Enolase Activates Homotypic Vacuole Fusion and Protein Transport to the Vacuole in Yeast*

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Membrane fusion and protein trafficking to the vacuole are complex processes involving many proteins and lipids. Cytosol from Saccharomyces cerevisiae contains a high M, activity, which stimulates the in vitro homotypic fusion of isolated yeast vacuoles. Here we purify this activity and identify it as enolase (Eno1p and Eno2p). Enolase is a cytosolic glycolytic enzyme, but a small portion of enolase is bound to vacuoles. Recombinant Eno1p or Eno2p stimulates in vitro vacuole fusion, as does a catalytically inactive mutant enolase, suggesting a role for enolase in fusion that is separate from its glycolytic function. Either deletion of the non-essential ENO1 gene or diminished expression of the essential ENO2 gene causes vacuole fragmentation in vivo, reflecting reduced fusion. Combining an ENO1 deletion with ENO2-deficient expression causes a more severe fragmentation phenotype. Vacuoles from enolase 1 and 2-deficient cells are unable to fuse in vitro. Immunoblots of vacuoles from wild type and mutant strains reveal that enolase deficiency also prevents normal protein sorting to the vacuole, exacerbating the fusion defect. Band 3 has been shown to bind glycolytic enzymes to membranes of mammalian erythrocytes. Bor1p, the yeast band 3 homolog, localizes to the vacuole. Its loss results in the mislocalization of enolase and other vacuole fusion proteins. These studies show that enolase stimulates vacuole fusion and that enolase and Bor1p regulate selective protein trafficking to the vacuole.

Because band 3, an integral membrane protein, binds glycolytic proteins to the membrane of human erythrocytes (9), we looked for a similar protein in yeast that might be responsible for the localization of cytosolic enolase to the vacuole. Bor1p has sequence homology to band 3, but little is known of its function (10). We show here that Bor1 is localized to vacuoles and that its loss results in defects in the localization of proteins, including enolase, to the vacuole.

EXPERIMENTAL PROCEDURES

Vacuoles were isolated from yeast strains BJ3505 (MATa ura3-52 trp1-101 his3-D4 200 lys2-801 trpl-D 101 (gal3) can1 gal2 prb1-D1.6R pep1::HIS3) (11) and DKY6281 (Mata ura3-52 leu2-3 leu2-112 trpl-901 his3-200 lys2-801 suc2-9 pho8::TRP1) (12) or derivatives of R11258 (URA::CMV-tTA MATa his3-1 2-leu2-0 met15-0) (OpenBiosystems) for in vitro fusion reactions. The R11258 derivative YSC1180-7429015 (pENO2::kan-5-tet07-TATA URA3::CMV-tTA MATa his3-1 2-leu2-0 met15-0) (OpenBiosystems) was used to generate BDY3 (YSC1180-7429015 pep1::leu eno1::met15) and BDY4 (YSC1180-7429015 pho8::leu eno1::met15). The integrating vector pRS403 (13) containing LEU1 flanked by upstream and downstream regions of either the PEP1 or PHO8 gene was transformed into the YSC1180-7429015 strain (14). The resulting strains, BDY1 and BDY2, respectively, were confirmed by PCR and immunoblotting (14). BDY1 and BDY2 were then transformed with the integrating vector pRS403 containing MTE15 flanked by upstream and downstream regions of ENO1. The resulting strains, BDY3 and BDY4, respectively, were confirmed by PCR and immunoblotting.

Vacuole Isolation and In Vitro Fusion Assay—Unless otherwise indicated, vacuoles were isolated from yeast strains BJ3505 and DKY6281 for in vitro fusion (12). Where indicated, vacuole suspensions were mixed with 0.2 volume of 50% glycerol and 10 mM Pipes-KOH, pH 6.8, 200 mM sorbitol to final concentration of 3 μg/ml and frozen dropwise in liquid N2. Fusion reactions (40 μl) contained 3 μg of vacuoles lacking the protease Pep1p, 3 μg of vacuoles from cells without Pho8p, 5 mM MgCl2, 10 mM Pipes-KOH, pH 6.8, 1 mM ATP, 40 mM creatine phosphate, 0.5 mg/ml creatine kinase, and 10 μM coenzyme A. Fusion reactions with frozen vacuoles were incubated at 30°C. Fusion was monitored by immunoblotting using antibodies to alkali phosphatase (6).

Recombinant Proteins—ENO2 and ENO1 sequences were amplified from genomic DNA by PCR using oligonucleotide primers containing flanking BamH1 and XhoI sequences. Digested PCR products were ligated into parallel vectors (25) for fusion to GST (GST-Eno1 and GST-Eno2) or His6 (His6-Eno1 and His6-Eno2). GST-H159A Eno2 was made using the QuikChange site-directed mutagenesis kit (Stratagene). Expression of Escherichia coli was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (3 h, 37°C). The cells were harvested and frozen in liquid nitrogen. Frozen pellets were resuspended in 2 ml of lysis buffer.
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TABLE 1
Purification of HMA

| Step               | Volume | Protein | Fusion stimulatory activity | Specific activity | Purification |
|--------------------|--------|---------|----------------------------|-------------------|--------------|
| I. Cytosol         | 38     | 1188    | 14454                      | 98                | 8.0*         |
| II. HR400          | 69     | 148     | 16596                      | 97                | 7.8          |
| III. Lyophilization| 8      | 171     | 119                        | 119               | 9.5          |
| IV. FFQ            | 8      | 77      | 13846                      | 374               | 29.9         |
| V. Butyl-Sepharose | 13     | 37      | 2293                       | 50956             | 4076.5       |
| VI. Green 19       | 5      | 0.045   |                            |                   |              |

*It is assumed that all HMA is recovered by gel filtration and separated from other activities (such as LMA).

To identify factors involved in yeast vacuole fusion, we fractionated yeast cytosol and monitored the ability of fractions to stimulate in vitro vacuole fusion. Although freshly isolated vacuoles do not require cytosol or added components for fusion, the harsher procedure used to make frozen vacuoles (4) yields vacuoles, which are more responsive to cytosol and added purified components. Freezing the vacuoles may remove or denature fusion factors or may partially permeabilize the vacuoles; cytosolic factors could rescue such a defect.

Gel filtration of cytosol yields two peaks of activity, of high and low $M_r$ which stimulate in vitro fusion of frozen vacuoles (4). We have previously identified the low $M_r$ activities as thioredoxin and J2 (4, 9). To identify the HMA, proteins from K-91 yeast cytosol were resolved (Table 1) by HR400 gel filtration. Fractions were tested for their ability to stimulate in vitro vacuole fusion. HMA emerged after the bulk of cytosolic proteins near 60 kDa (data not shown). This fraction was concentrated 10-fold by lyophilization and further separated by Q-Sepharose Fast Flow. The HMA activity was in the unbound fraction. HMA was further resolved by butyl-Sepharose 4 Fast Flow and by Green 19 chromatography. A silver-stained gel showed a dominant band in the active fractions (Fig. 1). This band was excised and identified by reverse-phase HPLC nano-electrospray tandem mass spectrometry as a mixture of enolase 1 and enolase 2.

It is surprising that enolase appears to be involved in membrane fusion, as its recognized cellular role is in glycolysis and its localization is cytosolic. To determine whether enolase is also on the vacuole, we performed immunoblot analysis of isolated vacuoles using anti-Enol1-His$_6$ antibody (which recognizes both Eno1p and Eno2p), anti-Vam7 antibody, and anti-Cdc48 antibody. As expected, Vam7p is predominantly on the vacuole, whereas Cdc48p is largely absent from vacuoles. Using scanning densitometry, we found enolase to be present on vacuoles (Fig. 2) at ~1 ng/100 ng of total vacuolar protein.

To directly test whether enolase is HMA or a contaminant that had co-purified with HMA, we tested recombinant enolase for its capacity to stimulate in vitro vacuole fusion (Fig. 3A). Eno1p-His$_6$, Eno1p-GST, Eno2p-His$_6$, and Eno2p-GST stimulated in vitro vacuole fusion in a dose-dependent manner. H159A Eno2p-GST, a point mutant deficient in glycolytic enzymatic activity (15), has comparable activity to the wild type Eno2p-GST (Fig. 3B). As controls we note that adding similar amounts of bovine serum albumin gave no stimulation and adding Vam7p, a known vacuolar fusion protein, stimulated in vitro fusion 5-fold (data not shown). As further confirmation that enolase is not
acting through glycolysis, we noted that fusion was not affected by 2-phosphoglycerate (an enolase substrate), other glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, or pyruvate kinase), or inhibitors of glycolysis (2-deoxy-D-glucose or quercetin) (data not shown). Thus enolase is the active component in the purified HMA fraction but does not act through glycolysis.

To test the role of enolase in vacuole fusion in vivo, we assessed the ability of cells lacking enolase to maintain normal vacuole structure. Because Eno2p is essential for cell viability, we obtained a strain with a tetracycline-repressible promoter substituted for the native ENO2 promoter to assess how the loss of Eno2p affects vacuole fusion. When grown in the presence of varied concentrations of tetracycline, these cells showed a dose-dependent decrease in the amount of enolase protein, as monitored by immunoblot (Fig. 4A). This decrease in enolase was accompanied by vacuole fragmentation (Fig. 4B). Cells in which the tetracycline-repressible promoter was inserted into the promoters of known fusion factors, Erg25p (an ergosterol biosynthetic enzyme) and Sec17p, showed increased vacuole fragmentation during tetracycline repression (Fig. 5). Strains in which ENO1 is deleted are deficient in vacuole fusion, resulting in a fragmented vacuole phenotype in 41% of
Without added inhibitor, 26% of cells from the eno2-tetR strain have fragmented vacuoles, reflecting the fact that the tetracycline-repressible promoter is considerably weaker than the normal ENO2 promoter. When the loss of Enolase is combined with a deficiency in Enolase (eno1Δ, eno2-tetR), 97% of the cells show highly fragmented vacuoles (Fig. 6, A and B). This clearly indicates an in vivo role for Enolase in maintaining normal vacuole structure. Vacuoles from pep4Δ and pho8Δ derivatives (BDY3 and BDY4, respectively) of the eno1Δ,
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FIGURE 7. The role of enolase in vacuole protein targeting. Parental and eno1Δ eno2-tetR strains were grown in 2% YPD without tetracycline. The indicated μg of total cell homogenate and isolated vacuoles were assayed for specific proteins by immunoblot. Total cell lysates of 10, 15, and 20 μg and vacuoles of 1, 2.5, and 5 μg were analyzed by SDS-PAGE and probed with antibody to CPY, Ypt7p, and Vam6p. Total cell lysates and vacuoles (1, 5, and 10 μg) were assayed for Pep4 and Vps33p. Total cell lysates (0.1, 0.5, and 2.5 μg) and vacuoles (0.4, 2, and 10 μg) were assayed for Sec17p, Eno1, 2p, and Nyv1p. Total cell lysates (0.8, 4, and 20 μg) and vacuoles (0.4, 2, and 10 μg) were assayed for Sec18p and Vam3p. Total cell lysates (4 and 20 μg) and vacuoles (0.4, 2, and 10 μg) were assayed for Vti1p.

FIGURE 8. Bor1p regulates the abundance of several vacuole proteins. A, Bor1-GFP is enriched on vacuoles. Total cell homogenate (0.2, 1, and 5 μg) or purified vacuoles (1, 5, and 25 μg) were assayed for Bor1p by immunoblot, and the bands were quantified by scanning densitometry. B, Bor1Δ strains have fragmented vacuoles. The vacuoles of parental and bor1Δ strains were visualized using the vacuolar dye FM4 – 64. Fragmented vacuoles are seen in 95% of bor1Δ cells. C, enolase and other fusion components are lacking on vacuoles from a bor1Δ strain. Vacuoles were isolated from wild type or a bor1Δ strain and assayed for the indicated proteins by immunoblot. Protein abundance was quantified by scanning densitometry.

with vacuoles? Band 3 has been shown to localize glycolytic enzymes to the membranes of human erythrocytes (9). The yeast protein Bor1p has sequence homology to band 3 (10). To test for Bor1p on the vacuole, total cell extracts and vacuoles from strains containing a Bor1-GFP fusion protein were immunoblotted for GFP. Bor1-GFP is enriched on vacuoles as compared with the total cell lysate (Fig. 8A). The importance of vacuolar Bor1p is supported by the vacuolar phenotype of a bor1Δ strain. Approximately 90% of cells lacking Bor1p have fragmented vacuoles (Fig. 8C). To determine whether Bor1p is needed for enolase localization to the vacuole, immunoblots were performed on vacuoles from the bor1Δ strain (Fig. 8B). Vacuolar markers ALP and CPY were present in amounts comparable to their abundance on vacuoles from the wild type strain, whereas Enol1p, Pep4p, Ypt7p, and Vam3p were severely lacking on vacuoles from the bor1Δ strain. These results establish that yeast Bor1p is localized to vacuoles and show a novel role for Bor1p in the trafficking of proteins to the vacuole.

DISCUSSION
We have purified a HMA that stimulates vacuole fusion and identified that activity as enolase 1 and enolase 2. Enolase is an abundant cytosolic protein and a glycolytic enzyme. To test whether enolase was a contaminant in HMA and not the active protein per se, recombinant proteins were tested for stimulation of the in vitro fusion assay. Either recombinant Enol1p or Enol2p stimulate in vitro vacuole fusion, confirming that the activity in the fractions purified from yeast are not due to a minor component. The ability of both Enol1p and Enol2p to stimulate fusion is not surprising because they are 95% identical (7, 8).

Many glycolytic proteins have functions in addition to their roles in glycolysis. Aldolase binds actin and supports protein trafficking to the
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plasma membrane (16, 17); hexokinase-2 and lactate dehydrogenase participate in transcriptionsal regulation (18, 19); glyceraldehyde-3-phosphate dehydrogenase functions in tubulin binding, nuclear RNA export, phosphorylation, membrane fusion, and transcriptional regulation (20), and glucose-6-phosphate isomerase supports cell motility and proliferation (21).

Enolase too has a role that is separate from its function in glycolysis. Vacuole fusion is not stimulated by the addition of the substrate or product of enolase nor by the addition of other glycolytic enzymes and is not inhibited by the addition of glycolytic inhibitors. A catalytically inactive enolase mutant stimulates in vitro fusion as well as wild type enolase ([15], Fig. 2B). Thus enolase participates in glycolysis and in vacuole fusion by distinct mechanisms.

Enolase-deficient cells (i.e. those lacking Eno1p, those deficient in Eno2p, and those lacking Eno1p and deficient in Eno2p) exhibit vacuole fragmentation (Fig. 6B), indicating a role for enolase in vacuole fusion, in trafficking fusion factors to the vacuole, or both. Indeed, enolase deficiency causes a decrease in vacuolar levels of other proteins required for fusion (Fig. 7) demonstrating a role for enolase in trafficking of proteins to the vacuole in addition to its role in fusion.

Why does recombinant enolase stimulate the in vitro fusion of wild type vacuoles but not enolase-deficient vacuoles? One would expect that adding recombinant enolase to enolase-deficient vacuoles would rescue fusion if there were no secondary defects in vacuolar protein composition when enolase is deficient. However, we have found that enolase-deficient vacuoles have the secondary defect of lacking proteins that are required for fusion. This explains the inability of enolase to rescue the fusion of vacuoles from enolase-deficient cells.

Enolase has been reported to bind to a subunit of the adaptor protein complex 3 (22), a complex that provides vesicle structure and cargo specificity for vesicles moving between the Golgi and vacuole (22, 23). Both ALP and Vam3p, which traffic using the adaptor protein 3 complex-containing vesicles, (23), are deficient on vacuoles when enolase is limited (Fig. 7).

Sequence comparisons of yeast proteins to human band 3 protein, which localizes glycolytic enzyme complexes to membranes, identified Bor1p as the yeast homologue of band 3 (9, 10). Little is known about Bor1p in yeast other than the enhanced efflux of boron when Bor1p is overexpressed (24). To determine whether Bor1p is involved in enolase localization to the vacuole, we examined vacuoles from bor1Δ cells.

These vacuoles are deficient in enolase and other proteins involved in fusion (Fig. 8C). Thus Bor1p is also involved in the trafficking of proteins to the vacuole, in accord with the fragmented vacuolar phenotype common to both enolase-deficient strains and bor1Δ strains (Fig. 8B). Understanding the roles of Eno1p and Bor1p in trafficking will likely require analysis of in vitro reactions that reflect trafficking from the Golgi to the vacuole.

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