-IV* in each panel, *open triangles*). To the last two aliquots, buffer was added and one aliquot was placed at 25°C for 110 min as a control for maximal fusion during the second incubation (Fig. 6, reactions *II-IV, filled circles*) while the other reaction was transferred to ice as a control for the signal generated during stage I (*filled squares*). To the main reaction, cytosol was added to allow stage II to proceed. After an incubation of 20 min at 25°C, an aliquot was again removed and made into a complete reaction by adding ATP. The same eight reactions were prepared, including the two control reactions, and incubated at 25° or 0°C for an additional 90 min to allow stage III and IV to occur (Fig. 6, *III-IV*). In the meantime, ATP was added to the main reaction, and stage III was allowed to proceed for 30 min. After this third incubation, inhibitors were added and the incubation continued for an additional 60 min to allow stage IV to be completed (Fig. 6, *IVa*). The remaining main reaction was centrifuged to reisolate the stage III vacuoles. These vacuoles were resuspended in buffer, and inhibitors were added to aliquots before an additional incubation of 60 min at 25°C (Fig. 6, *IVb*). With this experiment, we determined to what degree a reagent could after each stage, still block any subsequent fusion reaction when compared to the inhibition caused by transferring to ice. The data presented are average values (and deviations) of alkaline phosphatase activities obtained from duplicate reactions in one representative experiment. Similar results were seen in several independent experiments. The signal obtained for the control reaction decreases in the Fig. 6, II-IV, III-IV, and IVa and IVb reactions when compared to the unstaged reaction. This is due to the shorter incubation periods in the presence of all components in these III-IV and IV reactions and to some loss in efficiency when performing the in vitro reaction in a staged manner. Furthermore, the vacuoles of reaction IVb were reisolated before the last incubation which causes some loss of vacuolar membranes and therefore additional loss of signal. The activities obtained for the ice reactions after the various stages are very low until stage III. At the beginning of stage III, ATP is added to the reaction which allows fusion initiation and alkaline phosphatase maturation. This causes the increased alkaline phosphatase activity of the ice control after stage III.

Each of the agents tested inhibited the reaction when present throughout stages I-IV, or even when only added after the incubation with salt (i.e., Fig. 6, during stages *II-IV*), or after the incubation with salt and cytosol (during stages *III-IV*). Sensitivity to these reagents is therefore not limited to stages I and II of the reaction. A distinct pattern of inhibition was seen during stage IV, and this pattern was, for some inhibitors, influenced by whether vacuoles were incubated in isolation (Fig. 6, reactions *IVb*) or in the continued presence of the other reaction components (*IVa*). Apyrase, bafilomycin

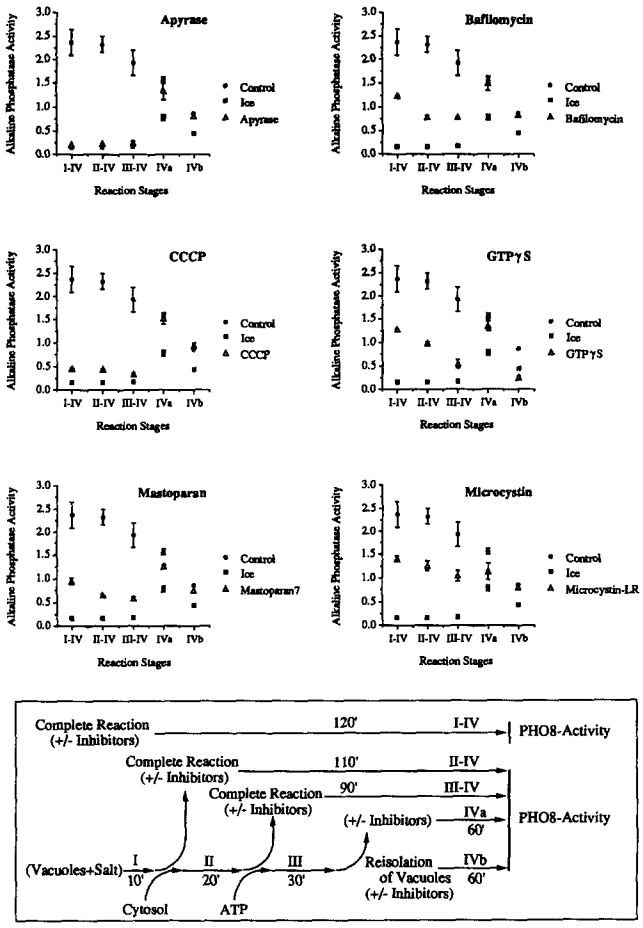


Figure 6. Inhibitors block distinct stages of the vacuole inheritance reaction. As a control for the experiment which was performed in a staged manner, eight complete reactions were prepared as described in Materials and Methods. To two of these, buffer was added, and one reaction was transferred to ice whereas the other reaction was incubated at 25°C for 2 h (*I–IV*). To the rest of the reactions, inhibitors were added at the following concentrations: 16.7 U/ml apyrase, 10 μM bafilomycin A_1, 20 μM CCCP, 1 mM GTPγS, 15 μM Mas7, and 15 μM microcystin-LR. These reactions were also incubated for 2 h at 25°C (*I–IV*) before being stopped by transferring to ice. For the staged incubation, a reaction (32 standard aliquots) containing vacuoles and salt was prepared and incubated for 10 min at 25°C after which an aliquot of eight reaction volumes was removed. This aliquot was made complete by adding cytosol and ATP. The aliquot was divided into eight reactions and the same reactions were prepared as for the control experiment: to two samples, buffer was added and one of these was put on ice, to the other six samples, individual inhibitors were added. These reactions were incubated at 25°C for an additional 110 min (*II–IV*). To the main reaction, cytosol was added. After a second incubation for 20 min at 25°C an aliquot of eight samples was again removed. ATP was added to this aliquot which was then divided into eight samples. The same eight reactions were prepared as described above and incubated for an additional 90 min (*III–IV*). To the main reaction, ATP was added. After a third incubation of 30 min, the reaction was split into two aliquots of eight reactions each. One aliquot was divided into eight reactions, and buffer and inhibitors were added to these before a fourth incubation at 25°C for 60 min. (*IVa*). The vacuoles of the other aliquot were sedimented, resuspended in isolation buffer, and divided into eight reaction aliquots. To these, buffer and inhibitors were added which was followed by an additional incubation of 60 min at 25°C (*IVb*). The reactions were stopped and the alkaline phosphatase activity was determined. The data shown are alkaline phosphatase activities and represent average values and deviations of duplicate reactions from one representative experiment. Depending on the experiment and the vacuole preparation, the alkaline phosphatase activity obtained for the control reaction of the unstaged experiment (*I–IV*) varied from 1.146 to 2.576 U and for the ice control from 0.145 to 0.184 U.

A_1, and CCCP had little effect during the stage IV reaction. Stage III of the reaction apparently alters the sensitivity of the reaction to these agents. In contrast, microcystin-LR, mastoparan, and GTPγS all show some inhibition during stage IV, and this inhibition was influenced by the presence or absence of the other reaction components. Compared to the inhibition caused by GTPγS in the complete, unstaged reaction (Fig. 6, *I-IV*), the reaction is more sensitive to GTPγS when added after stage I (*II-IV*), and even more so when added after stage II (*III-IV*) in the staged reaction. We do not know why the staged reaction becomes more sensitive to GTPγS during stage I and II. Data presented in the accompanying paper (Haas et al., 1994) suggest that multiple G proteins might be involved during vacuole segregation in vitro. It is possible that G proteins have stimulatory as well as inhibitory activities with respect to the in vitro reaction which might explain the complex behavior observed in the presence of GTPγS. After stage III (Fig. 6, *IVa* and *IVb*), the addition of GTPγS has no effect on the further reaction if the soluble reaction components are present during stage IV (*IVa*). If these components are removed by reisolating the vacuoles after stage III (Fig. 6, *IVb*), GTPγS strongly inhibits stage IV. We have not yet further investigated the basis for this inhibition. Microcystin-LR inhibits ~50% of the reaction when added to the unstaged reaction (*I-IV*), after stage I (*II-IV*), or after stage II (*III-IV*). If the soluble components are removed after stage III, stage IV is resistant to microcystin-LR (Fig. 6, *IVb*), but if the components are left in the reaction, stage IV is still sensitive to this reagent (*IVa*). In summary, the data obtained indicate that stages I and II of the reaction do not cause resistance to the agents tested and suggest that stages III and IV are the stages actually being affected by the inhibitors.

To further investigate the effects of microcystin-LR on stage IV, complete reactions were incubated at 25°C to generate stage IV-competent vacuoles. This incubation, during which only ~50% of the total signal was generated, resulted in the completion of stages I-III. This was indicated by the observation that, after reisolation, these vacuoles could complete the reaction in the absence of any soluble reaction components, such as medium ionic strength (*salt*), cytosol, and ATP, which is characteristic of stage IV (Fig. 7, *vacuoles* only, *filled bars*). The addition of either ATP or cytosol to the stage IV-competent vacuoles during the second incubation had little effect on the stage IV reaction while the addition of salt had a modest stimulatory effect.

To determine which of the soluble reaction components caused susceptibility to microcystin-LR during stage IV, we added microcystin-LR to stage IV-competent vacuoles in the presence of all soluble components (salt, cytosol, and ATP), in the presence of either ATP, cytosol, or salt, or in the absence of all soluble components, i.e., to the stage IV-competent vacuoles only. The stage IV reaction is very sensitive to

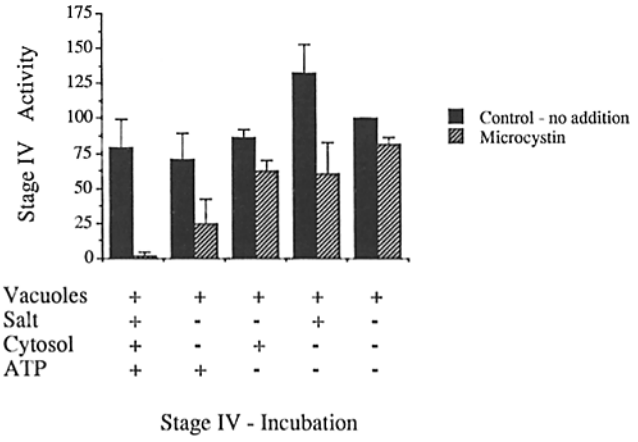


Figure 7. Stage IV sensitivity to microcystin-LR. A large complete reaction corresponding to 20 reaction aliquots was prepared as described in Materials and Methods and incubated for 30–45 min at 25°C. Vacuoles were collected by centrifugation, resuspended in buffer, and then split into five reactions of three aliquots each. To these either all soluble components (ATP, cytosol, and salt) of the reaction were added, only one at a time was added (ATP, cytosol, or salt) or none were added (vacuoles only). Each of these five reactions was then split into three aliquots. To two aliquots, buffer was added and either returned to 25°C or transferred to ice for an additional 75–90 min. To the remaining aliquot 10 μM microcystin-LR was added before returning the reaction to 25°C for an additional 75–90 min. The reactions were stopped by transferring them to ice. The PHO8 signal obtained for the (vacuoles only) control reaction which was returned to 25°C after the first incubation (1.10 U) was set to 100% Stage IV Activity after subtracting the PHO8 signal obtained for the control reaction which was transferred to ice instead (0.58 U). The data presented is expressed as percentage of Stage IV Activity and represents average values and SD of four independent experiments.

microcystin-LR in the presence of the complete set of reaction components but not in their absence (Figs. 6 and 7). In the presence of salt, microcystin-LR causes a 50% inhibition of the stage IV reaction. The addition of cytosol has only a minor effect. The strongest effect is seen with the addition of ATP, which allows >70% inhibition of the stage IV reaction by microcystin-LR. Complete susceptibility is only seen in the presence of all components, suggesting that they might act in synergy to cause sensitivity to microcystin-LR. These results suggest that a type 1 or 2A protein phosphatase activity is required during stage IV in complete reactions but not in reactions with vacuoles only. A protein which is associated with the vacuoles after stage III and which is required in its dephosphorylated state for stage IV could be a target for a cytosolic kinase activity in complete reactions. The presence of microcystin-LR in complete reactions would therefore cause an increase in the phosphorylated form of this protein which, after reaching a critical level, could inhibit the stage IV reaction. That some of this kinase activity might actually be associated with the vacuolar membrane after reisolation is suggested by the observation that the addition of ATP (which stimulates kinase activity) causes partial susceptibility of the reaction to microcystin-LR.

The knowledge of the susceptibilities of stage IV vacuoles to phosphatase inhibitors will allow us to develop assays to determine the nature of the factors involved in these specific, fusion-inhibiting reactions.

Discussion

The process of vacuole inheritance involves the formation of membranous, tubulovesicular structures and a membrane fusion event. At the cellular level, vacuole inheritance therefore resembles the vesicular trafficking events of the secretory and endocytic pathway. The reconstitution of these transport events in vitro have led to the characterization and isolation of soluble and membrane-bound factors involved in various stages of intracellular transport and have illuminated their basic mechanisms (Gruenberg and Howell, 1989; Schmid, 1992; Rothman and Orci, 1992; Pryer et al., 1992). The identification of subreactions and intermediates, and the characterization of their susceptibilities to inhibitors, was a major step towards the functional isolation and characterization of required factors (Balch et al., 1984; Melançon et al., 1987; Groesch et al., 1990; Beckeres et al., 1990; Rexach and Schekman, 1991; Schmid and Smythe, 1992, Davidson and Balch, 1993). These include NSF, the *N*-ethylmaleimide-sensitive factor (Block et al., 1988; Malhotra et al., 1988), SNAPs, the soluble NSF-attachment proteins (Weidman et al., 1989; Clary et al., 1990), and the isolation of various functional *SEC* proteins from *S. cerevisiae* (Hicke et al., 1992; Pryer et al., 1993).

The in vitro reaction of vacuole inheritance can be staged into four biochemically distinct subreactions. The overall staged reaction proceeds with an efficiency of 40–60% when compared to a complete, standard reaction. It is not established, however, whether these defined stages occur in this order in vivo.

The requirements for the various components of the in vitro reaction of vacuole inheritance (salt, cytosol, ATP, and concentrated vacuoles) can be kinetically separated. Solutions of moderate ionic strength are required during stage I. The incubation of vacuoles in such a solution appears to "prime" the vacuoles, possibly by influencing the electrochemical potential across its membrane, by activating a component of the organelle, or by promoting the dissociation of an inhibitory component. This priming step might allow the subsequent binding of a cytosolic component or the assembly of a complex of cytosolic factors ("pre-fusion" complex) on the vacuolar membrane during stage II. Alternatively, cytosolic components may not bind or assemble during stage II, but may activate certain vacuolar proteins. An exogenous energy source is not required during stage I and stage II, though low amounts of nucleotides present in the cytosol could serve a regulatory function during stage II. Stage III requires energy, provided by ATP. Until stage III, very little mature alkaline phosphatase is generated when the reaction is performed in a staged manner as shown in Fig. 6. This is supported by the microscopic observation that vacuoles do not undergo noticeable enlargements until stage III (unpublished observations). ATP therefore appears to be required for the initiation of vacuole-to-vacuole fusion, possibly by activating pre-fusion complexes on the vacuole. Alternatively, ATP might drive the electrochemical potential across the vacuolar membrane and therefore directly or indirectly activate vacuoles for the fusion reaction. Stage IV of the overall reaction represents the stage during which the

actual fusion event occurs. Though independent of added soluble components, stage IV is sensitive to vacuole dilution. This suggests that activated pre-fusion complexes, which are able to induce fusion, assemble on the vacuoles during stages II and III. Through these complexes, vacuoles interact with each other and eventually fuse during stage IV. The dilution sensitivity indicates that these vacuole–vacuole interactions can be dissociated until the actual fusion event.

Similar studies have led to the characterization of subreactions in transport between the ER and the Golgi, or within the Golgi apparatus in mammalian cells and in *S. cerevisiae* (Beckers et al., 1990; Balch et al., 1984; Groesch et al., 1990; Rexach and Schekman, 1991). Recent studies indicate that an early stage of these in vitro reactions, the formation and budding of transport vesicles, requires active membranes, cytosolic factors, and GTP as a regulatory component (Orci et al., 1993; Ostermann et al., 1993; Salama et al., 1993). ATP does not appear to be required as an energy source during this early stage. A second stage in ER to Golgi and inter-Golgi transport, the targeting and docking of transport vesicles to the acceptor membrane, requires the presence of additional cytosolic components which are recruited onto the vesicles during transport or after attachment (Bacon et al., 1989; Baker et al., 1990; Rexach and Schekman, 1991; Griff et al., 1992; Söllner et al., 1993*a*,*b*). The actual fusion process of transport vesicles with the acceptor membranes requires these bound cytosolic factors and ATP (Rexach and Schekman, 1991; Griff et al., 1992; Wilson et al., 1992; Söllner et al., 1993*a*,*b*).

Stage II of the vacuole-to-vacuole fusion reaction requires salt primed, stage I vacuoles, and cytosol. This stage might therefore correspond to the first step in ER to Golgi and inter-Golgi transport which is the budding step and which in the case of vacuole inheritance could represent the stage during which segregation structures are formed. GTP which is necessary for this step in the secretory pathway, could be provided by the unfiltered cytosol (S100) in our system. In contrast to the reaction of ER to Golgi and inter-Golgi transport, which require additional cytosolic factors during later stages, stage II fulfills the cytosol requirement of the overall vacuole inheritance reaction. It is possible that all cytosolic factors required for the vacuole inheritance reaction are able to assemble into pre-fusion complexes on the vacuole during stage II and that a step equivalent to the targeting step in ER to Golgi and inter-Golgi transport might therefore be included in stage II. Alternatively, the in vitro reaction of vacuole inheritance might not require additional cytosolic factors during later stages.

The actual vacuole-to-vacuole fusion events are activated during stage III of the vacuole inheritance reaction and proceeds during stage IV. These stages are similar to the third stage of ER to Golgi and inter-Golgi transport in that they represent the stages during which energy is required in the form of ATP and during which fusion occurs. Whereas the ATP requiring stage and the stage during which fusion occurs cannot be kinetically separated in ER to Golgi and inter-Golgi transport, they represent distinct stages in the vacuole inheritance reaction. It is possible that an ATP-hydrolyzing enzyme, whose activity is required for fusion, binds ATP during stage III. During stage IV, the hydrolysis of the bound ATP could provide energy required for the subsequent fusion event. NSF which is involved during membrane fusion in various vesicular trafficking events (Rothman and Orci, 1992; Pryer et al., 1992), has been shown to be an ATPase (Tagaya et al., 1993). So far, we have no evidence for the involvement of sec18, the *S. cerevisiae* NSF homologue, in vacuole inheritance in vitro (unpublished observation). Stage IV of the vacuole inheritance reaction is independent of any added soluble components, but is still sensitive to vacuole dilution. The pre-fusion complexes assembled and activated during stage II and III can persist on the membrane during vacuole reisolation and therefore appear to be quite stable. The observation that stage IV nevertheless is dilution sensitive indicates that the activation of prefusion complex does not cause stable vacuole–vacuole interactions.

The four stages defined for the in vitro reaction of vacuole inheritance are susceptible to different reagents. The reaction is insensitive to bafilomycin A_1 and CCCP after completion of stage III. Bafilomycin A_1 and CCCP affect the electrochemical potential across the vacuolar membrane (Anraku et al., 1989; Bowman et al., 1989). These results suggest that an active vacuolar ATPase and a potential across the vacuolar membrane are important for the overall reaction until the completion of stage III, a stage which represents the initiation stage of vacuole fusion. During stage IV, the dissipation of the electrochemical potential no longer has an inhibitory effect on the fusion reaction. When added to complete reactions, as shown in the accompanying paper, bafilomycin A_1 and CCCP cause the vacuoles to aggregate (Haas et al., 1994). Considering the results we obtained from the staging experiments (Figs. 5 and 6), these aggregates might represent vacuoles which associate through prefusion complexes and accumulate because fusion-initiation (stage III) is blocked. The potential across the vacuolar membrane is therefore likely to play a role in fusion initiation (stage III), but not in the actual membrane fusion event (stage IV). It has recently been shown that vacuolar ATPase activity is also required for the in vitro formation of vesicular intermediates in early to late endosome transport in mammalian cells (Clague et al., 1993). Along with the data presented here, this suggests that vacuolar ATPase activity is required for efficient transport between endocytic compartments. The reaction is only partially inhibited (20%) by apyrase after stage III in a complete reaction, supporting the finding that the addition of ATP is no longer required after stage III.

Studies with mastoparans and GTPγS suggest that G proteins might be involved in the vacuole inheritance reaction in vitro (Haas et al., 1994). We now show that these reagents interfere with specific stages of the in vitro reaction. The amphiphilic peptide mastoparan still inhibits ~40% of the reaction during stage IV in a complete reaction, suggesting that the mastoparan target is probably involved in both early and late stages of the reaction. Its target might be involved in the assembly of pre-fusion complexes by providing an essential component or by regulating the assembly process. When mastoparan is present in complete reactions, vacuoles initiate the formation of segregation structures like vacuoles in control reactions but fail to undergo fusion (Haas et al., 1994). Instead, continuing structure formation eventually leads to the complete fragmentation of the organelles. This observation supports the idea that the target of mastoparan might be involved in regulating the transition from stage II to stage III. As discussed in the accompanying paper, this

target is likely to be heterotrimeric G proteins which, in a number of systems, have been implicated in the regulation of intracellular trafficking processes in vivo (Stow et al., 1991; Leyte et al., 1992). In addition, mastoparan has been shown to inhibit various transport reactions in vitro (Barr et al., 1991; Donaldson et al., 1991; Colombo et al., 1992; Ktistakis et al., 1992; Schwaninger et al., 1992; Pimplikar and Simons, 1993; Carter et al., 1993). Kinetic studies of ER to Golgi transport in mammalian cells have indicated that mastoparan acts during an early stage of the reaction, possibly the stage of vesicle formation, and that the reaction becomes resistant to the addition of the peptide after this early stage (Schwaninger et al., 1992). Our data suggests that mastoparan interferes with the regulation of the formation of segregation structures, which is equivalent to the step of transport vesicle formation. In contrast, the vacuole inheritance reaction remains sensitive to mastoparan at later stages. The mastoparan target could therefore be involved in both the regulation of segregation structure formation and in the actual initiation of fusion.

In ER to Golgi and inter-Golgi transport, coated transport vesicles accumulate in the presence of GTPγS (Melançon et al., 1987; Baker et al., 1988; Ruohola et al., 1989, Beckers and Balch, 1989). The formation of transport vesicles itself, as well as the actual fusion reaction, do not appear to be affected. Even after removal of GTPγS, these vesicles remain inactive and cannot fuse with the acceptor membranes. It has therefore been suggested that coats formed in the presence of GTPγS are irreversibly assembled and cannot be uncoated (Melançon et al., 1987; Orci et al., 1989), causing a permanent inactivation of the transport vesicles. GTPγS inhibits the vacuole inheritance reaction, both when assayed after stage I and even more so when assayed after stage II, suggesting that the system becomes more sensitive to GTPγS during stage II. As discussed above, we suggest that cytosolic factors required for the targeting and/or fusion of segregation structures assemble into pre-fusion complexes on the vacuolar membrane during stage II. These factors could include components of a structural coat which might cause the increase in susceptibility to GTPγS after stage II. In the presence of GTPγS, vacuoles appear to form segregation structures (data not shown) and then aggregate (Haas et al., 1994). Similar to the accumulation of coated transport vesicles, these aggregates could represent "coated" segregation structures blocked in their ability to fuse. The reaction is resistant to GTPγS after stage III, indicating that a reaction similar to an uncoating reaction might occur during stage III. In order to acquire resistance, the reaction requires the presence of the soluble reaction components. We do not yet know the chemical basis for this observation.

The reaction of vacuole inheritance can also be blocked by inhibitors of protein phosphatases of type 1 and 2A, indicating that a phosphoprotein in its dephosphorylated form might be involved in the reaction. Various transport reactions have been shown to be sensitive to inhibitors of protein phosphatases and protein kinases (Lucocq et al., 1991; Woodman et al., 1992; Davidson et al., 1992; Stuart et al., 1993; Davidson and Balch, 1993). It has been shown, for example, that microcystin-LR blocks an early step (possibly the step of transport vesicle formation or of vesicle targeting) of both ER to Golgi and inter-Golgi transport in vitro in mammalian cells (Davidson et al., 1992; Davidson and Balch, 1993). The kinetic data presented here indicate that microcystin-LR inhibits a late stage of the vacuole inheritance reaction, possibly by blocking fusion during stage IV (Figs. 5 and 6). When assayed in the staged experiment (Fig. 6), stage IV was resistant to microcystin-LR when the reaction components were removed from the reaction. When present, stage IV was very sensitive to microcystin-LR, which is in contrast to all the other reagents investigated. The susceptibility to microcystin-LR of complete reactions during stage IV can be partially mimicked by the addition of ATP as shown in Fig. 7. As discussed above, we propose that a phosphoprotein which is required in its dephosphorylated state for fusion during stage IV and which is associated with the vacuole is the target of opposing phosphatase and kinase activities.

As in control reactions, vacuoles form segregation structures in the presence of microcystin-LR. Some vacuoles appear to undergo fusion, but fusion does not occur to the extent found in control reactions, confirming that microcystin-LR blocks a late stage of the fusion reaction (data not shown).

We have been able to define four biochemically distinct subreactions occurring during vacuole inheritance in vitro. These stages might correspond to morphological changes which vacuoles undergo during vacuole inheritance in vivo and in vitro. The defined stages have different requirements and are susceptible to various reagents which will allow us to develop assays for the isolation of specific enzymatic activities required during the various subreactions.

We thank Marilyn Leonard, Douglas Geissert, and Karen Douville for excellent technical support and Drs. R. Arkowitz, T. Economou, and Z. Xu for many helpful discussions. We are grateful to Drs. K. Altendorf (Universität Osnabrück, Osnabrück, FRG) for providing bafilomycin A_1, Y. Kaneko (Institute for Fermentation, Osaka, Japan) for the strain K91-1A, and D. Klionsky (University of California, Davis, CA) for strain DKY6281.

This work was supported by a predoctoral fellowship of the Studienstiftung des deutschen Volkes to B. Conradt, a postdoctoral fellowship of the Deutsche Forschungsgemeinschaft to A. Haas, and a grant from the National Institute of General Medical Science.

Received for publication 20 December 1993 and in revised form 4 April 1994.

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