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A Truncated Form of the Pho80 Cyclin of *Saccharomyces cerevisiae* Induces Expression of a Small Cytosolic Factor Which Inhibits Vacuole Inheritance

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Vacuoles project streams of vesicles and membranous tubules into the yeast bud where they fuse, founding the daughter cell organelle. *vac5-1*, which encodes a truncated form of the Pho80 cyclin, inhibits normal vacuole inheritance. An in vitro inheritance assay which measures the fusion of vacuoles serves as a model for several steps of this process. We find that cytosol isolated from the *vac5-1* mutant is unable to promote the fusion of wild-type vacuoles in the in vitro assay. Wild-type vacuoles are irreversibly inactivated in a time- and temperature-dependent manner if preincubated with *vac5-1* cytosol and ATP, suggesting the presence of a soluble inhibitory factor. When mixed with wild-type cytosol, *vac5-1* cytosol inhibits the activity of wild-type cytosol. *vac5-1* cytosol treated with trypsin or papain is still able to inhibit the activity of wild-type cytosol. Partial fractionation of *vac5-1* cytosol reveals that the protein fraction (G25 void volume) can promote fusion if wild-type small molecules are included in the fusion reaction. In contrast, the *vac5-1* small-molecule fraction retains the full ability to inhibit fusion. Thus, the *vac5-1* allele of *PHO80* induces the synthesis of a small molecule that is an inhibitor of vacuole inheritance.

Organelle partitioning during cell division is critical for cell growth and survival (for a review, see reference 19). The yeast vacuole forms tubular or vesicular segregation structures during early S phase which deliver maternally derived material into the bud until late G_2 phase (3, 6). In vitro assays which reflect the steps of in vivo inheritance were developed (3, 7) to explore the mechanisms of vacuole partition and fusion during cell division.

Segregation structures can be viewed microscopically in semi-intact cells or isolated vacuoles (3). Formation of these structures requires ATP and cytosol and is time and temperature dependent. After structure formation, isolated vacuoles fuse and enlarge up to fivefold in diameter. This increase in size is due to vacuole-to-vacuole fusion. This fusion can also be assayed biochemically by measuring maturation of vacuolar proenzymes such as procarboxypeptidase Y or proalkaline phosphatase upon mixing of vacuole components. Homotypic fusion apparently reflects at least the last stage in vacuole segregation, whereby newly delivered vesicular material in the bud fuses into a single organelle. A quantitative colorimetric assay measuring alkaline phosphatase activity (7) has enabled more sophisticated dissection of intervacuolar fusion. This assay is performed by mixing vacuoles isolated from a *pho8*Δ strain with vacuoles isolated from a *pep4*Δ strain in the presence of ATP and cytosol. Upon fusion, the Pep4 proteinase (proteinase A) in the *pho8*Δ vacuoles can mature pro-Pho8p (alkaline phosphatase) in the *pep4*Δ vacuoles by removing the pro-peptide at the pro-Pho8p C terminus. Pho8p activity can be measured by providing a substrate, *p*-nitrophenyl phosphate, which turns yellow upon hydrolysis.

This quantitative assay has facilitated study of the vacuole fusion event. The effects of several pharmocological reagents have provided some insight into the types of proteins involved in fusion. Inhibition of vacuole-to-vacuole fusion by okadaic acid and microcystin suggests that type 1 and/or 2A serine/threonine phosphatases are involved in intervacuolar fusion (3). These types of phosphatases appear to have a role in regulation of the fungal cell cycle (1, 4). Mutations in the PP1 gene or disruption of PP2A in *Schizosaccharomyces pombe* causes mitotic defects (12, 16). The SIT4 gene encodes a PP1/PP2A-like phosphatase which plays a role in the G_1/S phase transition in *Saccharomyces cerevisiae* (18). Though okadaic acid and microcystin inhibit a wide range of phosphatases, their effects in the in vitro inheritance assay suggest that phosphorylation/dephosphorylation events are involved. GTP-hydrolyzing proteins also appear to be important for intervacuolar fusion. Fusion can be inhibited by either mastoparans, nonhydrolyzable GTP analogs, or benzalkonium chloride (7). Further evidence for G protein involvement is provided by studies which demonstrate that Ypt7p, a small Ras-like GTPase which is localized to the vacuole, is required for fusion (8). In addition, an electrochemical potential is also required since CCCP, a proton ionophore, and bafilomycin, a vacuolar ATPase inhibitor, block vacuole-to-vacuole fusion (2).

Mutants which are defective in vacuole inheritance, such as *vac1-1* and *vac2-1*, are defective in the in vitro assay (3, 7). The defect in these strains appears to be due to a change in the vacuoles rather than in the cytosol. Recently a new mutant, *vac5-1*, was characterized in vivo and the *VAC5* gene was cloned and shown to be identical to *PHO80* (15). *PHO80* encodes a cyclin which acts as a negative regulator of phosphate metabolism (10). Although recessive, the *vac5-1* allele of *PHO80* encodes a truncated form of the cyclin which appears to be a gain-of-function mutation. This truncated form of Pho80p is missing the carboxy-terminal third of the protein,

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which is similar to truncations seen in other cyclins such as CLN2 and CLN3 (9, 14). Genetic analysis demonstrates that the *vac5* truncated cyclin requires its partner kinase, Pho85, and transcription factor targets, Pho4 and Pho2, to affect vacuole inheritance. In order to determine its mode of action, the *vac5-1* mutant was characterized by using the in vitro assay. We now report that the cytosol of *vac5-1* has a dominant negative-like defect in the in vitro assay and that this inhibitory activity is due to a small molecule.

MATERIALS AND METHODS

Yeast strains. Cytosol was prepared from derivatives of TN.2B (*vac5-1 ura3-52*) and TN.2C (*VAC5 ura3-52*) crossed into K91-A (*pho8*::pAL134 *pho13*::pPH13 *ura3-52*), an *S. cerevisiae* strain previously described by Haas et al. (7). Vacuoles were prepared from BJ3505 (*pep4*::HIS3) and DKY6281 (*pho8*::TRP1) (7, 11).

Microscopic and spectrophotometric vacuole fusion assays. Isolation of vacuoles and cytosol was done as previously described (3). All preparations were made from yeast cell cultures in log phase (1×10^7 cells/ml). Cytosol was prepared by vortexing yeast cells in the presence of glass beads and buffer [20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-KOH, pH 6.8, 150 mM potassium acetate (KOAc), 0.25 M sorbitol, 5 mM magnesium acetate ($Mg(OAc)_2$)]. Samples were centrifuged (3,000 × *g*, 10 min) to remove intact cells, and the supernatant was then transferred to new tubes and centrifuged (150,000 × *g*, 1 h, 4°C) to remove cell debris. When the cytosol isolated from mutant strains was cloudy, additional centrifugation was used to remove lipids. Protein was assayed by determining the A_{280}. Vacuoles were isolated by lysing oxalyticase-generated yeast spheroplasts in 10 mM PIPES-KOH, pH 6.8–0.2 M sorbitol–15% Ficoll with DEAE-dextran. The lysate was then layered beneath a Ficoll density gradient (15 to 0%) and centrifuged (150,000 × *g*, 1 h, 4°C). Vacuoles which floated to the 0%/4% interphase of the gradient were collected and used immediately.

The visual assay of vacuole fusion was done as previously described (3). CDCFDA-stained wild-type vacuoles isolated from BJ3505 and DKY6281 (final concentration, 0.25 mg/ml) were labeled in vitro with 50 μM CDCFDA (10 min, 30°C) and mixed in equal proportions with cytosol at a final concentration of 2.0 mg/ml and an ATP-regenerating system (3) with 1 mM ATP. In all experiments, samples had a final volume of 30 μl comprising 20 μl of vacuoles, 6 μl of ATP reaction buffer [final concentration, 20 mM PIPES-KOH, pH 6.8, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 0.3 M sorbitol, 40 mM creatine phosphate, 0.2 mg of creatine kinase per ml, 1 mM ATP], and 4 μl of cytosol. Incubations were at 25°C for 2 h. Labeled vacuoles were viewed with a fluorescein filter (absorption wavelength 450 to 490 nm). The colorimetric assay described by Haas et al. (7) was performed as previously described and used to measure the maturation of the pro-alkaline phosphatase present in BJ3505 vacuoles.

Protease treatment of cytosol. Wild-type or *vac5-1* cytosol was treated with 1.0 mg of trypsin per ml, (final concentration, 1.0 mg/ml) for 10 min at 30°C and incubated with a 5× molar excess of trypsin inhibitor for 5 min at 30°C, and the mixture was added to a complete reaction of vacuoles plus ATP and incubated at 25°C for 2 h. Other portions of cytosol were incubated with papain bound to beads (Sigma) for 60 min at 30°C. Papain protease was removed by sedimenting the beads in a microcentrifuge. In each case, 45 μg of treated (or untreated) wild-type or *vac5-1* cytosol was added to a complete reaction with 30 μg of wild-type cytosol (final total concentration of cytosol, 2.5 mg/ml). The total volume was 30 μl.

Fractionation of cytosol. G25 resin was equilibrated with cytosol buffer [20 mM PIPES-KOH, pH 6.8, 150 mM KOAc, 0.25 M sorbitol, 5 mM $Mg(OAc)_2$] in 3-ml prepacked columns (Bio-Rad). The excess buffer was removed by sedimentation in a clinical centrifuge for 2 min. Cytosol (50 to 75 μl) was layered on approximately 1 ml of packed resin and centrifuged for 2 min. Bradford assays were performed on the void volume fraction.

Small molecules were collected by adding cytosol at equal concentrations (15 to 20 mg/ml) to Centricon-10 ultrafiltration devices (Amicon), which are designed to allow molecules with masses of less than 10 kDa to partition to a lower chamber during centrifugation. In addition, partitioning with Centricon-3 ultrafiltration devices (with a 3-kDa cutoff) was performed, followed by fractionation with a spin column packed with G10 resin prepared as described above.

RESULTS

The in vitro inheritance assay requires three components: vacuoles, an ATP-regenerating system, and cytosol. Cytosols from *vac1-1* and *vac2-1* strains and from various class D vacuolar protein-sorting mutants (which have a vacuole segregation defect in vivo) are functional in the inheritance assay (3; unpublished observations). In contrast, the inability of *vac5-1* components to fuse in vitro is due to an altered cytosolic component.

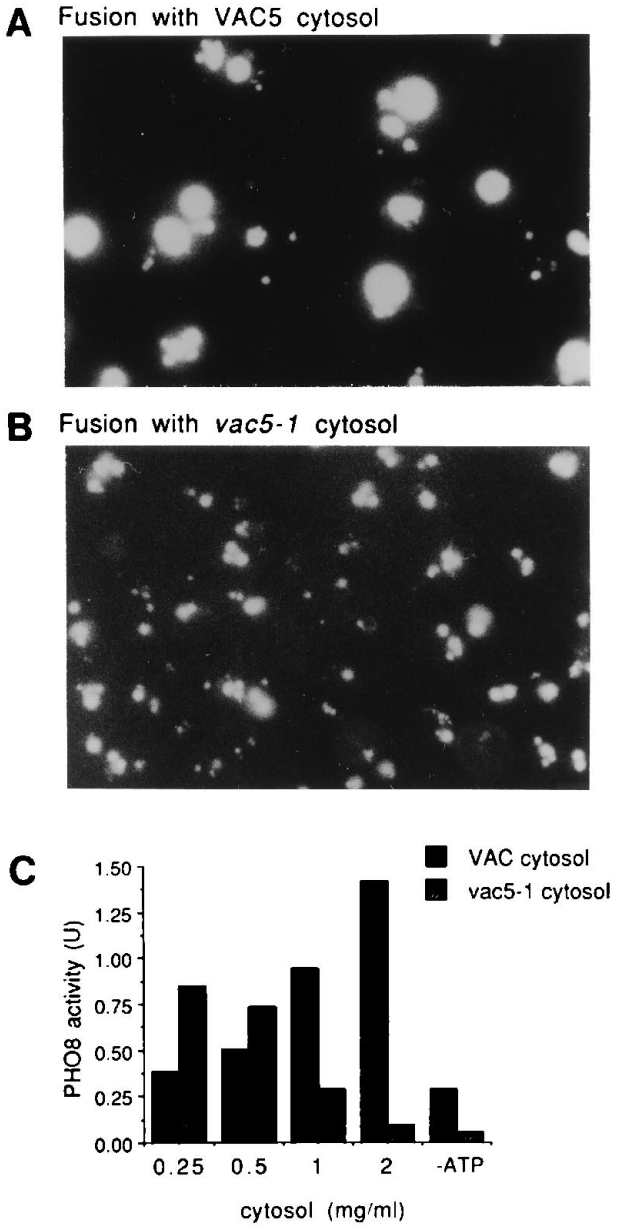

FIG. 1. *vac5-1* cytosol is defective in promoting vacuole-to-vacuole fusion. (A) Wild-type cytosol (2 mg/ml), 1 mM ATP, and CDCFDA-labeled wild-type vacuoles (0.25 mg/ml) were incubated at 25°C for 120 min. (B) CDCFDA-labeled wild-type vacuoles (0.25 mg/ml) were incubated at 25°C for 120 min with *vac5-1* cytosol (2 mg/ml) and 1 mM ATP. (C), Concentration curve of wild-type versus *vac5-1* cytosol using the pro-alkaline phosphatase maturation assay. Incubations were performed as described for panels A and B. (The value of a complete reaction from which cytosol was omitted was 0.106 U.)

Figures 1A and B show the microscopic assay of intervacuolar fusion in reactions with either wild-type or *vac5-1* cytosol. Incubation of CDCFDA-labeled wild-type vacuoles with wild-type cytosol promotes fusion and gives rise to large vacuoles which are bigger than the starting size. In contrast, incubation with *vac5-1* cytosol does not promote fusion and vacuoles either remain the same size or fragment. Defective fusion was seen with several different strain backgrounds containing the *vac5-1* mutation (data not shown). Increasing amounts of wild-type cytosol, up to 2.0 mg/ml, yield a proportionately higher

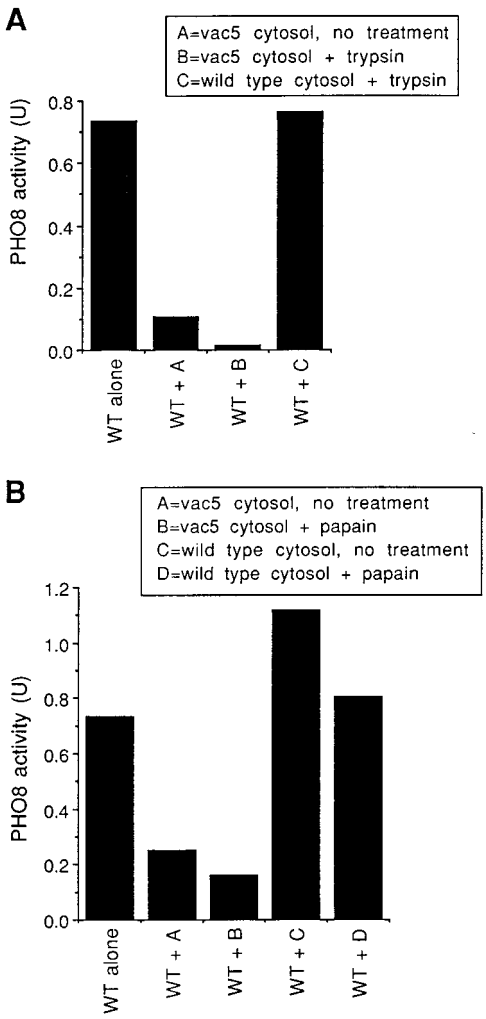

FIG. 2. The inhibitory activity of *vac5-1* cytosol is not sensitive to proteases. (A) Treatment of *vac5-1* cytosol with trypsin does not remove inhibitory activity. Wild-type or *vac5-1* cytosol was incubated with trypsin (final concentration, 1 mg/ml) for 10 min at 30°C. Trypsin inhibitor was then added (5 min at 30°C), and the mixture was incubated with a complete reaction of wild-type vacuoles and ATP and 1-mg/ml wild-type untreated cytosol for 2 h at 25°C. (B) Treatment of *vac5-1* cytosol with papain does not remove inhibitory activity. Wild-type or *vac5-1* cytosol was incubated for 60 min at 30°C with papain anchored to beads. The beads were sedimented, and the supernatant was then added to a complete reaction as described for panel A. In each experiment, 1.5 mg of protease-treated (or untreated) cytosol per ml as well as 1 mg of untreated cytosol per ml, was added to the incubations with vacuoles and ATP. The background of 0.394 U from incubations with ATP but no cytosol was subtracted.

signal in the Pho8p maturation assay (Fig. 1C). In contrast, *vac5-1* cytosol is more active than wild-type cytosol at low concentrations (Fig. 1C). At these lower concentrations, the vacuoles fuse normally and increase in size (data not shown). As increasing amounts of *vac5-1* cytosol are added, the signal which represents vacuole fusion decreases to the level obtained in a control reaction without ATP.

The vacuole fusion obtained with 1.0-mg/ml wild-type cytosol alone (Fig. 2A and B, WT alone) is decreased by the addition of 1.5 mg/ml *vac5-1* cytosol (Fig. 2A and B, WT + A). In contrast, additional wild-type cytosol increases the signal (Figure 2B, WT + C). Previous experiments have shown that trypsin pretreatment of wild-type cytosol inhibits the ability of the cytosolic components to promote intervacuolar fusion (3). This suggests that at least some of the cytosolic components required for fusion are proteinaceous. Protease digests were performed to determine whether the *vac5-1* inhibitory factor is proteinaceous. Pretreatment with either trypsin or papain did not affect the ability of *vac5-1* cytosol to inhibit the activity of wild-type cytosol (Fig. 2A and B, WT + B). In control reactions, the same protease treatment of wild-type cytosol did not yield inhibitory activity (Fig. 2A, WT + C, and 2B, WT + D). The signal obtained in these controls is approximately the same as those obtained in reactions with wild-type cytosol alone. Separate assays showed that the proteases were active.

To determine whether the inhibition is reversible, wild-type vacuoles were preincubated with *vac5-1* cytosol and then reisolated for a second incubation with wild-type cytosol (Fig. 3). Exposure of wild-type vacuoles to wild-type cytosol and ATP during the first 0 to 30 min did not affect their ability to fuse (Fig. 3A). The control aliquots, transferred to ice at the indicated times, show the amount of fusion achieved in the first incubation. Maximal signals were obtained if wild-type cytosol was added in the second incubation. Addition of *vac5-1* cytosol in the second incubation had the greatest inhibitory effect at the earlier time points. In contrast to the wild-type control, preincubation with *vac5-1* cytosol plus ATP for only 15 min irreversibly inactivated the wild-type vacuoles (Fig. 3B).

Since protease treatment did not abolish the inhibitory activity of *vac5-1* cytosol (Fig. 2B), partial fractionation was performed to determine the size of the *vac5-1* factor. *vac5-1* cytosol was separated into a protein fraction and a small-molecule fraction by using Sephadex G25 gel filtration and an ultrafiltration membrane with a cutoff at 10 kDa (see Materials and Methods). The activity of the G25 void volume fraction was variable but was always less than the activity of unfractionated cytosol (data not shown). Figure 4A shows the effects of adding back the small-molecule fraction (mass, <10 kDa) from wild-type cytosol or *vac5-1* cytosol to a complete fusion reaction with the *vac5-1* protein fraction. The first two bars represent signals obtained with wild-type (dots) or *vac5-1* (hatched) G25 protein fractions alone. As more wild-type small molecules are added to *vac5-1* proteins, the signal increases and reaches a plateau. In contrast, as more *vac5-1* small molecules are added to *vac5-1* proteins, the signal rises initially and then decreases, similar to the pattern obtained with unfractionated *vac5-1* cytosol (Fig. 1C). The inhibition by the *vac5-1* small-molecule fraction is maximal at 4 μl, a volume which corresponds to the volume of unfractionated *vac5-1* cytosol required for comparable inhibition.

Further size fractionation of *vac5-1* small molecules demonstrates that the *vac5-1* factor is bigger than 0.7 kDa and smaller than 3.0 kDa. *vac5-1* cytosol was first passed through an ultrafiltration membrane with a cutoff at 3 kDa, and the filtrate was then passed through G10 resin to remove small ions and molecules. Figure 4B shows the results of adding either wild-type or *vac5-1* small molecules (C3/G10 fractions; masses between 3.0 and 0.7 kDa) to wild-type G25 void volume proteins. The wild-type small-molecule fraction still stimulates the fusion activity of the protein fraction, whereas the *vac5-1* small molecule fraction inhibits activity.

DISCUSSION

The in vitro inheritance assay measures intervacuolar fusion, a late step in the vacuole segregation process, and *vac5-1* cytosol is defective in promoting this step. In vivo, many *vac5-1* mutant cells exhibit single, round mother cell vacuoles without either segregation structures or vacuolar material in the bud (15). If stained with fluoroscein isothiocyanate, many mutant

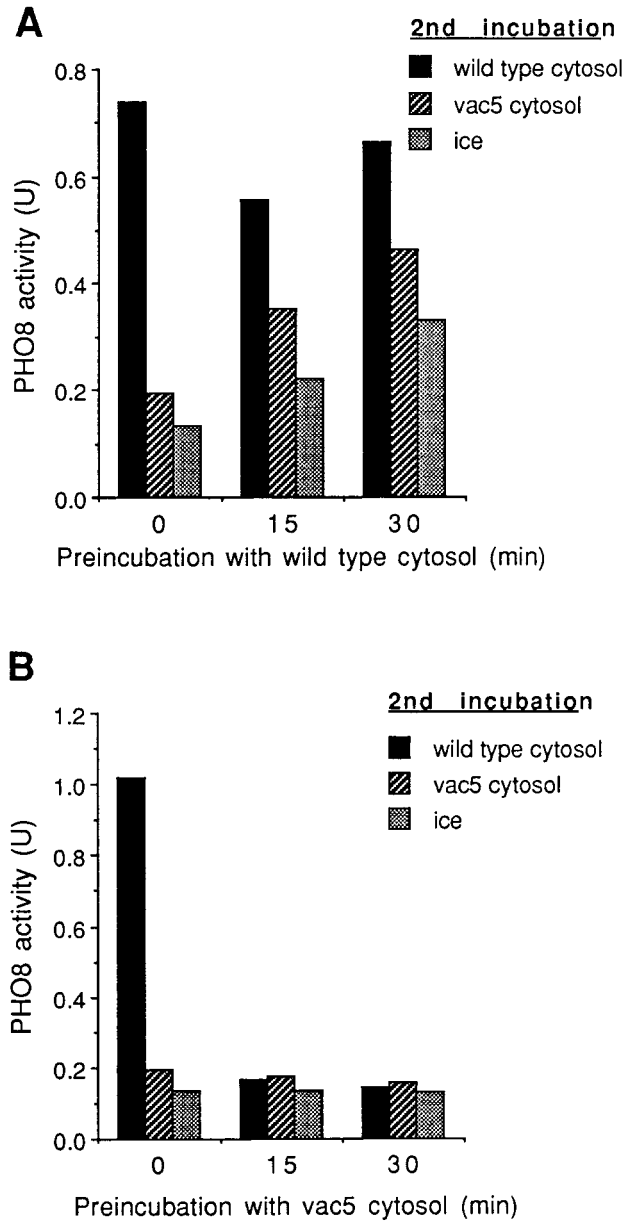

FIG. 3. Rapid inactivation of vacuoles by *vac5-1* cytosol. (A) A complete reaction of wild-type cytosol, ATP, and vacuoles was performed at 25°C. Aliquots were removed, and the vacuoles were sedimented (10,000 × *g*, 1 min, 4°C) and resuspended to the same concentration in isolation buffer (0.2 M sorbitol, 10 mM PIPES-KOH, pH 6.8). These samples were divided into three aliquots, and a second incubation was performed with the indicated cytosols plus ATP for a total of 120 min (the third aliquot was immediately placed on ice). (B) A complete reaction of *vac5-1* cytosol, ATP, and vacuoles was prepared and treated as described for panel A.

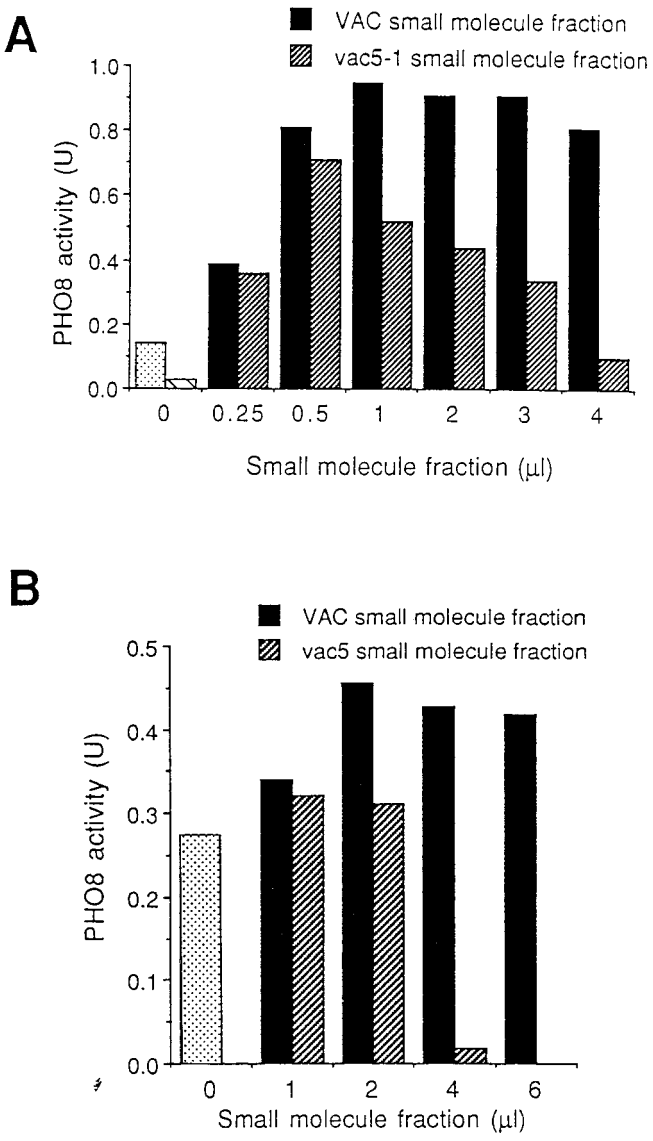

FIG. 4. Size of the *vac5-1* factor. (A) Fractionation of *vac5-1* cytosol reveals that the cytosolic inhibitor is a small molecule. A wild-type or *vac5-1* small-molecule fraction obtained by ultrafiltration with a Centricon-10 filter (<10-kDa cutoff) was added to a *vac5-1* G25 void volume fraction containing proteins and molecules of >3 kDa (1 mg/ml) and assayed in the presence of ATP and vacuoles. The total volume of each reaction was 30 μl. The first two bars compare fusion activity of wild-type (dots) and *vac5-1* (wide hatch) G25-purified proteins alone. (B) The cytosol from wild-type and *vac5-1* cells was fractionated by passage through a Centricon-3 filter (<3 kDa cutoff) and applied to G10 resin spin columns (0.7-kDa cutoff). The void volume from the G10 resin was added to the G25 void volume protein fraction of wild-type cytosol (1 mg/ml). The first bar (dots) indicates the signal obtained with wild-type G25 proteins alone. Samples were incubated with wild-type vacuoles and ATP at 25°C for 2 h. The assay value of a complete reaction from which cytosol was omitted (for panel A, 0.190; for panel B, 0.269) was subtracted from each.

cells display buds which contain a single small vesicle. Taken together, these cytological observations suggest that mutant cells either do not form vacuolar segregation structures or only manage to partition a small amount of material to the bud. This is consistent with our in vitro findings that isolated wild-type vacuoles, if incubated with *vac5-1* cytosol, either remain the same size or tend to fragment (Fig. 1).

Concentration curves of *vac5-1* cytosol reveal an interesting pattern of fusion activity that is greater than wild-type activity at low concentrations and inactive at high concentrations. This suggests that a factor which normally promotes fusion is present at higher levels in *vac5-1* cytosol. Too much of this factor may inhibit intervacuolar fusion in the same way that adding too much wild-type cytosol can inhibit fusion. If one adds a final concentration of 4 mg/ml or more of wild-type cytosol to a complete reaction, then fusion activity starts to decrease (6a). In addition, excess stimulatory factor may en-

able *vac5-1* to inhibit the activity of wild-type cytosol. Alternatively, *vac5-1* may induce the expression of a distinct inhibitor, although this model is not consistent with the observation that *vac5-1* cytosol has higher-than-normal activity at low concentrations.

Partial fractionation and protease treatment of *vac5-1* cytosol suggests that the *vac5-1* factor is a small, protease-resistant molecule with a size of between 0.7 and 3 kDa. These results suggest that the *vac5-1* factor is neither a small polypeptide nor a small ion. The *vac5-1* inhibitory factor(s) appears to be an overproduced component normally involved in fusion. Since metabolic phosphatase (e.g., Pho5) expression increases in *vac5-1* strains, it seemed possible that an increase in phosphate might have been the cause of inhibition of vacuolar membrane fusion. However, this is very unlikely, since the fractionation with G10 resin removes most of the P_i. Furthermore, the amount of P_i that is present in the 3.0- to 0.7-kDa, *vac5-1* small-molecule fraction as measured by a malachite green assay is too low to account for an inhibitory effect in the vacuole fusion assay (unpublished observations).

The requirement for small molecules other than peptides or ions has been shown in other reconstituted systems measuring membrane fusion. One example is palmityl-coenzyme A, which stimulates *N*-ethylmaleimide-sensitive factor-dependent *cis* Golgi to *trans* Golgi fusion (5, 17). A second example is arachidonic acid, which has an annexin-dependent role in endosome fusion (13). The present study provides an assay for the purification of the small molecule which is overproduced in *vac5-1* strains. This is an essential step towards evaluating its function in the regulation of vacuole inheritance.

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