In Vitro Reactions of Vacuole Inheritance in *Saccharomyces cerevisiae*

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Abstract. Vacuole inheritance is temporally coordinated with the cell cycle and is restricted spatially to an axis between the maternal vacuole and the bud. The new bud vacuole is founded by a stream of vacuole-derived membranous vesicles and tubules which are transported from the mother cell into the bud to form the daughter organelle. We now report in vitro formation of vacuole-derived tubules and vesicles. In semi-intact cells, formation of tubulovesicular structures requires ATP and the proteins encoded by *VAC1* and *VAC2*, two genes which are required for vacuole inheritance in vivo. Isolation of vacuoles from cell lysates before in vitro incubation reveals that formation of tubulovesicular structures requires cytosol as well as ATP. After forming tubulovesicular structures, isolated vacuoles subsequently increase in size. Biochemical assays reveal that this increase results from vacuole to vacuole fusion, leading to mixing of organellar contents. Intervacuolar fusion is sensitive to the phosphatase inhibitors microcystin-LR and okadaic acid, suggesting that protein phosphorylation/dephosphorylation reactions play a role in this event.

CYTOPLASMIC organelles are inherited rather than synthesized de novo. To ensure that each daughter cell receives each type of organelle, special mechanisms must coordinate organelle division with the cell cycle and ensure proper spatial partitioning of organelles between cells (Birkey, 1983; Warren, 1985). Cytological studies in mammalian cells revealed that low copy number organelles like the endoplasmic reticulum and the Golgi apparatus vesiculate at the start of mitosis (Zeligs and Wollman, 1979; Lucocq and Warren, 1987). The vesicles partition and fuse to reconstitute their organelle of origin in the daughter cells after mitosis (Lucocq et al., 1989; Ho et al., 1989). In the case of mammalian lysosomes and endosomes, a dispersal of the otherwise clustered, perinuclear organelles is observed early during cell division, followed by a rapid reclustering in the daughter cells at the end of mitosis (Matteoni and Kreis, 1987).

Similar studies have been performed in the budding yeast *Saccharomyces cerevisiae* (Yaffe, 1991). In *S. cerevisiae*, as in mammalian cells, the endoplasmic reticulum and the Golgi apparatus are low copy number organelles. However, a cell cycle–regulated fragmentation and reassembly of these organelles is not observed (Preuss et al., 1991; Redding et al., 1991). Cytological and genetic studies have also been performed in *S. cerevisiae* on the inheritance of mitochondria (Stevens, 1981; McConnell et al., 1990; Pon and Schatz, 1991) and on the inheritance of vacuoles, the acidic lysosomal-like compartments of these cells (Weisman et al., 1987; Weisman and Wickner, 1988; Weisman et al., 1990; Raymond et al., 1990; Shaw and Wickner, 1991; Gomes de Mesquita et al., 1991). Mutants have been isolated which are defective in the inheritance of either mitochondria or vacuoles (McConnell et al., 1990; Steward and Yaffe, 1991; Weisman et al., 1990; Shaw and Wickner, 1991; Weisman and Wickner, 1992). Detailed cytological studies revealed that the yeast vacuole projects tubular and vesicular structures which enter the emerging bud during S phase (Weisman and Wickner, 1988). Maternal, vacuolar material is transported via these "segregation structures" until G2/M phase.

We have characterized two mutants which are defective in vacuole inheritance. *vac*1-1 was isolated from a collection of mutants that secrete the soluble, vacuolar protein carboxypeptidase Y (CPY)[1] (Weisman et al., 1990). The *vac*1-1 mutation affects both vacuolar protein targeting and vacuole inheritance at 23 and 37°C. The inheritance phenotype of *vac*1-1 is the formation of a large bud which fails to inherit vacuolar material from the mother cell. The organelle inheritance defect in *vac*1-1 cells is specific for the vacuole, as the Golgi apparatus, mitochondria, and nucleus sort normally into the bud (Weisman et al., 1990; Weisman and Wickner, 1992). In addition to *vac*1-1, several other vacuolar protein sorting mutants are also reported to have vacuole inheritance defects (Raymond et al., 1990; Herman and

1. *Abbreviations used in this paper*: CDCFDA, 5 (and 6-)-carboxy-2',7'-dichlorofluorescein diacetate; CPY, carboxypeptidase Y; PP1 and PP2A, protein phosphatases type 1 and 2A; PrA, proteinase A.

Emr, 1990; Banta et al., 1990). In contrast, another mutant, *vac*2-1, is specifically defective for vacuole inheritance at 37°C and is distinguished from *vac*1-1 and other vacuolar protein sorting mutants in that it has no obvious defect in the sorting of soluble proteins to the vacuole (Shaw and Wickner, 1991).

We now report assays, using semiintact yeast cells and isolated vacuoles, which reflect two individual steps in the vacuole inheritance pathway. Our criterion of authenticity has been the requirement of these reactions for the *VAC1* and *VAC2* encoded proteins. In these assays, vacuoles form tubulovesicular structures in vitro which resemble the vacuolar segregation structures observed in vivo. In an in vitro assay based on semiintact cells, the formation of these structures requires ATP and *VAC1* and *VAC2* function. Using isolated vacuoles, formation of tubulovesicular structures is shown to also require cytosol. In addition, isolated vacuoles can undergo a second reaction: intervacuolar fusion. We propose that the two reactions reconstituted in these cell-free assays represent two discrete steps of the process of vacuole inheritance.

Materials and Methods

Yeast Strains

For in vivo studies, *S. cerevisiae* strain X2180-1A [*Mata suc2 mal mel gal2 CUP1*] was used (Yeast Genetic Stock Center, University of California, Berkeley, CA). Strains X2180-1A, LWY147 [*Mata ade2-101 ura3-52*], LWY148 [*Matα ade2-101 ura3-52 vac1-1*] (Weisman et al., 1990), JSY102 [*Mata ura3-52 lys2-801 ade2-101*], and JSY103 [*Mata ura3-52 lys2-801 ade2-101 vac2-1*] (Shaw and Wickner, 1991) were used for in vitro microscopic assays with semiintact cells and isolated vacuoles. For in vitro studies on intervacuolar fusion, BJ3501 [*Matα pep4::HIS3 prb1-Δ1.6R his3-Δ200 ura3-52 GAL can1*] (Moehle et al., 1986) and SEY2108 [*Matα ura3-52 leu2-3 leu2-112 prc1::LEU2 suc2-Δ9*] (Bankaitis et al., 1986) were used. ABYS1 (*Mata pra1 prb1 prc1 cps1 ade*$^-$) was used for cytosol preparations (Achstetter et al., 1984). All strains were grown in YPD, a rich medium. X2180-1A, BJ3501, SEY2108, and ABYS1 were cultured at 30°C. The strains LWY147, LWY148, JSY102, and JSY103 were grown at 23°C.

Materials

Tri-X pan400 and TMAXp3200 films (Eastman Kodak Co., Rochester, NY) were used for photography. 5 (and 6-)-carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA) was from Molecular Probes Inc., (Eugene, OR). Oxalyticase was purchased from Enzogenetics, Corvallis, OR and DEAE-dextran was from Pharmacia LKB Biotechnology Inc., (Piscataway, NJ). Immunofluorescence slides (Teflon coated; 25 × 75 mm; 6-mm wells) were obtained from Polyscience (Niles, IL). Protein assay reagents were from Bio-Rad Laboratories (Richmond, CA). Creatine phosphokinase and creatine phosphate were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). The anti-CPY antibody was kindly provided by Dr. T. Stevens (University of Oregon, Eugene, OR). Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corporation, Freehold, NJ. Okadaic acid and microcystin-LR were from Calbiochem-Behring Corp. (San Diego, CA). Protein concentrations were determined with Bio-Rad assay reagents or by the method of Lowry et al. (1951).

Fluorescent Labeling of Vacuoles In Vivo

Vacuoles were labeled according to Pringle et al. (1989). A culture of X2180-1A was grown to early-log phase in YPD. Cells were collected from 1 ml of culture by centrifugation (12,000 *g*, 1 min, 23°C) and resuspended in 1 ml of YCM medium, pH 4.5 (Rogers and Bussey, 1978). CDCFDA was added from a 10-mM stock in DMSO to a final concentration of 50 μM. The cell suspension was incubated at 30°C for 10 min. Cells were collected as above, resuspended in 1 ml of YPD, and examined by fluorescence microscopy.

Preparation of Semiintact Yeast Cells

Semiintact cells were prepared as described by Baker et al. (1988) with the following modifications. Cells were grown in YPD to early-log phase (0.5–1.0 OD_{600}). Cells were collected (1,000 *g*; 5 min; 23°C) and resuspended to an OD_{600} of 10 in 100 mM Pipes-KOH, pH 9.4, 10 mM DTT. The cell suspension was incubated at 30°C for 10 min. Cells were collected as above and resuspended to an OD_{600} of 25–40 in spheroplasting buffer (YP, 0.2% glucose, 50 mM KPO_4, pH 7.5, 0.6 M sorbitol, 10 mM DTT). Oxalyticase was added from a 40,000-U/ml stock in 50 mM KPO_4, pH 7.5, to a final concentration of 20–40 U/OD_{600}. The suspension was incubated at 30°C for 10–15 min. Spheroplasts were collected (1,000 *g* for 2 min, and then 1,400 *g* for 15 s at 23°C) and carefully resuspended in YP, 1% glucose, 0.7 M sorbitol to an OD_{600} of 6. Spheroplasts were incubated for 20 min at 30°C, collected as above, and resuspended in YPD with 50 mM Na citrate, pH 4.0, 0.6 M sorbitol to an OD_{600} of 25. CDCFDA was added from a 10 mM stock in DMSO to a concentration of 50 μM. The suspension was incubated at 30°C for 10–15 min. Labeled spheroplasts were collected as above and resuspended in YP, 1% glucose, 0.7 M sorbitol to an OD_{600} of 6. Labeled spheroplasts were incubated at 30°C for 10–15 min. Spheroplasts were collected (1,000 *g* for 2 min, 1,400 *g* for 15 s, 4°C), twice resuspended to an OD_{600} of 25 in cold permeabilization buffer (20 mM Pipes-KOH, pH 6.8, 150 mM K(OAc), 2 mM $Mg(OAc)_2$, 0.4 M sorbitol), collected by sedimentation as above, and resuspended to an OD_{600} of 250 in cold permeabilization buffer with 0.5 mM EGTA. Aliquots of 25 OD_{600} units of cells were slowly frozen for 45–60 min in 1.5-ml microcentrifuge tubes 8–10 cm above liquid N_2 in a sealed styrofoam container and stored at −70°C.

Structure Formation in Semiintact Cells

Aliquots of semiintact cells were thawed at 25°C and transferred to ice. 0.9 ml of cold 0.25 M sorbitol buffer (0.25 M sorbitol, 20 mM Pipes-KOH, pH 6.8, 150 mM K[OAc], 5 mM $Mg[OAc]_2$) was added and cells were resuspended by swirling on ice. After 3–5 min, cells were collected by centrifugation (12,000 *g*, 10 s, 4°C). The cell pellet was twice resuspended in 1 ml of cold 0.25 M sorbitol buffer, incubated on ice for 3–5 min, and collected by centrifugation (12,000 *g*, 10 s, 4°C). The semiintact cells were resuspended in 200 μl of cold 0.25 M sorbitol buffer to a final protein concentration of 3–6 mg/ml.

In vitro reactions (30 μl) contained 20 μl of semiintact cell suspension, cytosol (1–2 mg/ml), 1 mM ATP, and an ATP-regenerating system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase). Throughout all the experiments reported, ATP and the regenerating system were always provided together. Reactions were incubated in 1.5-ml microcentrifuge tubes and analyzed by fluorescence microscopy. The percentage of semiintact cells with tubulovesicular structures was determined by counting the total number of vacuoles and the number of vacuoles with tubulovesicular structures in random fields. Experiments were performed double-blind. 100–150 vacuoles were counted for each data point.

Preparation of Vacuoles

Vacuoles were prepared as described by Bankaitis et al. (1986) with the following modifications. Cells were grown in YPD to an OD_{600} of 0.5 to 1.2 and collected by centrifugation (1,000 *g*, 5 min, 23°C). Cells were resuspended in 100 mM Pipes-KOH, pH 9.4, 10 mM DTT to an OD_{600} of 10 and incubated at 30°C for 10 min. Cells were collected and converted to spheroplasts as described above. Spheroplasts were collected (1,000 *g* for 2 min, 1,400 *g* for 15 s, 4°C) and carefully resuspended in cold 15% Ficoll buffer (15% Ficoll, 10 mM Pipes-KOH, pH 6.8, 0.2 M sorbitol) to an OD_{600} of 70. 75–100 μl of a 0.4-mg/ml DEAE-dextran solution in 15% Ficoll buffer was added for each 100 OD_{600} units of spheroplasts. The suspension was incubated on ice for 1 min and at 30°C for 5 min. $MgCl_2$ was added to 1.5 mM and vacuoles were isolated by flotation through a discontinuous Ficoll step gradient. Lysate (3.5 ml) was transferred to an SW41 tube (Beckman Instrs., Inc., Fullerton, CA); overlayed with 3 ml of 8% Ficoll, 4 ml of 4% Ficoll, and 1 ml of 0% Ficoll (all with 10 mM Pipes-KOH, pH 6.8, 1.5 mM $MgCl_2$, 0.2 M sorbitol); and centrifuged (110,000 *g*, 90 min, 4°C). Vacuoles (200–400 μl, 0.2 to 0.5 mg/ml) were collected from the 0%/4% interphase.

In Vitro Assays with Isolated Vacuoles

The sorbitol concentration of the isolated vacuoles was adjusted to 0.3 M. For the microscopic in vitro assays, vacuoles were labeled as follows:

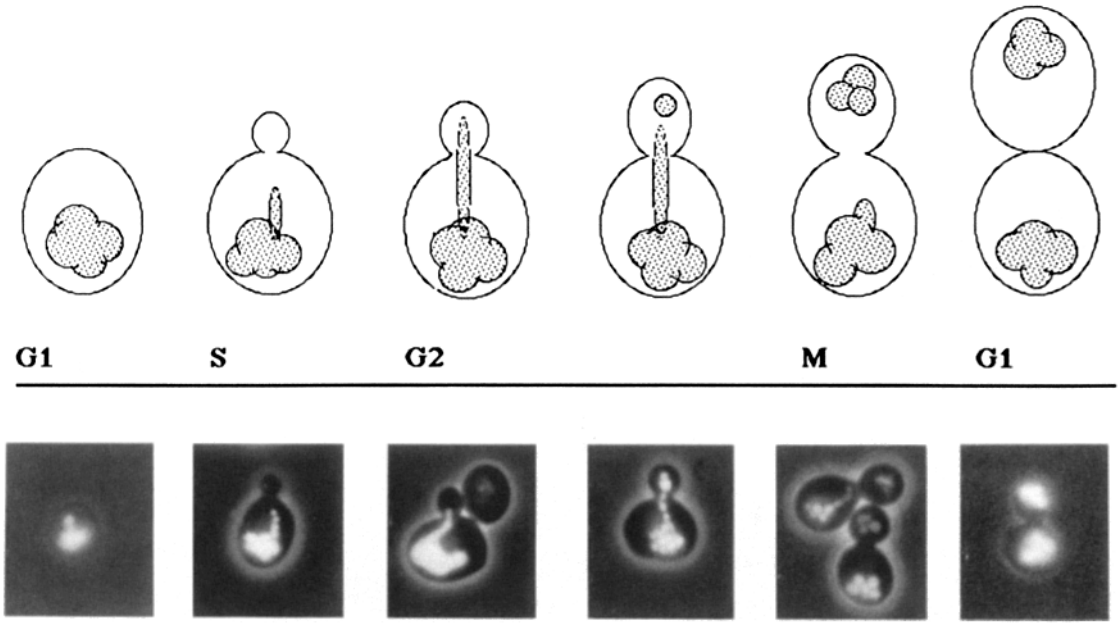

Figure 1. Vacuole morphology during the cell cycle in *S. cerevisiae*. *S. cerevisiae* strain X2180-1A was grown and labeled with CDCFDA as described in Materials and Methods. Labeled cells were examined using a microscope from Carl Zeiss, Inc. (Thornwood, NY) and photographs were taken with an F3 camera (Nikon Inc., Melville, NY) using Tri-X pan400 and TMAXp3200 film (Eastman Kodak Co.).

CDCFDA was added to the isolated vacuoles from a 1-mM stock solution in 10% DMSO to a final concentration of 50 μM. This suspension was incubated at 30°C for 10 min and transferred to ice. In vitro assays (30 μl) contained vacuoles (20 μl), 20 mM Pipes-KOH, pH 6.8, 150 mM K(OAc), 5 mM $Mg(OAc)_2$, cytosol (2–5 mg/ml), 1 mM ATP, and an ATP-regenerating system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase). For the microscopic assay, reactions were incubated in 1.5-ml microcentrifuge tubes and analyzed by fluorescence microscopy using immunofluorescence slides (see Materials). The percentage of vacuoles with tubulovesicular structures was determined by counting the total number of vacuoles (100–200) and the number of vacuoles with structures in random fields. For the biochemical assay, reactions were incubated for 3 h and then analyzed by SDS-PAGE (10% acrylamide; Deshaies and Schekman, 1987) and immunoblot (Towbin et al., 1979) using an anti-CPY antibody with a chemiluminescent development (Amersham Corp., Arlington Heights, IL).

Cytosol Preparation

Cytosol was prepared according to Baker et al. (1988) with the following modifications. Cells were grown in YPD to an OD_{600} of 1.0–2.0, collected (1,000 *g*, 5 min, 4°C), and resuspended in cold 0.25 M sorbitol buffer (0.25 M sorbitol, 20 mM Pipes-KOH, pH 6.8, 150 mM K[OAc], 5 mM $Mg[OAc]_2$) to an OD_{600} of 40. Cells were collected (1,000 *g*, 5 min, 4°C) and resuspended in a minimal volume of cold 0.25 M sorbitol buffer with 1 mM DTT and 0.5 mM PMSF. Cells (2,000 OD_{600} units) were lysed by 15–20 cycles of vortexing (30 s) in a 15-ml glass corex tube with 2 g of acid-washed glass beads (0.5-mm-diam) and chilling on ice (30 s). The extent of lysis was determined by methylene blue exclusion and was routinely 80–90%. The cell lysate was clarified (3,000 *g*, 5 min, 4°C) and the supernatant was centrifuged (150,000 *g*, 30 min, 4°C). The S150 (20–30 mg/ml) was frozen in liquid nitrogen and stored at −70°C.

Results

To study vacuolar segregation structures in vivo, we labeled dividing wild-type cells (strain X2180-1A) with the fluorescein derivative CDCFDA, a fluorophore which specifically stains the yeast vacuole (Pringle et al., 1989). In interphase cells (Fig. 1, G1 phase), CDCFDA stains a cluster of organelles, which is typical for the vacuole of strain X2180-1A in rich medium. During early S phase, when cells possess a small bud, the maternal vacuoles form a tubulovesicular structure which is directed toward the emerging bud (S phase). Throughout S and G2 phase, material can be seen to be transported from the mother cell vacuole into the bud along this pathway (G2 phase). During mitosis, this transport is disrupted (M phase). The vacuolar material that enters the bud from the mother cell forms the new bud vacuole (G1 phase). A quantitative analysis of this in vivo inheritance pathway has been presented elsewhere (Weisman et al., 1987; Weisman and Wickner, 1988; Weisman et al., 1990; Gomes de Mesquita et al., 1991).

Vacuole Segregation Structure Form In Vitro

Yeast spheroplasts can be permeabilized through a combination of osmotic shock and slow freezing (Baker et al., 1988). The resulting semiintact cells retain much of their organellar architecture. Because of their permeability to small molecules and, to a variable extent, to proteins and other macromolecules, semiintact cells have been used for the in vitro reconstitution of various cellular reactions. To reconstitute aspects of vacuole segregation, semiintact cells were prepared from the strain X2180-1A after labeling vacuoles in vivo with the fluorophore CDCFDA. Upon incubation at 30°C in the presence of ATP and cytosol, vacuoles form tubulovesicular structures (Fig. 2 *A*, compare left and right images). These structures resemble the vacuolar segregation structures observed in intact cells (Fig. 1). As in vivo, only one segregation structure forms per organelle in vitro. This reaction is energy dependent and is stimulated by the addition of exogenous cytosol (Fig. 2 *B*), though the degree of stimulation varies considerably between different preparations of semiintact cells (data not shown). This variability reflects the variable degree of cell permeabilization obtained

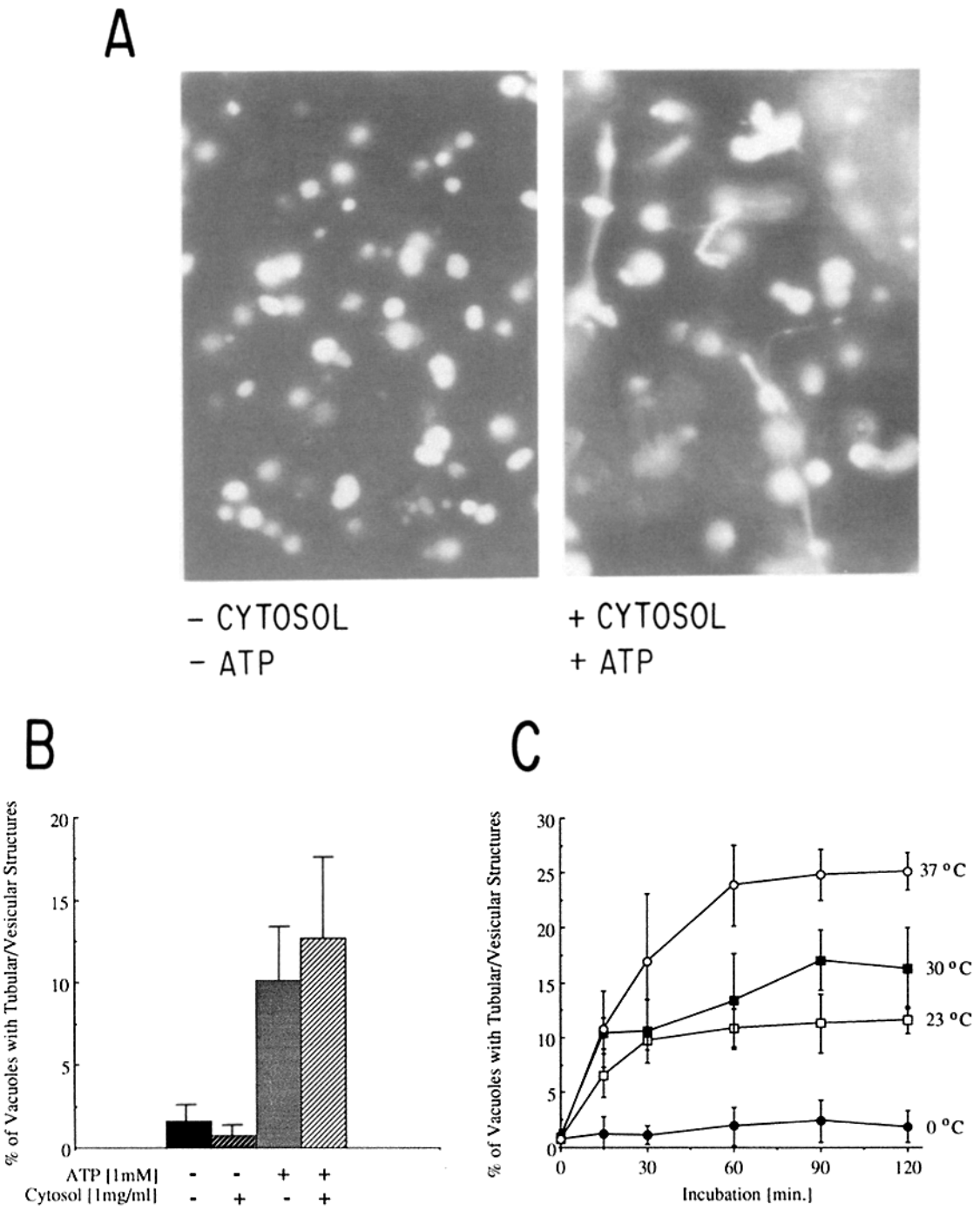

Figure 2. Formation of tubulovesicular structures derived from vacuoles in semiintact cells. CDCFDA-labeled semiintact cells (strain X2180-1A) were prepared as described in Materials and Methods. (*A*) Semiintact cells were incubated at 37°C for 60 min in 0.25 M sorbitol buffer with either no additions or with 1 mg/ml cytosol and 1 mM ATP. Reactions were examined as described in Materials and Methods. (*B*) Semiintact cells were incubated (60 min, 30°C) with 0.25 M sorbitol buffer only (column *1*), 1 mg/ml cytosol (column 2), 1 mM ATP (column *3*), or 1 mg/ml cytosol and 1 mM ATP (column *4*). The percentage of vacuoles with tubulovesicular structures was determined as described in Materials and Methods. The percentages indicated for the reactions are average values of six independent experiments. Error bars represent standard deviations. (*C*) CDCFDA-labeled semiintact cells were incubated with 1.0–1.5 mg/ml cytosol and 1 mM ATP. The percentage of vacuoles with tubulovesicular structures was determined as described. The data are averages of 3–4 experiments.

in different semiintact cell preparations. In addition, structure formation requires incubation at physiological temperatures (Fig. 2 *C*). The percentage of vacuoles with tubulovesicular structures increases linearly over the first hour of incubation, and then reaches a plateau. The percentage of vacuoles with structures depends on the incubation temperature and is maximal at 37°C (Fig. 2 *C*).

We exploited the *vac* mutants, which are defective in vacuole inheritance, to determine whether the in vitro formation of these structures reflects the process of vacuole segrega-

tion. Semiintact cells and cytosol were prepared from the mutants *vac*1-1 and *vac*2-1 and the isogenic background strains and incubated at either 23°C or 37°C in the presence of ATP. *vac*1-1 strains are defective in vacuole segregation in vivo at both 23°C and 37°C (Weisman et al., 1990). In the in vitro assay, *vac*1-1 cells incubated with *vac*1-1 cytosol did not form a significant number of structures at either temperature (Fig. 3, *top*). The mutant *vac*2-1 is temperature sensitive for vacuole inheritance, with 37°C being the nonpermissive temperature (Shaw and Wickner, 1991). *vac*2-1 semiintact cells incubated with *vac*2-1 cytosol produced tubulovesicular structures at 23°C but not at 37°C (Fig. 3, *bottom*). The background strains of *vac*1-1 and *vac*2-1 behaved like wild-type strain X2180-1A and formed structures at both temperatures (Fig. 3). Since the structures formed in vitro have a morphology which is similar to those involved in vacuole segregation in vivo, and require two proteins which have been shown to be necessary for vacuole inheritance in vivo, we refer to them as "segregation structures."

Isolated Vacuoles Form Tubulovesicular Structures

Purification of the proteins which support the formation of segregation structures requires a cell-free assay. Since we only observed a variable stimulation by cytosol of the formation of segregation structures in semiintact cells, we assayed segregation structure formation using purified vacuoles, isolated from cell lysates by flotation. Vacuoles were isolated from the wild-type strain X2180-1A, labeled with CDCFDA, and incubated at 30°C with ATP and cytosol. Segregation structures formed which resembled those seen in vivo and in semiintact cells (Fig. 4, *right column, middle image*, 30 min). Formation of these structures requires incubation at physiological temperatures and is completely dependent on the addition of both ATP and cytosol (Figs. 4 and 5). The highest efficiency is obtained at 30°C, where >15% of the vacuoles form structures. After 30 min of incubation, the percentage of vacuoles with segregation structures peaks and then actually begins to decline, reaching a plateau of about one half the maximal value (Fig. 5 *B*). During this time, an increasing number of very large vacuoles appear (Fig. 4, *right column, bottom image*, 120 min), and their size increases over the course of the experiment (data not shown). This reaction also required the presence of ATP and exogenous cytosol (compare the 120-min images). Similar reactions of segregation structure formation and increasing vacuole size are also seen with other strains (BJ3501 and SEY2108, data not shown).

To determine the role of the *VAC*1 and *VAC*2 encoded proteins in this cell-free assay, the assay was performed with isolated vacuoles and cytosol from *vac*1-1 and *vac*2-1 strains and their wild-type sister spores. Vacuoles of both wild-type sister spores formed segregation structures and subsequently large vacuoles when incubated at 23°C or 37°C with ATP and the corresponding cytosols (data not shown). Vacuoles of the two mutants, however, did not undergo the reactions observed with various wild-type strains. Incubations under the described conditions eventually resulted in the fragmentation of *vac*1 and *vac*2 vacuoles (Fig. 6). To test whether this fragmentation resulted from lysis of the mutant vacuoles, we measured the release of vacuolar carboxypeptidase Y (CPY) from wild-type and *vac*2 vacuoles during assay incubations.

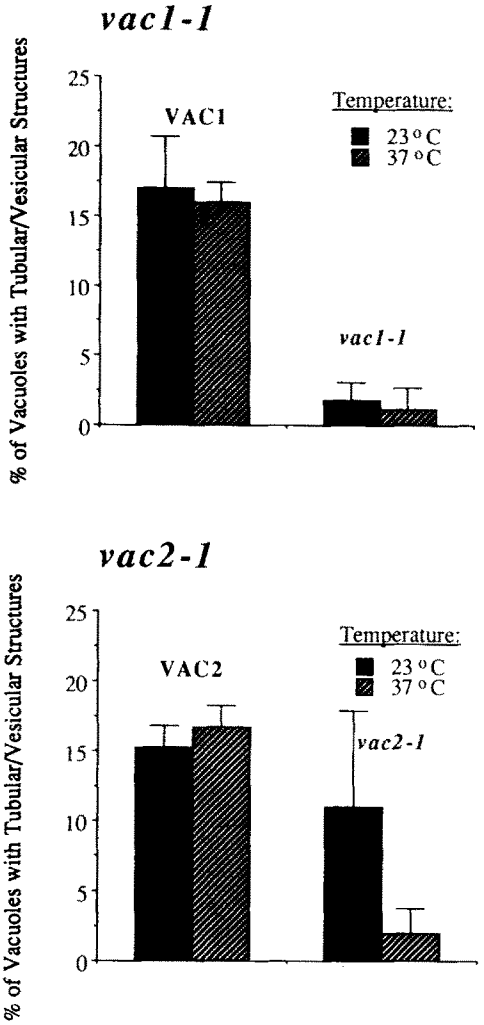

Figure 3. Semiintact *vac1-1* and *vac2-1* cells are defective for the formation of tubulovesicular structures. Cultures of *vac1-1*, *vac2-1*, and their wild-type sister spores were grown at 23°C. Cytosol and CDCFDA-labeled semiintact cells were prepared from these four strains as described in Materials and Methods. In vitro reactions were incubated at 23°C or 37°C for 60 min in the presence of 1 mM ATP and 1–2 mg/ml of the corresponding cytosol. The percentage of vacuoles with structures was determined as described in Materials and Methods. The data shown are averages of 4 (VAC1, *vac1-1*) and 3 (VAC2, *vac2-1*) experiments.

Comparable, small amounts were released (data not shown), indicating that the *vac2* mutation is not causing vacuole lysis.

Biochemical Assay for Intervacuolar Fusion

It seemed likely that the size increase of the isolated vacuoles in the cell-free assay might be the result of intervacuolar fusion. Such a fusion reaction, which is seen in vivo during vacuole inheritance in zygotes (Weisman and Wickner, 1988), would presumably lead to a mixing of the vacuolar contents, which could be measured by a protein maturation assay. To this end, vacuoles were isolated from two mutant strains. One strain has a deletion of the *PRC1* gene, which encodes CPY; its vacuoles are normal except for the absence of this protease, assayed by SDS-PAGE and immunoblot (Fig. 7 *A*; lane *1*, X2180-1A [wt] vacuoles and lane *2*,

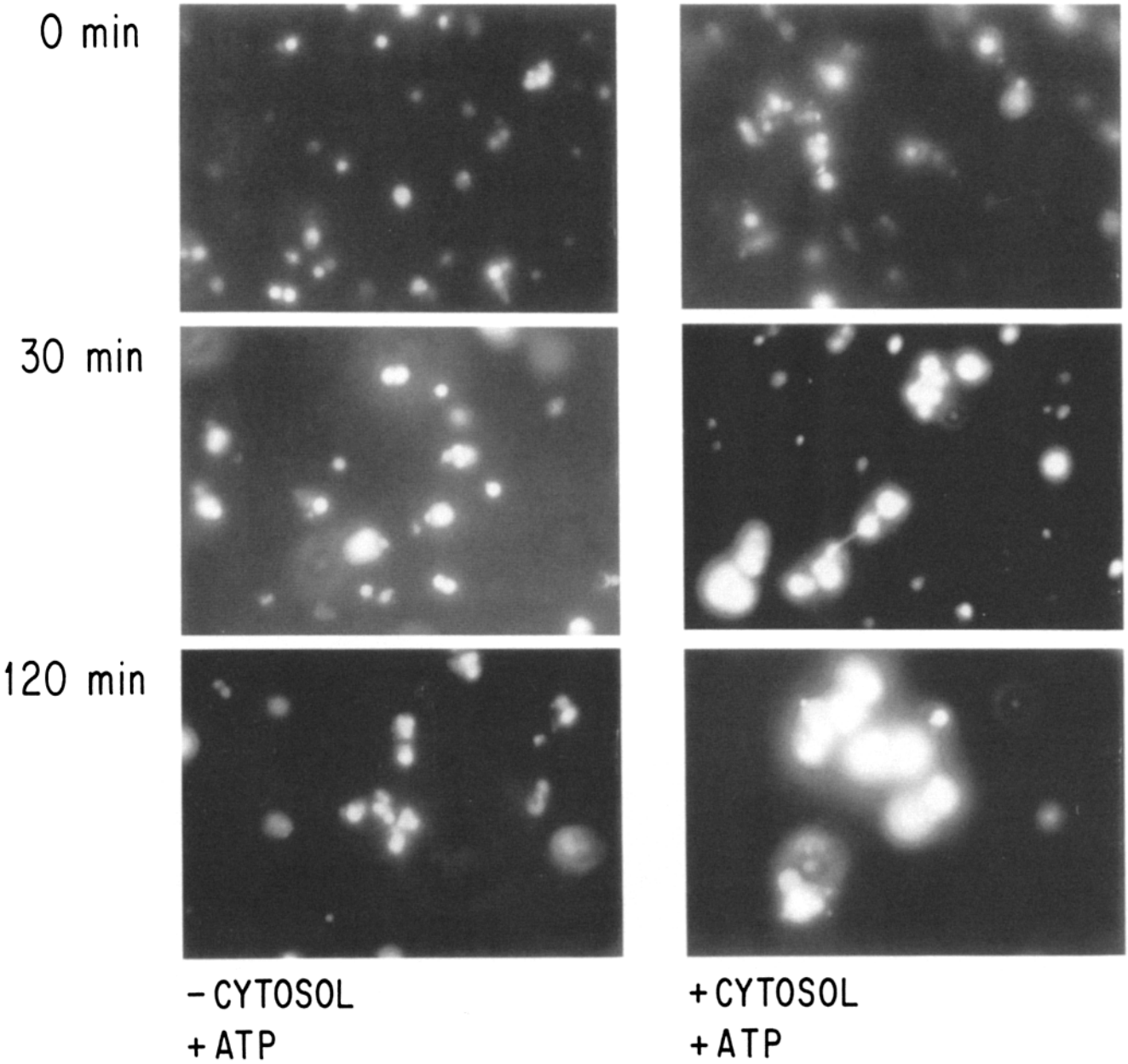

Figure 4. In the presence of ATP and cytosol, isolated vacuoles form tubulovesicular structures and then increase in size. Vacuoles were isolated from strain X2180-1A, labeled, and prepared for the microscopic in vitro reaction as described in Materials and Methods. Labeled vacuoles were incubated at 30°C with 1 mM ATP and either 5 mg/ml cytosol or buffer. Reactions were examined by fluorescence microscopy as described in Materials and Methods.

SEY2108 [CPY$^-$] vacuoles). The second strain has a deletion of the *PEP4* gene, encoding the vacuolar proteinase A (PrA). This proteinase is responsible for the maturation and activation of newly made pro-proteins as they reach the vacuole (Ammerer et al., 1986; Woolford et al., 1986). Vacuoles from this strain therefore accumulate the Golgi form of CPY, proCPY (lane *3*, vacuoles from BJ3501 [PrA$^-$]). Fusion between vacuoles of the two mutants would result in mixing of the contents, and the subsequent conversion of proCPY (69 kD) to mature CPY (61 kD) by the action of PrA. Indeed, when vacuoles from these two strains are artificially fused by exposure to pH 4.5 in a control experiment, maturation was observed (lane *5*). CPY maturation can therefore be used as an assay for intervacuolar fusion. To determine whether the observed enlargement of the vacuoles resulted from such intervacuolar fusion, we incubated vacuoles of the two mutants under the more physiological conditions used for the microscopic assay and analyzed the reactions for CPY maturation. CPY maturation occurred at neutral pH in a reaction requiring ATP, cytosol (Fig. 7 *B*, lanes *3–6*), and physiological temperatures, with 26°C being optimal (data not shown). Under these conditions up to 10% of proCPY is converted to mature CPY. In addition, maturation of CPY requires the presence of the PrA-containing vacuoles of strain SEY2108 (lanes *1* and *6*). To exclude the possibility that CPY maturation had occurred outside a membrane-bound compartment through leakage of PrA and proCPY from the vacuoles, we assayed the effect of vacuole lysis on

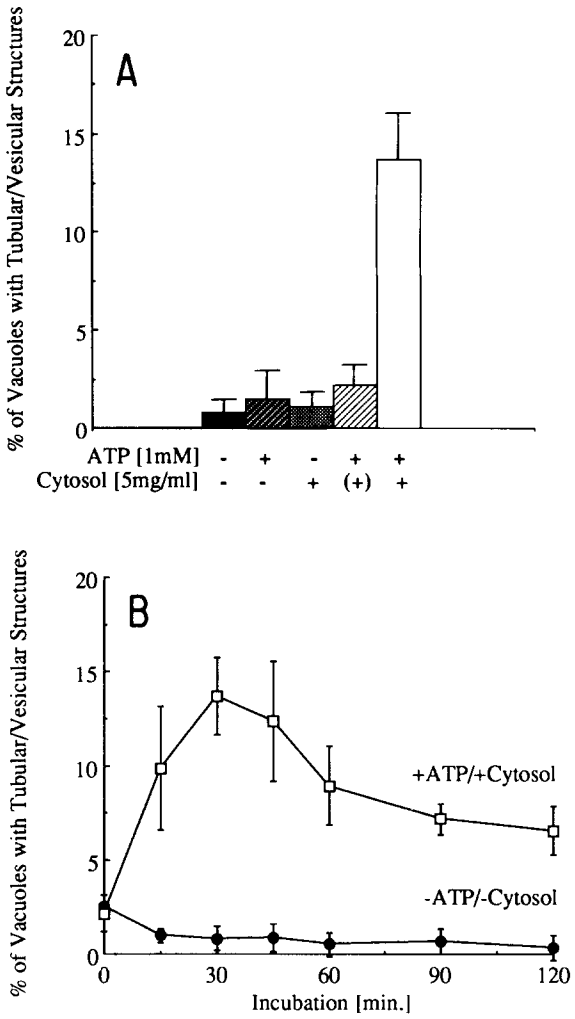

Figure 5. Formation of tubulovesicular structures from isolated vacuoles. X2180-1A vacuoles were isolated, labeled, and prepared for the microscopic in vitro reaction as described in Materials and Methods. (*A*) Vacuoles were incubated for 30 min at 30°C with 0.25 M sorbitol buffer only (column *1*), with 1 mM ATP (column *2*), with cytosol (5 mg/ml; column *3*), with ATP and boiled cytosol (column *4*), or with ATP and untreated cytosol (column *5*). The percentages shown are averages of several independent experiments. (*B*) Vacuoles were incubated at 30°C with 0.25 M sorbitol buffer only or with 1 mM ATP and 5 mg/ml cytosol. The percentage of vacuoles with structures was determined in independent experiments as described in Materials and Methods.

CPY maturation. Triton X-100 (0.01%) prevents maturation (Fig. 8 *A*; lane *4*), indicating that CPY maturation can only occur in intact vacuoles. In addition, we reisolated vacuoles after completion of an in vitro reaction by flotation and analyzed their contents. As expected, mature CPY formed in the initial in vitro reaction (Fig. 7 *C*; *ASSAY*, lane *2*) resides inside the reisolated, fused vacuoles (*RE-ISOLATION*, lane *2*). Whereas the efficiency of CPY maturation in the initial reaction is very low (*ASSAY*, lane *2*), ~60–70% of the CPY in reisolated vacuoles is matured (*RE-ISOLATION*, lane *2*). These data suggest that many of the vacuoles lyse during the assay, and that proCPY released from these lysed organelles cannot be matured. The actual efficiency of the reaction is therefore 60–70%, in agreement with the extent of vacuole fusion observed microscopically (Fig. 4). This efficiency confirms that the CPY maturation assay measures intervacuole fusion and not fusion between contaminating Golgi organelles and vacuoles.

CPY maturation requires proteins of both the cytosol and the vacuole surface. Boiling the cytosol decreases the amount of proCPY maturation to ~10–20% of that of control reactions (Fig. 8 *A*, lanes *2* and *3*), while the cytosol stimulation is completely abolished by pretreatment with trypsin (Fig. 8 *B*, lanes *1* and *2*). The vacuolar membranes can also be inactivated by trypsin treatment before the reaction (Fig. 8 *B*, lanes *3* and *4*).

We have recently used the PrA-dependent maturation of alkaline phosphatase, a vacuole membrane enzyme, to assay the effect of the *vac2* mutation on the intervacuole fusion reaction. As described above, vacuoles of this mutant fail to fuse and, eventually, fragment. In agreement with this, *vac2* vacuoles only showed 15% of the maturation of pro-alkaline phosphatase as was seen with wild-type vacuoles (Haas, A., B. Conradt, and W. Wickner, unpublished observations), indicating that *VAC2* is needed for the fusion reaction.

Intervacuolar Fusion Is Affected by Inhibitors of Protein Phosphatases of Type 1 and 2A

Inhibitors of protein phosphatases type 1 and 2A have been shown to block endocytic vesicle fusion and ER-to-Golgi transport in vitro (Woodman et al., 1992; Davidson et al., 1992). Okadaic acid and microcystin-LR, two potent inhibitors of these phosphatases (Cohen et al., 1990; Schoenthal, 1992; MacKintosh et al., 1990; Honkanen et al., 1990) are potent inhibitors of CPY maturation (Fig. 9). Half-maximal inhibition is obtained with 0.3 μM okadaic acid or 0.6 μM microcystin-LR. Comparable inhibitory potencies have been reported for other reactions of interorganelle traffic (Woodman et al., 1992; Davidson et al., 1992). Neither okadaic acid nor microcystin-LR (0.1–50 μM) had any direct inhibitory effect on the activity of PrA itself (data not shown), confirming that these drugs blocked CPY maturation by inhibiting the fusion reaction per se.

Discussion

Organelle inheritance has largely been studied at the morphological level in higher eukaryotic cells. We have previously reported cytological and genetic studies which helped to define the pathway of vacuole inheritance in *Saccharomyces cerevisiae* (Weisman et al., 1987; Weisman and Wickner, 1988; Weisman et al., 1990; Shaw and Wickner, 1991; Weisman and Wickner, 1992). We now report an in vitro, cell-free reaction which, by several criteria, assays individual steps during vacuole inheritance. Morphologically, it entails the formation of tubular and vesicular projections of vacuoles which resemble those seen in vivo during vacuole inheritance. Biochemically, it leads to membrane fusion and the mixing of lumenal vacuole contents. This has been observed during vacuole inheritance in budding zygotes. Using zygotes in which the vacuoles of the two mother cells had been differentially labeled, it was shown that tubules and vesicles originating from the two maternal organelles were delivered into the emerging bud and fused, leading to mixing of their contents (Weisman and Wickner, 1988). Intervacuolar fusion is likely to also be a step during vacuole in-

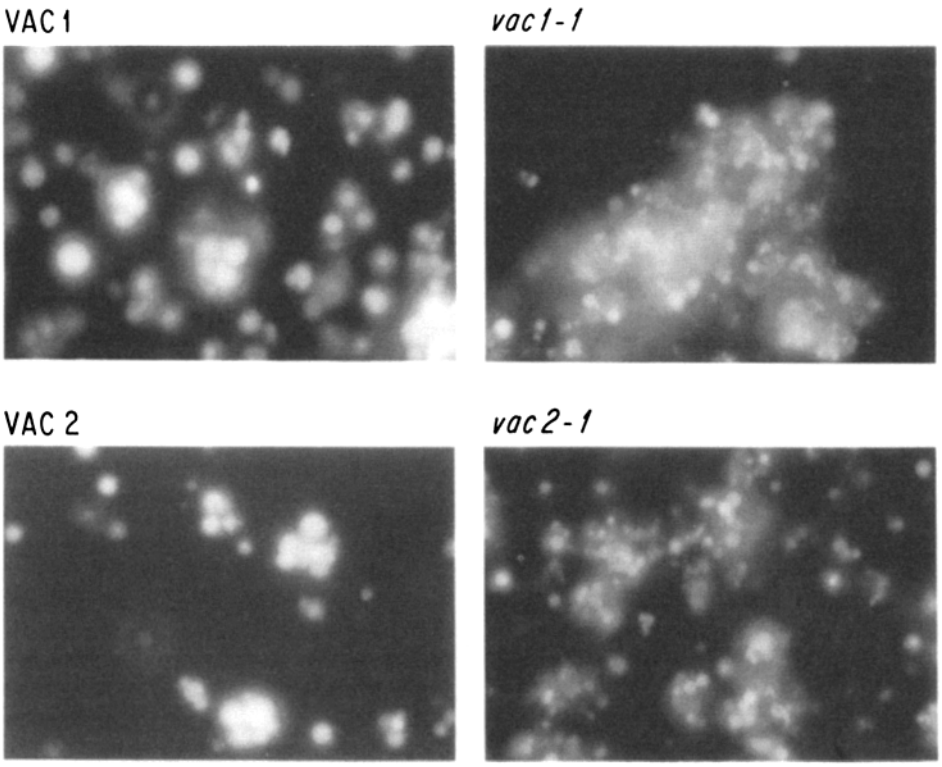

Figure 6. vac1-1 and *vac2-1* vacuoles are defective in the formation of large vacuoles. Strains *vac1-1*, *vac2-1*, and wild-type sister spores of *vac1-1* and *vac2-1* were grown at 23°C. Vacuoles were isolated and prepared for the microscopic in vitro reaction as described in Materials and Methods. Cytosol from these strains, grown at 23°C, was prepared as described. Vacuoles were incubated for 90–120 min at 23°C in the presence of 1 mM ATP and 4 mg/ml of the corresponding cytosol. Reactions were examined by fluorescence microscopy as described in Fig. 1.

heritance in the haploid, mitotic cell cycle. Fusion may occur in the bud after parental vacuolar material is delivered via segregation structures. The formation of tubulovesicular structures in semiintact cells and the fusion reaction seen with isolated vacuoles requires the *VAC1* and *VAC2* encoded proteins. This provides a strong genetic link between these cell-free reactions and vacuole inheritance in intact cells. An understanding of the roles of *VAC1* and *VAC2* proteins in the semiintact cell and purified vacuole reactions will await the isolation of these, and other, proteins in functional form. The in vitro reactions reveal an energy requirement for the reaction, and create the opportunity to purify the necessary components in the cytosol and on the vacuole membrane.

The reactions of intracellular, vesicular traffic which constitute the exocytic pathway (Rothman and Orci, 1992) and endocytic pathway (Gruenberg and Howell, 1989; Wessling-Resnick and Braell, 1990) provide instructive parallels to the pathway of vacuole inheritance. Each requires high-energy phosphate, cytosolic, and membrane proteins, and can be assayed by the resultant mixing of lumenal contents. Tubulovesicular structures, similar to the ones observed during vacuole segregation in intact yeast and in the in vitro reactions, have been described for other organelles and cell types. The endoplasmic reticulum, Golgi apparatus, lysosomes, and endosomes can form dynamic networks, composed of tubular and vesicular structures (Lee and Chen, 1988; Cooper et al., 1990; Knapp and Swanson, 1990; Tooze and Hollinshead, 1991). Some insight into the dynamics of these structures has come from studies using brefeldin A, a drug that affects membrane traffic and organelle structure (Pelham, 1991; Klausner et al., 1992). Brefeldin A blocks secretion (Misumi et al., 1986; Oda et al., 1987; Lippincott-Schwartz et al., 1989), possibly by inhibiting the assembly of coat proteins onto Golgi membranes (Orci et al., 1991; Robinson et al., 1992; Narula et al., 1992). This leads to a rapid and extensive formation of tubular structures, both in vivo and in vitro (Lippincott-Schwartz et al., 1990; Orci et al., 1991). It has been proposed that the formation of membranous, tubular structures is mainly driven by intrinsic membrane components, without the involvement of either coat proteins or microtubules (Orci et al., 1991; Klausner et al., 1992). The segregation structures formed early during vacuole segregation are also tubulovesicular and resemble the brefeldin A–induced structures. The in vitro formation of both vacuolar segregation structures and brefeldin A–induced, tubular networks from Golgi membranes appear to be microtubule independent and insensitive to low concentrations of nonhydrolyzable GTP (data not shown).

In mammalian cells interorganelle trafficking occurs throughout most of the cell cycle but is inhibited during cell division (Tuomikoski et al., 1989; Warren, 1989). This is due to the vesiculation of the endoplasmic reticulum and Golgi apparatus which follows the rearrangement of the cytoplasmic network of microtubules early in mitosis (Thyberg and Moskalewski, 1985; Kreis, 1990). In addition, it has been proposed that fusion of carrier vesicles with acceptor membranes is blocked during cell division (Warren, 1985, 1989; Tuomikoski et al., 1989). After mitosis, these organelles reassemble through membrane fusion, possibly mediated by microtubules (Lucocq et al., 1989; Ho et al., 1989). This fusion event is different from fusion events occurring during the various transport pathways in interorganelle trafficking in that it entails fusion between homologous membranes and is possibly coordinated with events of the cell division cycle. The fusion event reported here also occurs between homologous membranes and is likely to be under cell cycle control. It may therefore share features with the inheritance reactions of the endoplasmic reticulum and Golgi apparatus in mammalian cells.

Protein phosphatases may be involved in intervacuolar fu-

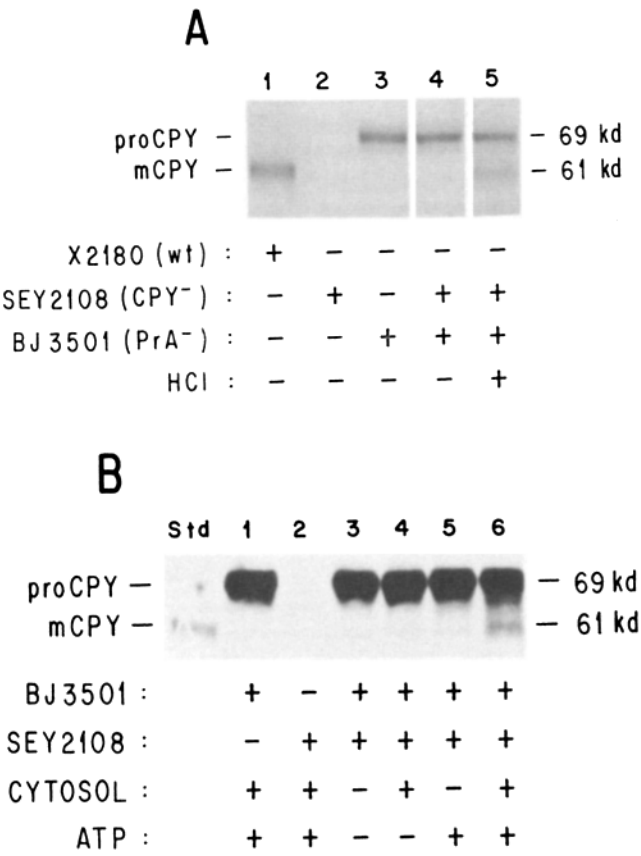

Figure 7. Biochemical assay of vacuole fusion. Vacuoles of strains X2180-1A (wt), SEY2108 (CPY⁻), and BJ3501 (PrA⁻) were isolated as described in Materials and Methods except that for *A*, Tris buffers instead of Pipes buffers were used and spheroplasting was performed with zymolyase (ICN Biomedicals, Inc., Costa Mesa, CA; 15 OD_{600} units of cells) in 0.6 M sorbitol, YPD, pH 7.0, 10 mM DTT. (*A*) Artificial vacuolar fusion. Vacuoles from 25 OD_{600} units of cells from the strains X2180-1A (lane *1*), SEY2108 (lane *2*), BJ3501 (lane *3*), or SEY2108 and BJ3501 (lanes *4* and *5*) were incubated for 30 min at 23°C in 1.5 ml of 20 mM Tris-malate, pH 5.0, 0.2 M sorbitol in the absence (lanes *1–4*) or presence (lane *5*) of 15 mM HCl. Reactions were TCA precipitated and analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods, except that for immunoblotting, a colorimetric developing method was used (Bio-Rad Laboratories). (*B*) Physiological vacuole fusion. Vacuoles from strains BJ3501 (lane *1*), SEY2108 (lane *2*), or BJ3501 and SEY2108 (lanes *3–6*) were incubated at 26°C for 3 h in buffer only (lane *3*), with 2.5 mg/ml cytosol (lane *4*), with 1 mM ATP (lane *5*), or with 2.5 mg/ml cytosol and 1 mM ATP (lanes *1*, *2*, and *6*). Reactions were analyzed as described in Materials and Methods. (*C*) Vacuoles (BJ3501 and SEY2108) were incubated with 2 mg/ml cytosol only (lane *1*) or with 2 mg/ml cytosol and 1 mM ATP (lane *2*). In vitro reactions (Materials and Methods) were scaled up 15-fold to a final volume of 450 μl. The reactions were incubated at 25°C for 2.5 h and then chilled on ice. A 30-μl aliquot of each reaction was mixed with SDS-PAGE sample buffer. One third of these samples was analyzed by SDS-PAGE and immunoblotting as described (*ASSAY*, lanes *1* and *2*). The rest of the two main reactions (420 μl) was mixed with 3.0 ml of 15% Ficoll, 10 mM Pipes-KOH, pH 6.8, 0.2 M sorbitol, and overlaid by a discontinuous Ficoll step gradient as described in Materials and Methods to reisolated intact vacuoles from the in vitro reactions by flotation. Vacuoles were recovered in a volume of 200 μl. 50 μl of 5 × sample buffer was added to these and 1/12 (*RE-ISOLATION*, lane *1*) and 1/5 (lane *2*) of the samples were analyzed as described.

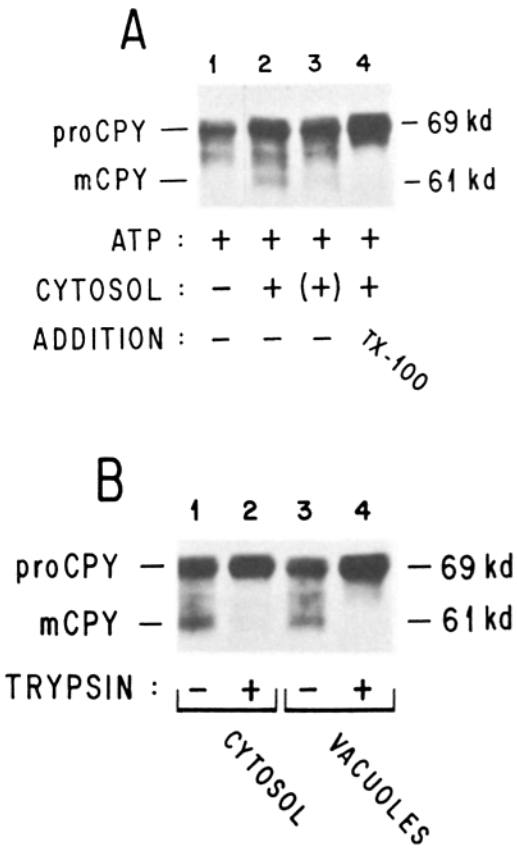

Figure 8. Requirements of the CPY maturation assay. (*A*) BJ3501 and SEY2108 vacuoles were incubated for 3 h at 26°C in the presence of 1 mM ATP with either no further addition (lane *1*), with 2.5 mg/ml of untreated cytosol (lane *2*), with an equivalent volume of boiled cytosol (lane *3*), or with 2.5 mg/ml untreated cytosol in the presence of 0.01% TX-100 (lane *4*). Cytosol was boiled for 5 min and then centrifuged for 5 min in a microcentrifuge at 4°C, and the supernatant used for the in vitro reaction. (*B*) Cytosol (lane *2*) or vacuoles (lane *4*) were preincubated for 30 min on ice with 1 mg/ml trypsin (*CYTOSOL*) or 0.01 mg/ml trypsin (*VACUOLES*). Soybean trypsin inhibitor was then added to 5 mg/ml (*CYTOSOL*) or 0.05 mg/ml (*VACUOLES*). The cytosol and vacuoles were incubated for an additional 10 min on ice before adding them to separate in vitro reactions. For mock treatment, cytosol (lane *1*) and vacuoles (lane *3*) were incubated for 40 min on ice in the presence of preincubated (10 min) trypsin and soybean trypsin inhibitor (1 mg/ml and 5 mg/ml for cytosol, 0.01 mg/ml and 0.05 mg/ml for vacuoles). The in vitro reactions were incubated for 3 h at 26°C in the presence of 1 mM ATP and the final cytosol concentration was 2.5 mg/ml. Reactions were analyzed by immunoblotting as described in Materials and Methods.

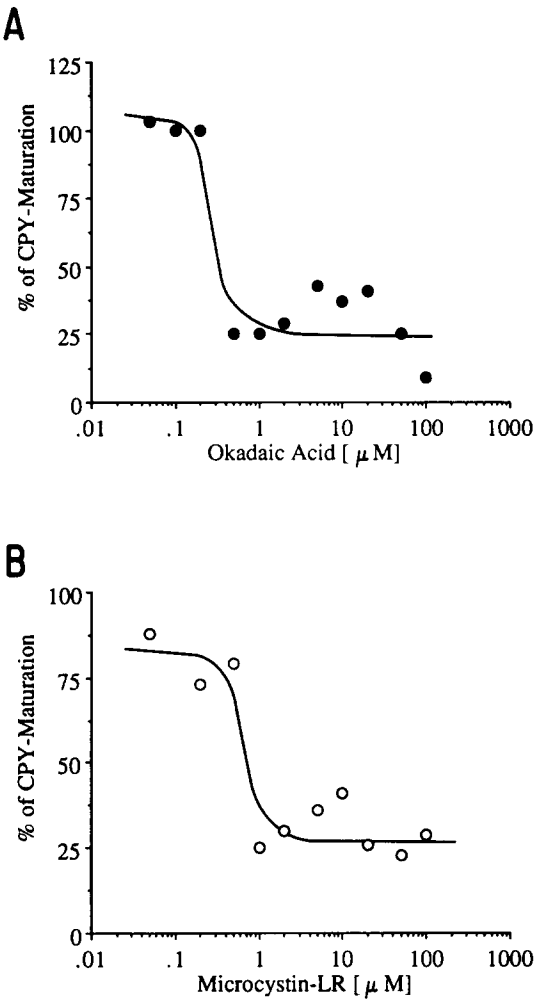

Figure 9. Okadaic acid and microcystin-LR, inhibitors of PP1 and PP2A, prevent CPY maturation. In vitro reactions with BJ3501 and SEY2108 vacuoles, 2.5 mg/ml cytosol, and 1 mM ATP were prepared as described in Materials and Methods. Various concentrations (0.02–100 μM) of okadaic acid (*A*) or microcystin-LR (*B*) were added to these reactions. Okadaic acid was added from a 1-mM stock in 10% DMSO and microcystin-LR from a 2-mM stock in H_2O. For each reaction, a control reaction was prepared with the corresponding concentration of solvent (DMSO or H_2O). In vitro reactions were incubated for 3 h at 26°C and analyzed by immunoblotting as described in Materials and Methods. The intensities of the mature CPY bands were analyzed by densitometry and normalized to the control reactions.

sion, since CPY maturation is blocked by low concentrations of okadaic acid and microcystin-LR, two structurally different inhibitors of the serine/threonine protein phosphatases type 1 and 2A (PP1 and PP2A). These results are in agreement with okadaic acid and microcystin-LR effects on reconstituted endocytic vesicle fusion and ER-to-Golgi transport which have been reported recently (Woodman et al., 1992; Davidson et al., 1992). PP1 and PP2A also seem to be involved in cell cycle regulation in many organisms, including fungi (Cyert and Thorner, 1989; Cohen and Cohen, 1989). Mutations in the structural gene for PP1 cause mitotic defects in *S. pombe* and *A. nidulans* (Doonan and Morris, 1989; Ohkura et al., 1989). The disruption of PP2A activity in *S. pombe* results in the formation of small cells, a pleiotropic phenotype that could result from mitotic arrest (Kinoshita et al., 1990). The simultaneous disruption of the PP2A genes in *S. cerevisiae* is lethal to the cells (Sneddon et al., 1990, Ronne et al., 1991). Recently, yet another protein phosphatase with a possible role in cell cycle regulation has been identified and cloned from *S. cerevisiae* (Arndt et al., 1989; Sutton et al., 1991). This phosphatase shows homologies to both the mammalian PP1 and PP2A and may be important for the G1/S phase transition. Our results suggest that protein phosphorylation reactions mediated by PP1 and PP2A may also be involved in regulating the intervacuolar fusion event described here.

The in vitro reactions described here provide a means to isolate and reconstitute proteins which catalyze and regulate vacuole inheritance. The interplay between this biochemical approach, and the isolation of *vac* mutants which affect this process in vivo, is essential to a molecular understanding of organelle inheritance.

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