Loss of Drs2p Does Not Abolish Transfer of Fluorescence-labeled Phospholipids across the Plasma Membrane of Saccharomyces cerevisiae

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The yeast DRS2 gene, which is required for growth at 23 °C or below, encodes a member of a P-type ATPase subgroup reported to transport aminophospholipids between the leaflets of the plasma membrane. Here, we evaluated the potential role of Drs2p in phospholipid transport. When examined by fluorescence microscopy, a drs2 null mutant showed no defect in the uptake or distribution of fluorescent-labeled 1-palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylether (NBD) detected by fluorescence microscopy, 1-myristoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylethanolamine. Quantification of the amount of cell-associated NBD fluorescence using flow cytometry indicated a significant decrease in the absence of Drs2p, but this decrease was not restricted to the aminophospholipids (phosphatidylserine and phosphatidylethanolamine) and was dependent on culture conditions. Furthermore, the absence of Drs2p had no effect on the amount of endogenous PE exposed to the outer leaflet of the plasma membrane as detected by labeling with trinitrobenzene sulfonic acid. The steady state pool of Drs2p, which was shown to reside predominantly in the plasma membrane, increased upon shift to low temperature or exposure to various divalent cations (Mn²⁺, Co²⁺, Ni²⁺, and Zn²⁺ but not Ca²⁺ or Mg²⁺), conditions that also inhibited the growth of a drs2 null mutant. The data presented here call into question the biochemical function of this group of enzymes as aminophospholipid translocases (12). The Na⁺/K⁺ ATPases, various Ca²⁺ ATPases of animal cells as well as the H⁺-ATPases of fungi and plants belong to this family of ion transporters, which share a characteristic set of conserved regions and a similar transmembrane topology (14). The members of the new subgroup differ from the ion-transporting ATPases in several amino acids within transmembrane segments critically involved in ion translocation. Apart from the bovine cDNA, the yeast DRS2 gene and four related yeast genes (13) as well as sequences from Plasmodium falciparum and Caenorhabditis elegans appear to carry these changes (12) whereby negatively charged residues have been replaced by bulky, hydrophobic groups. The observation of a defect in fluorescence-labeled PE (P-C₆-NBD-PE) internalization in a drs2 mutant at low temperature has been interpreted as evidence for the biochemical function of this group of enzymes as aminophospholipid translocases (12). In this report, we examined the potential role of Drs2p in phospholipid transport using fluorescence microscopy, flow cytometry, and TNBS labeling. The deletion of DRS2 had no effect on the uptake or distribution of fluorescent-labeled PS (P-C₆-NBD-PS) or PE (P-C₆-NBD-PE) detected by fluorescence microscopy, 1-myristoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]phosphatidylethanolamine; P-C₆-NBD-PS, 1-palmitoyl-2-[6-NBD-amino]caproyl]phosphatidylethanolamine; HA, hemagglutinin; kb, kilobase(s); YPAD, yeast extract/peptone/glucose; YDP, yeast extract/peptone/glucose.
microscopy. Quantification of the amount of cell-associated NBD fluorescence indicated a significant decrease in the absence of Drs2p, but this decrease was not exclusive to the aminophospholipids (PE and PS) and was dependent on culture conditions. The absence of Drs2p had no effect on the amount of endogenous PE exposed to the outer leaflet of the plasma membrane as detected by labeling with TNBS. The steady state pool of Drs2p, which was shown to reside predominantly in the plasma membrane, increased upon shift to low temperature or exposure to various heavy metal cations, conditions that inhibited the growth of a drs2 null mutant. The data presented here call into question the identification of Drs2p as the exclusive or major aminophospholipid translocase in yeast plasma membranes (12).

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—All yeast strains used in this study were derived from the S288C-related wild-type strain YR98 (MATa ade2 his3-110 leu2-3, 112 lys2-801 ura3-52) isogenic with strain AA255 (15). The drs2–1::URA3 strain YR884 was obtained by transforming YR884 with drs2–1::URA3 DNA as described below. The HA::DRS2 strain YR886 carrying an in-frame insertion of 27 base pairs encoding the HA epitope (16) was constructed by transforming YR884 with HA::DRS2 DNA as described below. All media were prepared according to standard protocols (17).

Construction of drs2–1::URA3 and HA::DRS2 Alleles—To construct drs2–1::URA3, the 5.9-kb EcoRI fragment carrying the entire DR52 gene was isolated from a drs2–1::URA3 genomic clone by digestion with EcoRI and NotI and subcloned into pUC19 (18). Subsequently, the 3.5-kb BstEII-HpaI fragment within the DR52 ORF was replaced with URA3 sequences by inserting the 1.1-kb HindIII fragment of URA3 via blunt end ligation. DnaI from the resulting plasmid was digested with EcoRI and used to transform YR98. The replacement of the DR52 gene by drs2–1::URA3 DNA was verified in one of the Ura+ transformants (YR884) by Southern blot analysis.

To obtain the HA::DRS2 allele, a 27-base pair insertion of HA encoding DNA (19) was introduced into the 1.1-kb EcoRI-Xhol fragment of DR52 by joining the polymerase chain reaction products of two independent polymerase chain reaction products on a DR52 template via recombinant polymerase chain reaction (20). One reaction utilized primers A (5'-ggcggcggaGATTCGAGCAAGAAGGTAAG, capital letters correspond to bases pairing with the DR52 ORF) and B (5'-AGGAGCTCCTGAGATCCTTACCAAGGGATGCTTACATGCCAAGAAGAAAGAAC, underlined letters correspond to bases encoding the 9-aminoo acid HA epitope); the other reaction used primers C (5'-TTATCCATAGCGCGAGGATGCAATGCTGATAGCTGACAACAGAGGAC) and D (5'-TTATCCATAGCGCGAGGATGCAATGCTGATAGCTGACAACAGAGGAC) (specific activity 1 Ci/mmol; NEN Life Science Products). Phospholipid concentrations were determined by a lipid phosphorus assay (21). Membrane, outer leaflet PE was labeled with TNBS as described previously (22). Cells were harvested by centrifugation and homogenized in ice-cold 40 mm NaCl, 120 mm NaHCO3, pH 8.4, resuspended in the same buffer containing 5 mm TNBS (Sigma), and immediately placed on ice for 1 h with periodic vortex mixing. After TNBS labeling, cells were washed by centrifugation three times in fresh buffer, pelleted, and disrupted by vortexing with glass beads. Cellular lipids were extracted with chloroform/methanol (2:1) and separated by two-dimensional TLC (1st solvent: chloroform/methanol/ammonium hydroxide (65:35:5); 2nd solvent: chloroform/methanol/acetone/acidic acid/water (50:10:20:10:5). Spots were identified by comparison with known standards. The percentages of PE, PS, trinitrophenylalkyl phospholipid-PE and trinitrophenylalkyl phospholipid-PS were quantified by phosphorimaging with a PhosphorImager SI scanning instrument (Molecular Dynamics, Sunnyvale, CA). The percent viability was determined by counting the number of cells labeled after dilution into 0.02% methylene blue with a hemocytometer and did not differ significantly between the two strains. The data are presented in the text as the average percentage of trinitrophenylalkyl phospholipid-PE or -PS relative to total cellular PE or PS ± S.D. for four trials.

RESULTS

Construction of the drs2–1::URA3 Null Allele—Using a drs2–1::TRP1 mutant allele, wherein the 1.4-kb BglII-NcoI fragment corresponding to the segment from positions 529 to 1201 within the DR52 open reading frame (1355 amino acids) was replaced by the yeast TRP1 gene (see Fig. 1), Ripmaster et al. (23) show that Drs2p is required for mitotic growth at 23 °C or below. Because these authors also reported that a 2.2-kb EcoRI-BglII fragment encoding the first 528 amino acids at the C terminus of Drs2p was still capable to complement a cold-sensitive drs2 mutant, we constructed a new drs2 mutant allele...
essentially lacking the entire DRS2 coding sequence. In this

\( \text{drs2-1::URA3} \) null allele, the 3.5-kb BstEII-HpaI fragment encoding amino acids 33 to 1206 of the DRS2 open reading frame was substituted by the yeast \( \text{URA3} \) gene. Transformation of a haploid wild-type strain (YR88) with \( \text{drs2-1::URA3} \) DNA produced the congeneric \( \text{drs2-1::URA3} \) strain YR884 used in all subsequent studies. Growth of YR884 was examined on different solid media (yeast extract/peptone/glucose (YPD), synthetic complete and minimal medium) at 23, 30, and 37 °C. On all media tested, this \( \text{drs2-1::URA3} \) strain grew well at 37 and 30 °C but was unable to grow at 23 °C (data not shown), as reported for the \( \text{drs2::TRP1} \) mutant (23).

Loss of DRS2 Does Not Alter the Internalization and Distribution of NBD-labeled Aminophospholipids Detected by Fluorescence Microscopy—The internalization and distribution of the NBD-labeled aminophospholipids, P-C6-NBD-PS and M-C6-NBD-PE, were observed by fluorescence microscopy in the \( \text{drs2-1::URA3} \) strain and its isogenic DRS2 parent (Fig. 2). No significant differences were detected between the two strains at 30 °C (permissive for growth) or at 23 °C (nonpermissive for growth). In previous experiments, it was concluded that M-C6-NBD-PE was internalized exclusively by inward-directed transport across the plasma membrane (flip), resulting in its distribution to the nuclear envelope, endoplasmic reticulum, and mitochondria (24). Internalized M-C6-NBD-PE was not degraded intracellularly, but was readily transported outward across the plasma membrane (flop), where it was degraded by periplasmic phospholipases (24). The similar pattern of fluorescence distribution observed for P-C6-NBD-PS and M-C6-NBD-PE (Fig. 2) suggests that both of these aminophospholipids are internalized and distributed by similar mechanisms in both the \( \text{drs2-1::URA3} \) and DRS2 strains.

To address the possibility that P-C6-NBD-PS was internalized by endocytosis, Tang et al. (12) labeled \( \text{drs2Δ} \) cells with high concentrations of probe solubilized in MeSO incubated on ice to inhibit endocytosis (12). Using our standard protocol to label cells on ice with P-C6-NBD-PS and M-C6-NBD-PE incorporated into liposomes resulted in no detectable fluorescence internalization using the sensitive SIT camera to capture images on the fluorescence microscope. We therefore followed the Tang et al. protocol for labeling cells on ice using high concentrations of MeSO-solubilized NBD-lipids. Following this labeling protocol, no P-C6-NBD-PS fluorescence could be detected. However, a very low level of M-C6-NBD-PE fluorescence was detected, but no differences were observed between the

\( \text{drs2-1::URA3} \) and DRS2 strains. Although detectable with the SIT camera, the fluorescence was too faint and diffuse to produce publishable images.

Thus, for all labeling conditions in which detectable amounts of NBD-aminophospholipid were obtained, the loss of Drs2p had no effect on their internalization and distribution. These observations are inconsistent with the previous study in which P-C6-NBD-PS internalization was abolished in a \( \text{drs2} \) mutant strain (12). The previous conclusion by Tang et al. (12) about the function of Drs2p was based on “back exchange” experiments and was not confirmed by direct observation of internalization and distribution by fluorescence microscopy. In the back exchange measurement, inward-directed transport (flip) is inferred from the amount of NBD-phospholipid that cannot be extracted from the surface of labeled cells by incubation with bovine serum albumin. This technique has been used successfully for many years to assay NBD-phospholipid transport in blood cells and reconstituted vesicles (1, 25). However, the report by Tang et al. (12) was the first use of back exchange to measure flip in yeast, and in the absence of proper controls, differences in the amount of NBD-phospholipid aggregates sticking to the cell wall or trapped in the periplasm or differences in the rate of NBD-phospholipid hydrolysis by periplasmic phospholipases may have been misinterpreted as differences in inward translocation. Direct observation of NBD-phospholipid internalization by fluorescence microscopy is not subject to these artifacts. One of the advantages of labeling cells with liposomes containing trace amounts of N-Rh-DOPC is that the rhodamine fluorescence can be used to determine the extent of cell-associated NBD fluorescence resulting from stuck vesicles (26, 24). This is not possible when cells are labeled with Me6SO-solubilized NBD-phospholipids.

Quantification of NBD-labeled Phospholipid Accumulation by Flow Cytometry—To make a more quantitative evaluation of the results obtained by fluorescence microscopy, cells were labeled with either P-C6-NBD-PS, M-C6-NBD-PE, or M-C6-NBD-PC, and the average cell-associated NBD fluorescence per cell was obtained by flow cytometry. Fluorescent lipid accumulation was measured for \( \text{drs2-1::URA3} \) and DRS2 strains grown at 30 °C in two different media (YPAD and SDC). Accumulation of the three NBD-phospholipids was compared at 30° and 23 °C, the nonpermissive growth temperature for \( \text{drs2-1::URA3} \). The ratio of the cell-associated fluorescence in \( \text{drs2-1::URA3} \) to that of DRS2 is presented in Table I for the two temperatures and growth media. Similar results were ob-
Fluorescence accumulation was measured by flow cytometry. A fluorescence intensity histogram was plotted for ~10,000 live cells, and the means and S.D. were calculated for each strain under the appropriate conditions. The percent average accumulation for the Δdrs2 strain relative to its isogenic parent is presented as ±S.D. The number of independent experiments (n) is in parenthesis. For those experiments with an n of 2, the mean ± the range is presented.

Table I

Percent NBD-phospholipid accumulation of drs2–1::URA3 strain relative to isogenic parent

| NBD-phospholipid | Growth media | 30 °C | 23 °C |
|------------------|--------------|-------|-------|
| P-C6-NBD-PS      | YPAD         | 51.3 ± 18.7 (5) | 53.7 ± 20.8 (5) |
|                  | SDC          | 116.3 ± 15.4 (3) | 118.6 ± 37.2 (3) |
| M-C6-NBD-PE      | YPAD         | 70.6 ± 19.6 (5) | 58.6 ± 17.1 (5) |
|                  | SDC          | 71.4 (1)        | 70.5 (1)        |
| M-C6-NBD-PC      | YPAD         | 66.4 ± 7.8 (2)  | 56.1 ± 8.3 (2)  |

* Cells were grown to early log phase in either YPAD or SDC at 30 °C.

† Cells were labeled by incubation with donor vesicles containing the appropriate NBD-phospholipid for 30 min at the stated temperature before washing 3 times with SDC/NaCl.

Drp2 Localizes to the Plasma Membrane—To facilitate detection of the Drs2p protein, we constructed a derivative of the Drs2p gene, HA::DRS2, harboring a 27-base pair sequence encoding the nine amino acid HA epitope (16, 19) inserted in-frame after the second codon (see Fig. 1). Haploid strain YR886 containing HA::DRS2 at the chromosomal Drs2 locus grew indistinguishable from Δdrs2 strains, indicating that the expressed HA-Drs2 protein was fully functional (data not shown). To determine the subcellular localization of HA-Drs2, extracts from strain YR886 (HA::DRS2) were fractionated by sucrose gradient centrifugation. All fractions collected from the gradients were tested by SDS-polyacrylamide gel electrophoresis and Western blotting for the presence of marker proteins specific for plasma membrane (H^+ -ATPase Pma1 (27)) and endoplasmic reticulum (dolichol phosphate mannosyltransferase Dpm1 (28–30)). Golgi and vacuolar membranes were identified by monitoring activities of GDPase (31) and α-mannosidase, Ams1 (32), respectively. As demonstrated by the data shown in Fig. 3, the bulk of HA-Drs2 co-fractionated with the plasma membrane ATPase, well separated from endoplasmic reticulum membranes and the bulk of GDPase or α-mannosidase activity. Consistent with this observation, cells expressing HA-Drs2p from the chromosomal locus exhibited indirect immunofluorescence microscopy a ring-shaped rim staining pattern, but unfortunately the signal was very low (data not shown). Attempts to increase expression of HA-Drs2p from a

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2 A. Grant and J. Nichols, unpublished data.

3 P. K. Hanson and J. Nichols, unpublished observation.

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**Fig. 3.** Fractionation of HA-Drs2p on sucrose gradients. Whole cell extracts of the HA::DRS2 strain YR886 were fractionated by density centrifugation as described (38). Aliquots of the gradient fractions were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-HA antibodies, anti-Pma1 antibodies, and anti-Dpm1 antibodies. The sizes of marker proteins are given in kDa; the fraction numbers are indicated. Activities for α-mannosidase (Ams1) (32) and guanosine diphosphatase (31) were determined as described and are given in arbitrary units. Density (% sucrose, w/w) and protein concentration (arbitrary units) are plotted against the fraction number.
multicopy plasmid yielded a more pronounced rim staining but, in addition, produced a prominent perinuclear staining pattern, suggesting that the bulk of HA-Drs2p remained in the endoplasmic reticulum under these conditions (Fig. 4). Taken together, our data strongly argue for a steady-state localization of Drs2p in the plasma membrane but indicate that Drs2p could also exert functions in membranes of secretory organelles along the pathway to the plasma membrane.

The Steady-state Level of Drs2p Is Dependent on Growth Phase and Temperature—In monitoring expression of HA-Drs2p in HA::DRS2 cells growing in YPD medium at 30 °C, we found a substantial alteration of the steady-state level of HA-Drs2p with the growth phase of the culture. Fig. 5A (top panel) shows a Western blot analysis of crude membranes prepared from HA::DRS2 cells at various stages of growth (A600 of 0.6, 1.1, and 2.0). HA-Drs2p was fairly abundant during early and mid logarithmic growth but essentially disappeared as cells entered stationary phase. In contrast, the steady-state level of the plasma membrane H+-ATPase (Pma1p), which like Drs2p, is a member of the P-type ATPase family, did not show such a dramatic decrease, although Pma1p appeared somewhat reduced in cells approaching stationary phase (Fig. 5A, bottom panel). However, as demonstrated in Fig. 5B, cells entering stationary phase while growing at 23 °C retained a substantial amount of HA-Drs2p. This finding was consistent with the observed requirement of DRS2 for growth at this temperature (23). Taken together, these data suggested an important function for Drs2p, particularly during early growth phases at 30 °C and during growth at lower temperatures.

The Steady-state Level of Drs2p Increases upon Treatment with Heavy Metals—In the course of our genetic analysis, we discovered a hypersensitivity of dras2–1::URA3 mutants toward various heavy metals. As displayed in Fig. 6, serial dilutions of a dras2–1::URA3 culture were spotted onto solid minimal media containing the indicated concentrations of manganese (II), cobalt (II), nickel (II), or zinc (II) chloride. Evidently, the dras2–1::URA3 strain was particularly sensitive to Zn2⁺ and Co2⁺, and the hypersensitivity toward Mn2⁺ and Ni2⁺ was less pronounced. This growth inhibition by divalent cations was apparently restricted to transition elements, because dras2–1::URA3 grew like wild type in the presence of high concentrations of Mg2⁺ and Ca2⁺ (see Fig. 6).

We also examined the steady-state levels of HA-Drs2p accumulating in cells during growth in the presence of various metal cations. To this end, HA::DRS2 cells were inoculated into YPD media to which the indicated amounts of metal chlorides have been added. At an A600 of 0.6, 1.1, and 2.0, aliquots were withdrawn and analyzed for the presence of HA-Drs2p in crude membrane fractions. As seen in Fig. 7, all cations inhibitory to growth, i.e. Co2⁺, Ni2⁺, Mn2⁺, and Zn2⁺, also led to a pronounced accumulation of HA-Drs2p relative to the
control culture. In contrast, the presence of Mg\(^{2+}\), Ca\(^{2+}\), or monovalent cations had only slight effects on the steady-state level of HA-Drs2p. Both observations, the hypersensitivity of drs2::URA3 cells to heavy metals and the intracellular accumulation of HA-Drs2p triggered by the same divergent cations, indicated that cells required Drs2p function to effectively endure the toxic effects of these transition elements.

**DISCUSSION**

The current knowledge about the mechanisms leading to internalization of phospholipids into the yeast *S. cerevisiae* stems primarily from studies utilizing phospholipid molecules carrying one short acyl chain labeled with a fluorescent NBD group. Digital, video-enhanced fluorescence microscopy and spectrofluorometry have shown that at least two distinct pathways for phospholipid internalization exist: 1) transport by endocytosis to the vacuole, which partially accounts for the uptake of the NBD-labeled PC analog, M-C\(_6\)-NBD-PC (26) and 2) transport by a nonendocytic pathway to the nuclear envelope (24). The results do not exclude the possibility that Drs2p functions as a transporter of divalent cations or are indeed a consequence of the proposed function of Drs2p.

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At present, the mechanisms leading to the observed hypersensitivity of a drs2 null mutant toward some heavy metal cations, in particular Zn\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), and Ni\(^{2+}\), are not known. In our view, these phenotypes could either reflect a yet undiscovered role of Drs2p as a transporter of divergent cations or are indeed a consequence of the proposed function of Drs2p in aminophospholipid translocation. Two models, not necessarily exclusive, could provide an explanation for a link between aminophospholipid translocation and cation sensitivity. (i) An altered asymmetric distribution of lipids within some cellular membranes, presumably resulting from the loss of Drs2p, could compromise the activity of cation transporters embedded in the affected membranes. Given a prominent localization of Drs2p in the plasma membrane and the numerous exocytic and endocytic pathways leading to and originating from this mem-
brane, it seems possible that Drs2p function might impinge upon Golgi and vacuolar membranes, which both harbor numerous transport proteins engaged in the sequestration of valent cations within these organelles. (ii) Alternatively, an altered lipid distribution, i.e. a reduction in the amount of negatively charged PS in the cytoplasmic leaflets of some intracellular membranes, might directly result in altered cation binding properties of these membranes, thereby affecting some cation-dependent steps in membrane fusion reactions. Note-worthy, in vitro studies have shown that Zn$^{2+}$ ions are more effective than Ca$^{2+}$ to induce fusion of phospholipid vesicles with a low content of PS (35), and the depletion of Zn$^{2+}$ blocks endosome fusion in a cell-free system (36).

It should be noted that the loss of Drs2p function also impairs ribosome biogenesis. The DRS2 gene was first discovered in a search for mutants with an altered ratio of free 40 to 60 S ribosomal subunits or qualitative changes in polyribosome profiles. The dre2 mutant isolated in this screen processes the 20 S precursor of the mature 18 S rRNA slowly and is deficient in rRNA pairs ribosome biogenesis. The dre2 mutant isolated in this screen processes the 20 S precursor of the mature 18 S rRNA slowly and is deficient in rRNA pairs ribosome biogenesis. The dre2 mutant isolated in this screen processes the 20 S precursor of the mature 18 S rRNA slowly and is deficient in rRNA pairs ribosome biogenesis. Thus, the continuous functioning of the secretory pathway appears to be a prerequisite for the biogenesis of ribosomes (37). Evidently, the proposed role for Drs2p as a flippase affecting lipid distribution in vesicles of the late secretory pathway or at the plasma membrane would provide an intriguing explanation for the ribosome-related defects observed in the dre2 mutant (23).

Finally, we would like to emphasize that the previous demonstration of the abolishment of PS internalization in a yeast dre2 mutant (12) provided the sole functional data for the assignment of phospholipid translocase activity to the Drs2p subfamily within the class of P2 ATPases (13). Our inability to confirm a role of Drs2p in phospholipid translocation underscores the need for future experiments to carefully reevaluate this functional assignment.

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Loss of Drs2p Does Not Abolish Transfer of Fluorescence-labeled Phospholipids across the Plasma Membrane of Saccharomyces cerevisiae

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