Thioredoxin Is Required for Vacuole Inheritance in *Saccharomyces cerevisiae*

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Abstract. The vacuole of *Saccharomyces cerevisiae* projects a stream of tubules and vesicles (a "segregation structure") into the bud in early S phase. We have described an in vitro reaction, requiring physiological temperature, ATP, and cytosol, in which isolated vacuoles form segregation structures and fuse. This in vitro reaction is defective when reaction components are prepared from *vac* mutants that are defective in this process in vivo. Fractionation of the cytosol reveals at least three components, each of which can support the vacuole fusion reaction, and two stimulatory fractions. Purification of one "low molecular weight activity" (LMA1) yields a heterodimeric protein with a thioredoxin subunit. Most of the thioredoxin of yeast is in this complex rather than the well-studied monomer. Deletion of both *S. cerevisiae* thioredoxin genes causes a striking vacuole inheritance defect in vivo, establishing a role for thioredoxin as a novel factor in this trafficking reaction.

Low copy cytoplasmic organelles divide in synchrony with the cell cycle and are inherited in a spatially regulated manner (Warren, 1993). Cytological studies of mammalian cells have shown vesiculation of the nuclear, endoplasmic reticulum, and Golgi membranes at the onset of mitosis and the fusion of vesicles to reconstitute these organelles in the daughter cells after cytokinesis (Zeligs and Wollman, 1979; Lipsky and Pagano, 1985; Lucocq et al., 1987). Little is known of the biochemical mechanisms of these processes or how mitochondria and lysosomes divide and are inherited. Organelle inheritance has also been studied in *Saccharomyces cerevisiae* (Yaffe, 1991). The yeast nuclear membrane does not vesiculate during cell division ("closed mitosis"), and nuclear inheritance is blocked in mutants affecting the spindle (Berlin et al., 1990; Huffacker, 1992; Palmer et al., 1992; Eshel et al., 1993; Clark and Meyer, 1994; Muhua et al., 1994). Mutants have also been described in mitochondrial inheritance (*mdm* mutants), some affecting homologs of intermediate filament proteins (McConnell and Yaffe, 1992, 1993).

We have undertaken a cytological, genetic, and biochemical approach to study the inheritance of the vacuole (lysosome) in *S. cerevisiae.* Though the copy number of this organelle varies in different strains, exponentially growing cells typically have one to five vacuoles (Weisman et al., 1987). Early in S phase, the vacuole suddenly projects a stream of vesicles and membranous tubules into the bud (Weisman and Wickner, 1988). These vesicles fuse in the bud, founding the new (daughter) organelle. Inter-vacuole vesicular traffic continues until M phase, when it is interrupted by nuclear migration into the neck. We have isolated *vac* mutants in which this process is defective (Weisman et al., 1990; Shaw and Wickner, 1991; Nicholson et al., 1995). In these mutants, many large buds lack a vacuole entirely or have only an unusually small organelle. These cells pause at cytokinesis and many of the daughter cells inherit little or no vacuolar material. The daughter cells which do not receive a vacuole form a new one by unknown means before embarking on bud growth and a new cell cycle. Some *vac* mutants (e.g., *vac*1-1; Weisman et al., 1990) also have a *vps* (vacuolar protein sorting) defect in which proteins normally sorted from the Golgi to the vacuole are instead secreted to the cell surface. Other *vac* mutants (*vac* 2-1, *vac* 5-1; Shaw and Wickner, 1991; Nicholson et al., 1995) are not defective in sorting newly made proteins, establishing that vacuole inheritance is a distinct process from protein sorting. Strikingly, the deletion of some of these *VAC* genes is not lethal and still allows "leaky," low-level vacuole inheritance.

These cytological and genetic studies of vacuole inheritance have been complemented by the development of an in vitro reaction of inter-vacuole traffic (Conradt et al., 1992; Haas et al., 1994). In this reaction, isolated vacuoles incubated with ATP and cytosol form segregation structures and undergo fusion. The reaction is performed with vacuoles purified from two strains, one with normal vacuolar proteases but a deletion in the PHO8 gene encoding pro-alkaline phosphatase, the other with deletion of the genes for vacuolar proteases but normal PHO8. The latter vacuoles bear catalytically inactive pro-alkaline phosphatase

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which becomes proteolytically matured and catalytically activated when in vitro intervacuole fusion mixes the vacuole lumenal contents, allowing quantitative assay of vacuole fusion. This in vitro reaction occurs in four distinct stages in obligate sequence (Conradt et al., 1994). The first entails incubation of the mixed vacuoles at physiological ionic strength and temperature, the second requires cytosol, the third ATP, and the fourth needs concentrated vacuoles at physiological temperature but neither salt, ATP, nor cytosol. Inhibitor studies (Haas et al., 1994) have implicated G protein(s), phosphatases, and membrane potential requirements for this reaction, underscoring its inherent complexity.

We now report an initial scheme for fractionating the required cytosolic components of the reaction. Gel filtration reveals three activities, each capable of supporting the vacuole fusion reaction. One of these has been purified to homogeneity and is a novel dimeric form of thioredoxin.

Materials and Methods

Yeast Strains

S. cerevisiae strains used in this study were K91-1A (Matα *ura3 pho8::pAL134 pho13::pPH13 lys1*; from Dr. Y. Kaneko, Institute of Fermentation, Osaka, Japan), BJ 3505 (Matα *pep4::HIS3 Δprb1-1.6R HIS3 lys2-208 Δtrp1-101 ura3-52 gal2 can1*; Moehle et al., 1986), DKY 6281 (Matα *ura3-52 leu2-3 leu2-112 Δhis3-200 Δtrp1-901 lys2-801 Δsuc2-9 Δpho8::TRP1*; from Dr.D. Klionsky, University of California, Davis. CA), EMY21-1C (Mata *TRX1 TRX2 Δlys2::HIS3 ura3-1 Δade3-100 ade2 trp1-1 leu2-3 112 his3-11 can1-100*), EMY21-1D (Mata *TRX1 Δtrx2:LEU2*), EMY21-2C (Mata *Δtrx1::TRP1 TRX2*), and EMY21-8D (Mata *Δtrx1::TRP1 Δtrx2::LEU2*). These four EMY21- strains (Muller, 1991) were generously provided by Dr. E. Muller (University of Washington, Seattle, WA).

Materials

Oxalyticase was purchased from Enzogenetics (Corvallis, OR), DEAE-dextran, Ficoll 400, Sephadex G-25, Sephacryl S-100 HR, and Q Sepharose Fast Flow were from Pharmacia Fine Chemicals (Piscataway, NJ). Creatine phosphokinase, creatine phosphate, and Pefabloc SC were from Boehringer Mannheim Biochemicals (Indianapolis, IN). ATP, *p*-nitrophenyl phosphate (*p*NPP)[1], DTT, pepstatin A, leupeptin, 1,10-phenanthroline and Spirulina sp. thioredoxin were from Sigma Chemical Co. (St. Louis, MO). 5 (and 6-) -carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA), FITC, and *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM 4-64) were from Molecular Probes Inc. (Eugene, OR). Anti-TRX1 antiserum (Muller, 1995) was kindly provided by Dr. E. Muller at University of Washington (Seattle, WA).

Cytosol Preparation

Small scale cytosol preparation was performed according to Conradt et al. (1992) without modification. For large scale cytosol preparation, 75 liters of cells (strain K91-1A) were grown in YPD to an OD_{600} of 3 to 4 in a New Brunswick fermentor. The cells were collected by sedimentation (5,000 *g*, 10 min, 4°C) and suspended once with 3 liters cold water and once with 3 liters cold cytosol buffer (20 mM Pipes-KOH, pH 6.8, 150 mM KOAc, 250 mM sorbitol, and 5 mM $MgCl_2$). The cell pellet was then resuspended in 200 ml of the cytosol buffer. To this cell suspension, DTT and PMSF were added to final concentrations of 1 and 0.5 mM, respectively. The cells were lysed by 15 s homogenizations in a glass bead beater with equal volumes of glass beads and cell suspension and chilling on ice for 45 s. After 20 cycles, 80–90% of cells were lysed. The cell lysate was clarified (8,000 *g* 10 min, 4°C) and the supernatant was centrifuged (55,000 rpm; type 60 rotor, 90 min, 4°C; Beckman Instruments, Palo Alto, CA). The cytosol was collected and frozen in liquid nitrogen and stored at −80°C.

Vacuole Preparation

Vacuoles were isolated from the strains BJ3505 and DKY6281 as described previously (Haas et al., 1994; Conradt et al., 1994). To prepare salt washed vacuoles, the fresh isolated vacuoles were adjusted to a concentration of 0.3 mg/ml with "0% Ficoll buffer" (10 mM Pipes-KOH, pH 6.8, and 200 mM sorbitol). Equal amounts of vacuoles from BJ 3505 were mixed with those from DKY6281. KCl and KOAc were added to the mixture to final concentrations of 100 and 50 mM, respectively. Aliquots of 200 μl were incubated at 30°C for 10 min. After chilling on ice for 1 min, the aliquots were centrifuged at 10,000 rpm for 85 s at 4°C with a microcentrifuge. The supernatant was removed and the pellets were covered with 160 μl of 0% Ficoll buffer. These salt washed vacuoles can be used directly or stored at −20°C for two days and then transferred to −80°C for later use.

In Vitro Homotypic Vacuole Fusion Assay

The in vitro vacuole fusion reaction was carried out as described (Haas et al., 1994; Conradt et al., 1994). A 30 μl vacuole fusion reaction mixture contains 2.4 μg vacuoles from BJ 3505, 2.4 μg vacuoles from DKY6281, 200 mM sorbitol, 20 mM Pipes-KOH, pH 6.8, 150 mM KOAc, 5 mM $MgCl_2$, an ATP regenerating system (1 mM ATP, 40 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase), and either 0.5–1 mg/ml cytosol or fractions from the protein purification. In vitro reactions were incubated for 2 h at room temperature and stopped by chilling on ice.

Alkaline Phosphatase (PHO8) Activity

In vitro homotypic vacuole fusion was measured using the alkaline phosphatase maturation assay described by Haas et al. (1994). Alkaline phosphatase activity was determined according to Mitchell et al. (1981) with modifications by Haas et al. (1994). PHO8 substrate solution (470 μl of 250 mM TrisCl, pH 9.0, 0.4% Triton X-100, 10 mM $MgCl_2$, 1 mM *p*NPP) was added to each sample on ice. The reactions were transferred to 30°C and incubated for 5 min, and then stopped by adding of 500 μl of 1 M glycine-NaOH, pH 11.5. *p*-Nitrophenol (*p*NP) was determined by measuring the absorbance at 400 nm. 1 U of alkaline phosphatase activity (1 U PHO8 activity) corresponds to 1 μmol of *p*NP produced at 30°C/min/μg of BJ3505 vacuolar protein.

Purification of LMA1

All procedures were at 4°C. LMA1 activity was monitored by the in vitro homotypic vacuole fusion assay and alkaline phosphatase maturation assay (Haas et al., 1994). K91-1A yeast cytosol (15 ml × 40 mg/ml) containing a protease inhibitor cocktail (PIC) (0.1 mM Pefabloc, 0.5 μg/ml pepstatin A, 0.1 μg/ml leupeptin, and 0.5 mM phenanthroline) was applied to a Sephacryl S100 HR (Pharmacia Fine Chemicals) column (5 cm × 56 cm, 1,100 ml) equilibrated with 6 liters of buffer A (20 mM Pipes-KOH, pH 6.8, 5 mM $MgCl_2$, and 0.1 mM $MnCl_2$), and then one column volume of buffer A containing PIC. The column was eluted with buffer A containing PIC at a flow rate of 9 ml/cm²/h. Fractions (6.5 ml) were collected and aliquots (2 μl) of each fraction were used for assay of protein concentration and fusion activity. LMA1 peak fractions were pooled (32.5 ml). Portions of the LMA1 pool (8 ml) were adjusted pH to 8.8 with KOH and applied to a Q Sepharose Fast-Flow column (1.5 cm × 3 cm, 5.3 ml) equilibrated with 200 ml of buffer C (20 mM TrisAc, pH 8.8, 5 mM $MgCl_2$, and 0.1 mM $MnCl_2$) at a flow rate of 24.4 ml/cm²/h. After collecting the flow through, the column was washed with 16 ml of buffer C and the bound proteins were eluted with a 56 ml linear gradient of 0–1,000 mM KOAc in buffer C. Fractions (0.72 ml) were collected and aliquots (3 μl) were assayed for vacuole fusion activity. Fractions containing fusion activity were pooled (3.6 ml), diluted with 10.8 ml of buffer C, and applied with a flow rate of 0.5 ml/min to a Mono Q HR 5/5 column (Pharmacia Fine Chemicals) equilibrated with buffer C. The bound proteins were eluted with a 10 ml linear gradient of 0–1,000 mM KAc in buffer C. Fractions (0.3 ml) were collected and an aliquot (3 μl) of each fraction was assayed for its vacuole fusion activity. Fractions of peak activity were pooled (1.2 ml), mixed with 0.13 ml of 100% glycerol, and stored in aliquots at −80°C.

1. *Abbreviations used in this paper*: CDCFDA, 5 (and 6-)-carboxy-2',7'-dichlorofluorescein; HMA, high molecular weight activity, LMA, low molecular weight activity; pNPP, p-nitrophenyl phosphate.

Microscopy

Vacuoles were visualized with three different vital fluorophores. For labeling vacuoles with CDCFDA (Pringle et al., 1989; Weisman et al., 1990), cells were grown to early log phase in YPD and collected from 1 ml of culture by centrifugation (12,000 *g*, 1 min, 23°C). The cell pellet was resuspended in 1 ml of YCM medium (YPD + 50 mM citrate phosphate, pH 4.5) (Rogers and Bussey, 1978), mixed with 5 μl of 10 mM CDCFDA in DMSO, and incubated at 30°C for 10 min. Cells were collected by centrifugation, washed once with YPD and examined by fluorescence microscopy. For labeling vacuoles with FITC (Preston et al., 1987; de Mesquita et al., 1991), cells were grown, harvested, and resuspended in 1 ml YCM as described above and 10 μl of 4 mg/ml FITC was added. Cells were incubated at 37°C for 10 min, collected by centrifugation, washed once with YPD, and examined by fluorescence microscopy. For in vitro labeling of vacuoles with a lipophilic styryl dye, FM 4-64 (Vida and Emr, 1995), a 2 mM FM 4-64 stock in DMSO was diluted to 200 μM with 0% Ficoll buffer and 1 μl was added to a 30 μl fusion reaction. After incubation for 3 h at room temperature, an aliquot of the reaction was removed for fluoresence microscopy.

Other Methods

Protein concentration was determined by using Bio-Rad protein reagent (Bio-Rad Laboratories, Cambridge, MA) with BSA as a standard. SDS-PAGE was performed as described (Ito et al., 1980). In the "High Tris"system, the seperating gel contained 19.6% acrylamide, 0.216% bisacrylamide, 0.7 M TrisCl, pH 8.85, and 0.5 M NaCl, the stacking gel contained 5% acrylamide, 0.07% bisacrylamide, 125 mM Tris-HCl, pH 6.8, and the "running buffer" contained 1.4% glycine, 0.6% Tris-HCl, and 0.1% SDS. Sample buffer contained 2% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 1.5% β-mercaptoethanol, and 0.02% bromophenol blue. Transfer of proteins to PVDF membranes was performed with a semidry apparatus (American Bionetics). Immunoblot analysis of TRX proteins was performed as previously described (Haas et al., 1994). The anti-TRX1 antiserum was used at 1:2,000 dilution.

Results

Purification of LMA1

Cytosolic components were resolved by Sephacryl S100HR chromatography and fractions assayed for their ability to replace cytosol in the in vitro reaction of vacuole fusion. Three peaks of activity were obtained (Fig. 1 *A*), a high molecular weight activity (HMA) which emerged with the bulk cytosolic proteins and two partially overlapped peaks of low molecular weight activity (LMA1 and LMA2; arrows in the figure). These three fractions gave no more than additive activity in supporting the fusion reaction (data not shown). The LMA1 activity was stimulated by GSM, a small molecule fraction obtained by Sephadex G25 gel filtration, and by PFT, a flow-through fraction from phosphocellulose chromatography (Fig. 2 *A*). LMA1 was further purified by ion exchange chromatography (Table I), yielding a 4,800-fold purified fraction with an apparent molecular weight of 23 kD on gel filtration. The complete reaction with pure LMA1 has the same requirements (both types of vacuoles, ATP, and incubation at physiological temperature) as the reaction performed with cytosol (Fig. 2 *B*) and also has the same sensitivity (Fig. 2 *C*) to the protein phosphatase inhibitor microcystin-LR (Conradt et al., 1994). Microscopic examination (Fig. 3) directly confirms that LMA1 supports vacuole fusion. The purified LMA1 has two prominent polypeptides (Fig. 4), one of approximately 12 kD and the other of approximately 10 kD, which are enriched throughout the purification (lanes *1–4*) and co-chromatograph during the final chromatography (peak fractions are lanes *5–10*). Amino acid analysis showed that these two polypeptides had a 1:1 molar ratio and distinct amino acid compositions (data not shown). The identity of the lower molecular weight polypeptide, termed p10, is not known; it is currently under study. Two polypeptides from the

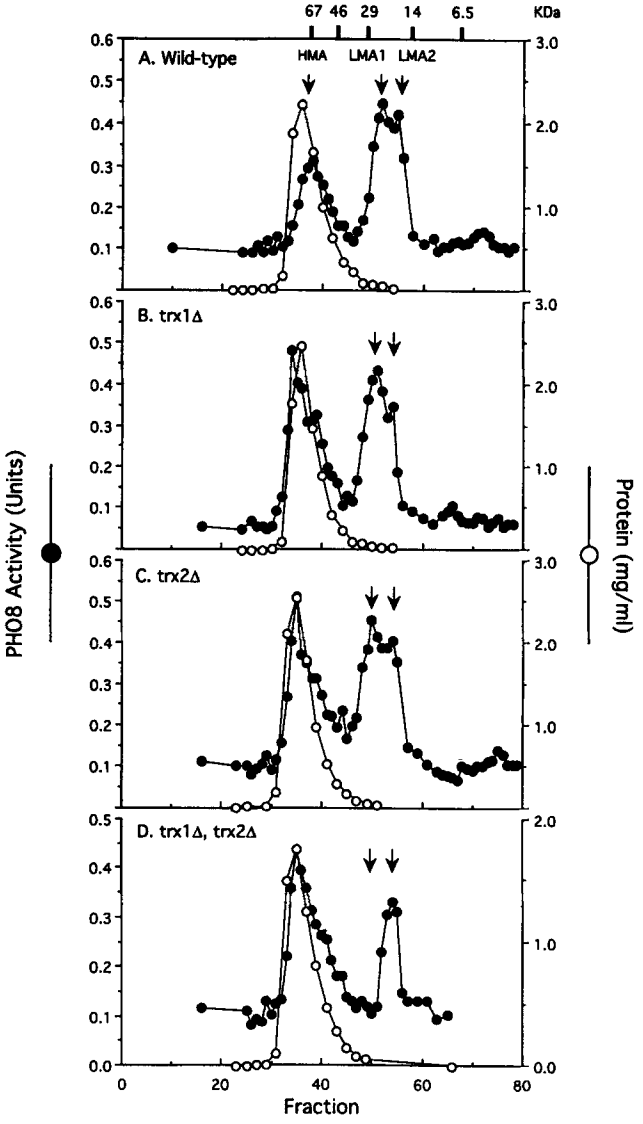


Figure 1. Sephacryl S100 HR gel filtration of yeast cytosol. Yeast cytosols prepared from *TRX1,TRX2* (*A*), *Δtrx1,TRX2* (*B*), *TRX1, Δtrx2* (*C*), and *Δtrx1, Δtrx2* (*D*) strains as described in Materials and Methods were mixed with 30× the concentrations of PIC components (Materials and Methods) and with 0.1 mM ATP, and then incubated on ice for 20 min followed by centrifugation (10,000 *g* 10 min) at 4°C. A 200-μl aliquot of the clarified cytosol was mixed with 3 μl of 20 mg/ml carbonic anhydrase and 20 mg/ml lysozyme as internal molecular mass markers. The sample was applied to a Sephacryl S100 HR column (1 cm × 26 cm, 20.4 ml) equilibrated in buffer C (20 mM TrisCl, pH 8.8, 5 mM $Mg(OAc)_2$, and 0.1 mM $MnCl_2$). The column was eluted (12 ml/cm^2/h) with buffer C. Fractions (300 μl) were collected. Protein concentration and fusion activity of the fractions were determined as described in the Materials and Methods. The internal molecular weight markers were visualized by SDS-PAGE and silver staining.

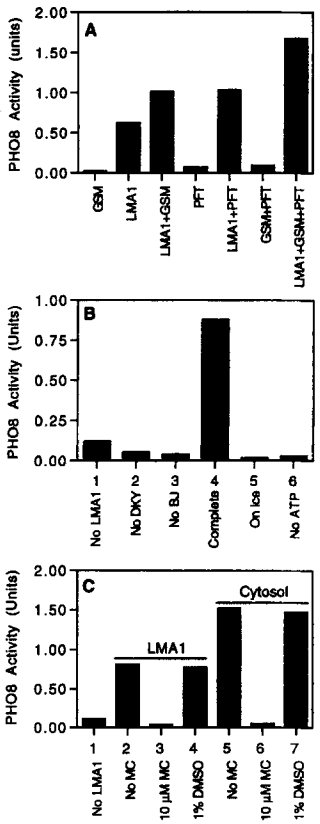


Figure 2. (*A*) Synergy between LMA1, GSM, and PFT. In a complete fusion reaction mixture, the cytosol portion was replaced by 2 μl of G25 small molecules (*GSM*), or 10 ng pure LMA1, or 2 μl of phosphocellulose flow through (*PFT*), or the different combinations as indicated in the figure labels. The fusion reaction and alkaline phosphatase assays were as described in Materials and Methods. Data were corrected for background activity (0.08 U/sample). (*B*) Requirements for vacuole fusion catalyzed by pure LMA1. Fusion reactions contained isolated vacuoles from BJ3505 (lane *2*), or DKY6281 (lane *3*), or both strains (lanes *1* and *4–6*). All the reactions contained 10 ng pure LMA1 except lane *1*. The reactions with (lanes *1–5*) or without (lane *6*) ATP (1 mM ATP and an ATP regenerating system) were incubated at room temperature (lanes *1–4* and *6*) or on ice (lane *5*) for 2 h followed by alkaline phosphatase assay as described in Materials and Methods. (*C*) Sensitivity to microcystin. Fusion reactions contained vacuoles from BJ3505 and DKY6281, ATP, salts, and 10 ng pure LMA1 (lanes *2–4*) or 15 μg cytosolic protein (lanes *5–7*) or no addition (lane *1*). Microcystin-LR was added to the reactions to a final concentration of 10 μM (lanes *3* and *6*) from a 2 mM stock solution in dimethylsulfoxide. Lanes *4* and *7* are the solvent (dimethylsulfoxide) only controls for lanes *3* and *6*, respectively. The reactions were incubated at room temperature for 2 h followed by alkaline phosphatase assay as described in Materials and Methods. Data were corrected for a background activity (0.08 U/sample).

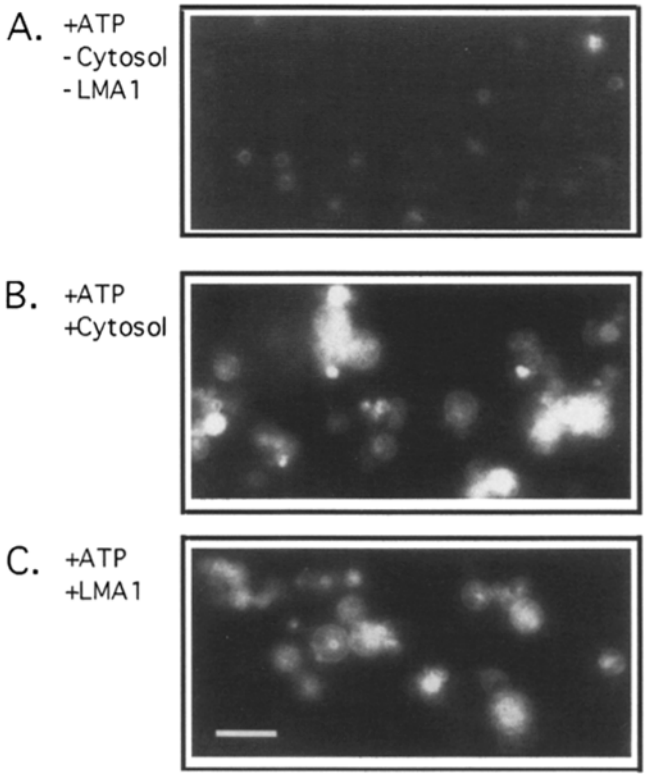


Figure 3. Microscopic analysis of vacuole fusion reactions. Vacuole fusion reactions contained equal amount of both BJ3505 and DKY6281 vacuoles, salt, and ATP only (*A*), or ATP plus 0.6 mg/ml cytosol (*B*), or ATP and 1 μg/ml pure LMA1 (*C*). The vacuoles were labeled with a lipophilic styryl dye FM 4-64 (Vida and Emr, 1995) as described in Materials and Methods. Bar, 4 μm.

larger subunit of LMA1, generated by trypsin digestion and separated by reverse phase HPLC, had the sequences: ThrAlaSerGluPheAspSerAlaIleAlaGlnAspLys and PheSerGluGlnTyrProGlnAlaAspPheTyrLys, identical to sequences of yeast thioredoxin 1 (Muller, 1991), the more abundant of the two yeast thioredoxins.

Thioredoxin and In Vitro Vacuole Fusion

To determine whether thioredoxin is a functional component of LMA1, cytosols were prepared from mutants with deletions in the genes for thioredoxin 1, thioredoxin 2, both thioredoxins, and their corresponding wild-type strain (Muller, 1991). Gel filtration of the wild-type cytosol (Fig. 1 *A*) showed HMA, LMA1, and LMA2 peaks of activity. Deletion of either thioredoxin alone (Fig. 1, *B* and *C*) had little effect, but deletion of both thioredoxin genes (Fig. 1 *D*) left the HMA and LMA2 peaks while obliterating activity from the LMA1 peak. Immunoblot analysis (Fig. 5) with antiserum to yeast thioredoxin confirmed that thioredoxin was entirely absent from the cytosol of the double deletion strain. For this analysis, equivalent amounts of wild-type and single-deletion cytosols were analyzed along with five times this amount of the double deletion cytosol. This antiserum, as well as one prepared to a peptide from the middle of the thioredoxin sequence (data not shown), did not react with the lower molecular weight subunit of LMA1, confirming that it is not a derivative of thioredoxin. Silver stained gels (Fig. 6) of these fractions show that both subunits of LMA1 were missing from the 23-kD position in the double deletion mutant. LMA1 is clearly a complex of thioredoxin with a second, smaller subunit p10.

Vacuole fusion activity was found in a pure thioredoxin from Spirulina, an unrelated alga, and this activity, though a hundredth the specific activity of LMA1, was also stimulated by the GSM and PFT fractions (Fig. 7). Taken together, these data show that yeast thioredoxin is largely found as a subunit of a dimeric complex and that the thioredoxin subunit has a primary role in its functional activity for the in vitro vacuole inheritance reaction.

Thioredoxin Is Needed for Normal Vacuole Inheritance

Since thioredoxin has not been reported to be involved in other organelle trafficking reactions, and the distinct HMA and LMA2 fractions are present and support vacuole inheritance in extracts of a double thioredoxin deletion mu-

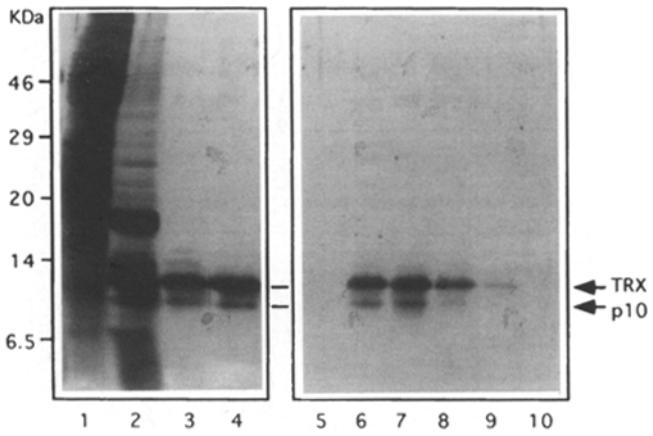


Figure 4. Purification of LMA1. Protein patterns of fractions at different stages of purification were visualized by high Tris SDS-PAGE and silver staining. K91-1A cytosol (lane *1*) was applied to Sephacryl S100 HR and the LMA1 peak was pooled (lane *2*) and subjected to Q Sepharose fast flow (lane *3*). Further purification was achieved on Mono Q (lane *4*). Lanes *5–10* show the protein patterns of LMA1 peak fractions of Mono Q. Lane *1* contained 120 μg cytosol protein. Lanes *2–10* were cold TCA precipitates from a 200-μl aliquot of their corresponding fractions.

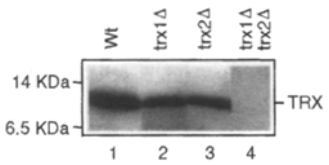


Figure 5. Immunoblot analysis of thioredoxin. Cytosols were prepared from wild-type (*Wt*), *Δtrx*1, *Δtrx*2, and *Δtrx*1,*Δtrx*2 strains as described in Materials and Methods. An aliquot of each cytosol was separated by high Tris SDS-PAGE followed by transfering to PVDF membrane and probing with anti-TRX1 antiserum.

tant, we examined the role of thioredoxin in vacuole inheritance in vivo (Fig. 8). The *Δtrx1Δtrx2* double *trx* deletion mutants show a clear *vac* phenotype of buds with no vacuole, whether vacuole staining was with CDCFDA (Fig. 8 *B*) or with the endogenous fluorophore of *ade2* strains (data not shown). While almost all wild-type cells with a bud diameter of at least half the maternal cell diameter had inherited a bud vacuole, 47% of double *trx* deletion cells had no vacuole in their bud (Table II). Those mutant cells which have a bud vacuole tend to have smaller bud vacuole volume than seen for the bud vacuoles of wild-type cells (Fig. 8 *C*). Some *trx* double deletion cells had buds with diameters equal to those of the mother cell (Fig. 8 *C*), whereas the wild-type cells did not, having undergone cytokinesis when the bud was distinctly smaller than the maternal cell (Hartwell and Unger, 1977). This delay in cytokinesis has also been reported for other *vac* and *mdm* mutants in vacuole and mitochondrial inheritance (Weisman et al., 1990; McConnell and Yaffe, 1992, 1993), suggesting a general requirement for complete organelle inheritance for cytokinesis. We also examined whether the *trx* double deletion mutant has a *vps* phenotype of secreting proteins which are normally localized to the vacuole; no such aberrant secretion was observed (data not shown). The strongly defective vacuole inheritance phenotype is not seen in either single *Δtrx* mutant, but is only seen in the double *trx* deletion mutants (Table II; and data not shown), indicating that the two thioredoxins are functionally interchangeable; thus *trx* mutants would not be found in a normal screen for *vac* mutants. These results provide a novel insight into thioredoxin structure and cellular function, and an important validation of this in vitro reaction of organelle inheritance.

Table I. Purification of LMA1

Step	Volume		Protein activity	Specific activity	Purification
	ml	*mg*	*U*	*U/mg*	*-fold*
I. Cytosol	130	5,200	2.14×10^5	41	–
II. S100	213	17	1.07×10^5	6,292	153
III. FFQ	115	0.34	2.27×10^4	66,700	1,620
IV. MonoQ	32	0.08	1.59×10^4	199,333	4,842

See text for details.

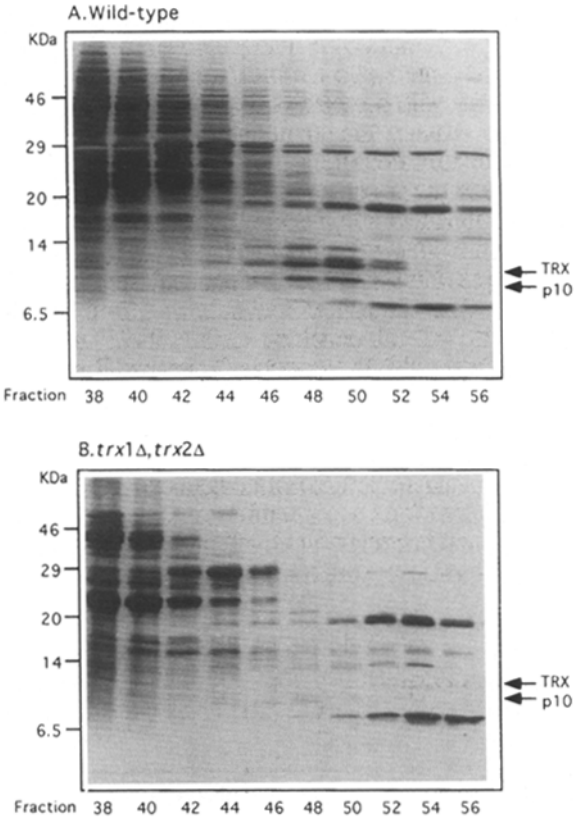


Figure 6. Both TRX and p10 bands are missing in the *Δtrx*1, *Δtrx*2 cytosol at the LMA1 position of S100 gel filtration. The protein pattern of Sephacryl S100 HR gel filtration of cytosol prepared from wild-type (*A*) or *Δtrx*1,*Δtrx*2 (*B*) strain was visualized by high Tris SDS-PAGE and silver staining. A 200-μl aliquot of cytosol samples was applied to a Sephacryl S100 HR column (1 cm × 24 cm, 18.8 ml) equilibrated in buffer C at 4°C. The column was eluted with buffer C at a flow rate of 12 ml/cm²/h. Fractions (300 μl) were collected. The LMA1 peak consisted of fractions from 46–52. The positions of TRX and p10 are indicated (*closed arrows*). The gray arrows on the right side of the figure indicate positions of internal molecular mass markers of 14 and 29 kD.

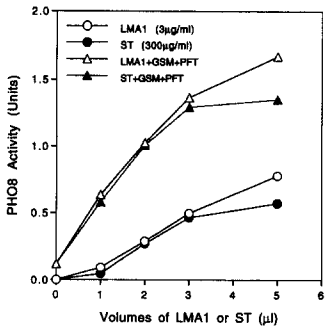
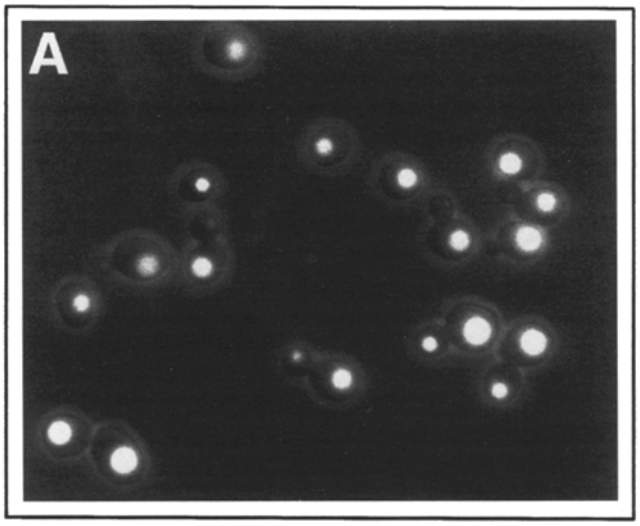


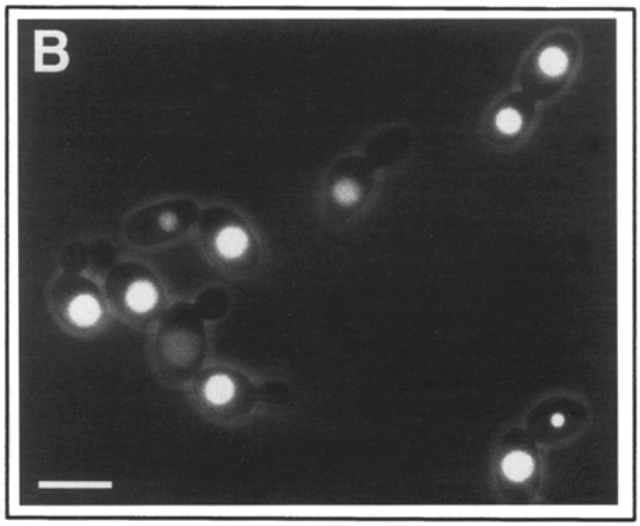
Figure 7. Spirulina thioredoxin catalyzes yeast vacuole fusion. Reactions contained vacuoles from both BJ3505 and DKY6281, salt, 1 mM ATP and an ATP regenerating system, and pure LMA1 (3 μg/ml) (*open circle* and *open triangle*) or Spirulina thioredoxin (*ST*) (300 μg/ml) (*closed cirle* and *gray triangle*) at the indicated amounts, with (*triangles*) or without (*circles*) GSM and PFT. The reactions were incubated at room temperature for 2 h followed by alkaline phosphatase assay as described in Materials and Methods. Data were corrected for background activity (0.07 U/sample).

Discussion

There are fundamental differences, and similarities, between vacuole inheritance and the well-studied secretory pathway. The vesicles which mediate the traffic of the secretory pathway can diffuse throughout the cytosol prior to being consumed at their target organelle (Novick and Schekman, 1979; Rothman et al., 1984) and this traffic continues throughout interphase as long as the donor and target organelle are intact. Vacuole inheritance is also mediated by tubulovesicular traffic (Weisman and Wickner, 1988; Raymond et al., 1992; Gomes de Mesquita et al., 1991) but the bud vacuole is founded at a precise time in the cell cycle by a spatially constrained stream of vesicles and membranous tubules (Weisman and Wickner, 1988). Like exocytic traffic, the targeting and homotypic fusion steps of vacuole inheritance require at least one G protein, Ypt7 (Haas et al., 1995). Unlike secretory traffic, there is a requirement for a membrane potential (Haas et al., 1994), sensitivity to the phosphatase inhibitor microcystin-LR (Conradt et al., 1994), and a requirement for a novel dimeric form of thioredoxin.

We now report the identification of LMA1, a protein which participates in vacuole inheritance but is not essential for the many membrane traffic events which underlie cell surface (bud) growth. Though vacuole inheritance is





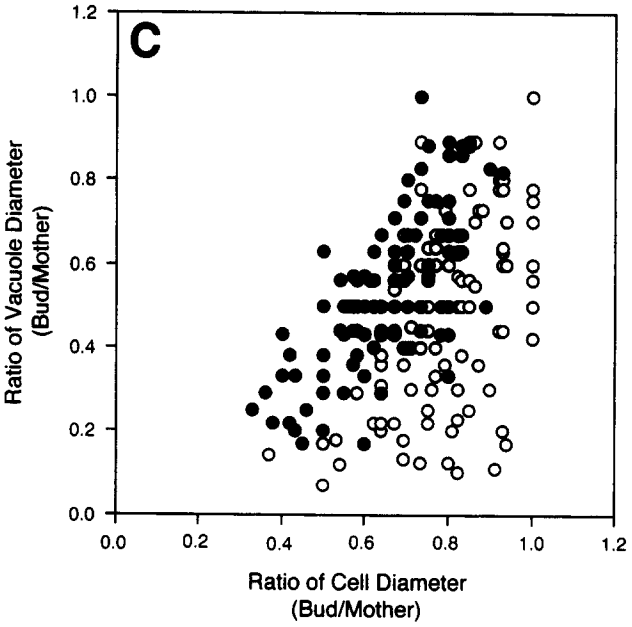


Figure 8. Deficiency of vacuole inheritance in *Δtrx1,Δtrx2* cells. Wild-type (*A*) or *Δtrx1,Δtrx2* (*B*) yeast cells were grown in YCM medium (Rogers and Bussey, 1978; Weisman et al., 1990) to early log phase. 1 ml of the culture was harvested by centrifugation and resuspended in 1 ml YCM with 10 μl of 4 mg/ml FITC. Cells were incubated at 37°C for 10 min, collected by centrifugation, resuspended in 50 μl of YPD, and examined by fluorescence microscopy. Bar, 4 μm. (*C*) Bud vacuoles in *Δtrx1,Δtrx2* cells were smaller than those of wild-type cells. Wild-type (*closed circle*) or *Δtrx1,Δtrx2* (*open circle*) yeast cells were grown, collected, and examined as described above. Photos were taken from random fluorescent fields and the diameters of cells and vacuoles were measured. The horizontal axis indicates the ratio of the diameter of bud cell to the diameter of its corresponding mother cell. The vertical axis indicates the ratio of the diameter of the bud vacuole to the diameter of its corresponding mother vacuole. Data represent 129 wild-type budding cells and 124 *Δtrx1,Δtrx2* budding cells with vacuoles in the buds, respectively.

Table II. A High Proportion of Double trx Deletion Cells Have No Visible Bud Vacuole

Strains	Bud diameter		
	Cell diameter >0.5	Bud with no Vacuole	
			%
Wild-type	123	3	2.4
*trx*1 deletion	74	4	5.4
*trx*2 deletion	91	3	3.3
double *trx* deletion	178	84	47.2

only initiated at a specific point in S phase, thioredoxin is not needed to initiate or pass through this part of the cell cycle. Thioredoxin-deficient yeast have a prolonged S phase (Muller, 1991); this may reflect roles for thioredoxin in DNA synthesis, vacuole inheritance, and other S phase–specific processes. Though our in vitro assay measures vacuole fusion, the in vivo phenotype of the loss of LMA1 is to diminish the inheritance of vacuole material into the bud rather than to merely inhibit the fusion in the bud of tubulovesicular inheritance structures. This, and our observations of the formation of striking tubulovesicular structures in our in vitro reaction, suggest that this in vitro reaction reflects more than the fusion step alone. It is a striking validation of this reaction that it not only is defective with vacuoles and cytosols prepared from *vac*1-1, *vac*2-1, or *vac*5-1 mutants but that its enzymological dissection has identified the redundant *TRX* genes as *VAC* genes. The more rigorous demands of a functional assay of organelle inheritance have guided us to a gentle purification procedure (which does not entail the customary thioredoxin purification steps (Holmgren, 1985) of exposure to heat and acid), revealing a novel dimeric form of thioredoxin.

Thioredoxins are a well-studied family of ubiquitous proteins which participate in redox cycles (Holmgren, 1985). Each thioredoxin has two cysteinyl residues in the highly conserved sequence TrpCysGlyProCysLys. These residues are reduced at the expense of $FADH_2$ in a reaction catalyzed by an NADPH-dependent thioredoxin reductase. These residues in turn reduce specific disulfide bonds of target proteins (such as ribonucleotide reductase). The binding affinity of thioredoxin for the target protein may strongly enhance this reduction reaction.

How does thioredoxin function in the vacuole inheritance reaction? Although there is a wealth of information concerning the structure and enzymatic properties of thioredoxin, the full range of its cellular functions has remained obscure. The most obvious possible function for thioredoxin in vacuole inheritance would be the reduction of an essential cysteinyl residue on a vacuole membrane surface protein. However, the vacuole inheritance reaction is not stimulated by NADPH or by powerful reductants such as dithiothreitol.

Deletion of both *TRX* genes strongly inhibits vacuole inheritance, yet half of the cells with large buds still receive a vacuole. This puzzle has a biochemical explanation, as any one of several proteins of different size (HMA, LMA1, and LMA2; see Fig. 1) can support the in vitro inheritance reaction. HMA and LMA2 may contain proteins (e.g., glutaredoxin) with similar catalytic mechanisms to LMA1, or with an entirely distinct mode of action. Despite its small genome and rapid growth rate, selective pressures on yeast maintain overlapping and partially redundant enzyme systems such as the two thioredoxins and the four cytosolic Hsp70 proteins.

The *trx*1*trx*2 double deletion mutant resembles previously described *vac* mutants in several regards: each shows large budded cells without vacuoles, yet all (daughter) cells have obtained a maternal vacuole by the time they again pass start and begin a new round of bud growth. Each has a striking delay of cytokinesis. Each has a primary defect whose cytological manifestation is a failure to deliver maternal vacuolar material into the bud rather than a block to the fusion step per se. The genetic relationships of bypass, suppression, and synthetic lethality among these *VAC* and *TRX* genes and their mutants should complement our biochemical fractionation of the encoded proteins.

The nature of the smaller subunit of LMA1 must await both cloning its gene, allowing functional studies in vivo through "reverse genetics," and further enzymological studies of how LMA1 promotes vacuole inheritance. We note that almost all of the yeast thioredoxin in our extracts is found in the LMA1 dimeric form, and none was recovered in the monomeric form (Porque et al., 1970) that has been the topic of many studies. Thioredoxin has been previously shown to be part of an essential heterodimeric complex in T7 phage-infected *Escherichia coli* (Mark and Richardson, 1976). Thioredoxin does not need to undergo redox cycles to support either T7 DNA replication (Russel and Model, 1986) or M13 phage assembly (Huber et al., 1986). It will be important to determine whether the thioredoxin of other organisms is also found in LMA1-like complexes, whether the extra, smaller subunit alters its target protein specificity and redox potentials, and whether it has a role in the inheritance of other organelles or in lysosome inheritance in higher eukaryotic cells.

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