Role for Drs2p, a P-Type ATPase and Potential Aminophospholipid Translocase, in Yeast Late Golgi Function

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Abstract. A DP-ribosylation factor appears to regulate the budding of both COPI and clathrin-coated transport vesicles from Golgi membranes. An arf1Δ synthetic lethal screen identified SWA3/DRS2, which encodes an integral membrane P-type ATPase and potential aminophospholipid translocase (or flippase). The drs2 null allele is also synthetically lethal with clathrin heavy chain (chl1) temperature-sensitive alleles, but not with mutations in COPI subunits or other SEC genes tested. Consistent with these genetic analyses, we found that the drs2Δ mutant exhibits late Golgi defects that may result from a loss of clathrin function at this compartment. These include a defect in the Kex2-dependent processing of pro-α-factor and the accumulation of abnormal Golgi cisternae. Moreover, we observed a marked reduction in clathrin-coated vesicles that can be isolated from the drs2Δ cells. Subcellular fractionation and immunofluorescence analysis indicate that Drs2p localizes to late Golgi membranes containing Kex2p. These observations indicate a novel role for a P-type ATPase in late Golgi function and suggest a possible link between membrane asymmetry and clathrin function at the Golgi complex.

Key words: adenosine diphosphate–ribosylation factor • aminophospholipid translocase • clathrin • DRS2 • trans-Golgi network

The directed movement of proteins between compartments of the secretory and endocytic pathways is driven by the formation of small coated transport vesicles that bud from a donor compartment, and then fuse with an acceptor compartment. COPI, COPII, and clathrin are the best characterized coat proteins involved in protein transport. COPI, a heptameric (α, β, β′, γ, δ, ε, ζ-COP) protein complex, coats vesicles that mediate protein transport from the Golgi to the ER and possibly between Golgi compartments. To initiate budding of COPI-coated vesicles from Golgi membranes, the small GTP-binding protein A DP-ribosylation factor (ARF) mediates the recruitment of COPI coats to primarly cis Golgi cisternae. ARF is also required to recruit clathrin/A P-1 coats to the trans-Golgi network. Clathrin coats are assembled from triskelions composed of three clathrin heavy chains and three light chains. The tetrameric adaptor protein (AP) complex, A P-1, links membrane proteins to clathrin at the TGN and appears to facilitate cargo delivery to endosomal compartments (reviewed in Schmid, 1997). Interestingly, the molecular basis for the ARF-dependent recruitment of COPI and clathrin/A P-1 to two different regions of the Golgi complex is not known. Clathrin/A P-2 vesicles mediate the internalization of proteins from the plasma membrane, although recruitment of this coat does not appear to require ARF. In addition, the recruitment of COPII to ER membranes is mediated by the small GTP-binding protein Sar1p rather than ARF.

In yeast Saccharomyces cerevisiae, ARF is encoded by two nearly identical genes, ARF1 and ARF2, that appear to be functionally redundant. Deletion of both ARF genes is lethal, but strains harboring either an arf1Δ or an arf2Δ allele are viable and grow nearly as well as an isogenic wild-type strain (Stearns et al., 1990a). However, deletion of the more highly expressed ARF1 gene causes a defect in the kinetics of protein transport through the secretory pathway, a reduction in Golgi-dependent glycosylation of secreted glycoproteins, and substantial changes in the morphology of Golgi cisternae and endosomes (Gaynor et al., 1998; Stearns et al., 1990b).

To gain a better understanding of the essential role that ARF plays in vivo, we have used a genetic screen to identify seven genes (SWA1–SWA7) for which mutant alleles...
exhibit synthetic lethality with arflΔ. This genetic interaction often predicts that the two gene products function in the same pathway or in parallel pathways. swa5-1 was previously identified as a new temperature-sensitive (ts) allele of the clathrin heavy chain gene (chc1-5), providing support for a functional interaction in vivo between clathrin and ARF (Chen and Graham, 1998). Deletion of the CHC1 gene can be lethal or viable depending on the strain background. Mutants harboring chc1Δ, if viable, exhibit slow growth, decreased rates of endocytosis, and a defect in retention of late Golgi membrane proteins (Payne et al., 1987, 1988; Payne and Schekman, 1989; Lemmon et al., 1990). Disruption of the clathrin light chain gene (CLC1) causes a reduced stability of Chc1p and results in phenotypes similar to that of chc1 mutants (Chu et al., 1996). Although mutations in AP-1 subunits (A ps1p, A pm1p, and A pl2p) do not result in any detectable phenotypes, a pairwise combination of apl1Δ, ampl1Δ, or apl2Δ with chc1-ts exacerbates defects in growth and retention of Golgi resident proteins relative to the chc1-ts mutant (Phan et al., 1994; R ad et al., 1995; Stepp et al., 1995).

Here we report that SWA3 is allelic to DRS2, which encodes an integral membrane P-type ATPase (Ripmaster et al., 1993) that is 47% identical to the mammalian ATPase II in these vesicles is not known. The mammalian ATPase II purified from chromaffin granules (Tang et al., 1996). ATPase II is thought to be an aminophospholipid translocase (A uland et al., 1994; Zachowski et al., 1989), capable of flipping phosphatidylserine (PS) or phosphatidylethanolamine (PE) from the external leaflet of a lipid bilayer to the cytoplasmic leaflet to generate an asymmetric distribution of these lipids in the two leaflets of a membrane. In mammalian cells, most of the plasma membrane PS is restricted to the cytoplasmic leaflet by the action of a similar aminophospholipid translocase, but a calcium influx induces the loss of plasma membrane lipid asymmetry and results in the exposure of PS on the cell surface. This event allows recognition and removal of apoptotic cells by phagocytes, and in blood cells activates the coagulation cascade (Williamson and Schlegel, 1994). Mammalian ATPase II purified from chromaffin granules (Tang et al., 1996). ATPase II is thought to be an aminophospholipid translocase (A uland et al., 1994; Zachowski et al., 1989), capable of flipping phosphatidylserine (PS) or phosphatidylethanolamine (PE) from the external leaflet of a lipid bilayer to the cytoplasmic leaflet to generate an asymmetric distribution of these lipids in the two leaflets of a membrane. In mammalian cells, most of the plasma membrane PS is restricted to the cytoplasmic leaflet by the action of a similar aminophospholipid translocase, but a calcium influx induces the loss of plasma membrane lipid asymmetry and results in the exposure of PS on the cell surface. This event allows recognition and removal of apoptotic cells by phagocytes, and in blood cells activates the coagulation cascade (Williamson and Schlegel, 1994). Mammalian ATPase II is found in chromaffin granules (Zachowski et al., 1989), synaptic vesicles (H icks and Parsons, 1992), and clathrin-coated vesicles (X ie et al., 1989), although the function of ATPase II in these vesicles is not known. While there is no evidence that Drs2p generates phospholipid asymmetry in the yeast plasma membrane, the drs2Δ mutant has been reported to exhibit a defect in translocation of a fluorescent PS derivative across the plasma membrane (Tang et al., 1996). However, this result has been disputed (S eymund et al., 1998), calling into question whether Drs2p functions as an aminophospholipid translocase at the plasma membrane.

In addition to the synthetic lethal interaction with arflΔ, we found that drs2Δ also exhibits a specific synthetic lethal interaction with chc1-ts alleles. Moreover, the drs2Δ mutant has a defect in late Golgi function and exhibits several phenotypes at the nonpermissive temperature that suggest a loss of clathrin function at the yeast Golgi in vivo. Consistent with these observations, immunofluorescence and subcellular fractionation studies indicate that Drs2p localizes to late Golgi membranes. These results argue that the primary site of Drs2p function is in the Golgi complex rather than the plasma membrane, and suggest a link between Drs2p and the ARF-dependent recruitment of clathrin to Golgi membranes.

Materials and Methods

Strains and Media

Yeast cells were grown in standard rich medium (YPD), sporulation, or SD minimal media containing required supplements and 0.2% yeast extract where indicated (Sherman, 1991). Strains used were SEY 6210 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 lys2-801 suc2-49; R binson et al., 1988), 6210 drs2Δ (SEY 6210 drs2Δ::TRP1), 6210 arflΔ (SEY 6210 arflΔ::HIS3; Gaynor et al., 1998), 6210 chc1-ts (SEY 6210 chc1-ts::URA3; Chen and Graham, 1998), PRY 6222 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 ade2-101 suc2-49 drs2Δ::TRP1), PY 1103 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 suc2-49 arflΔ::LEU2; Phan et al., 1998), EGY 101-16D (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 lys2-801 sec21-1; Gaynor et al., 1998), TBY 103 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 sec23-1; Gaynor et al., 1998), TGY 144 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 lys2-801 sec1-1), TGY 1906 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 suc2-49 pan1-20), TGY 1912 (MATα leu2-3,112 ura3-52 his3Δ200 trp1-901 lys2-801 suc2-49 end4-1), SEY 5185 (MATα leu2-3,112 ura3-52 sec3-181; BH Y 161 (SEY 6210 vps25Δ::HIS3), BH Y 163 (SEY 6210 vpl1Δ::HIS3), 6210 vps3Δ (SEY 6210 vps33Δ::HIS3), and 6210 vps35Δ (SEY 6210 vps33Δ::HIS3).

Cloning of SWA3 and Plasmid Construction

To clone SWA3, strain CCY 2808 (swa3-2; Chen and Graham, 1998) was transformed with a genomic library (H orazdovsky et al., 1994) and Leu R transformants were selected on synthetic dextrose-Leu media containing 4 μg/ml adenine. A fter 7–10 d of incubation at 30°C, 50 colonies that appeared to sector (out of 21,000 transformants) were streaked on 5-fluoro-orotic acid (FOA) plates. Library plasmids were rescued from the four strain that grew on FOA, and one plasmid was retransformed into CCY 2808. Only the transformants harboring pRP10 were able to sector on YPD at 30°C and were able to grow at 20°C. Partial sequencing indicated that the other three plasmids contained ARF2.

pRP10 contained a fragment of chromosome I from coordinates 94916-104338. Deletion of the BamHI fragment contained solely in DR5 (to produce pRP10b) destroys the complementing activity of pRP10. The full-length DR5 gene contained on a Spel-SnaB1 fragment was subcloned into SpeI-SnaB1 digested pRS515 or pRS425 to produce pRS515-DR5 and pRS425-DR5. Both plasmids complemented the cold-sensitive growth defect of CCY 2808. A drs2 deletion plasmid (pGR1) was constructed by replacing a BamH I-SnaB1 fragment in pRS426 with an ~11-kb BglII-PvuII fragment containing TRP1 from pJ 650 (J ones and Prakash, 1990). The plasmid pGR1 was linearized with SacI and Hpal and transformed into SEY 6210 to produce 6210 drs2Δ. The correct integration event was confirmed by PCR.

Site-directed mutagenesis of DR5 was performed by the megaprimer PCR method (Barkin and Galinski, 1991). To generate megaprimers containing either a D to N or D to E mutation at amino acid position 560, reverse primers TCTTCGTCTTTTCTTTGGATATATACT and TCTTCGTCTTTTTGATATATACT, respectively, were combined with forward primer TTTGTCACCGTGAAATATC into a PCR reaction mixture containing pRS515-DR5. The gel purified ~150-bp product was added to a second PCR reaction containing the reverse primer TTCATCATTCAATCTCTGGTCAAGA and pRS515-DR5. The resulting ~650-bp products were digested with BglII and BglI, and then ligated into BglI/BglII cut pRS515-DR5 to produce pDR5S (D560N) and pDR5E (D560E). Sequencing of the resulting plasmids indicated that the specific mutations had been introduced with no additional mutations.

The 2-μm KEX2 HA plasmid was constructed by subcloning a SalI/EagI digestion fragment of pSfN218 (Nothwehr et al., 1995), containing the KEX2-HA sequence, into SalI/EagI-digested pRS426 to produce pSfN246K. The H A.11 mouse monoclonal antibody (Covance) was used at a 1:250 dilution to detect Kex2-HA by immunofluorescence.

Cell Labeling and Ste3p Turnover

Cell labeling. Immunoprecipitation (Gaynor and Emr, 1997), FM 4-64 up-
take (Vida and Emr, 1995), and Ste3p turnover (Davis et al., 1993) experiments were performed as previously described. Ste3p-myc was detected with 9E10 c-myc antibody (Oncogene Research Products) and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) followed by the enhanced chemiluminescence detection (A mersham Corp.).

Subcellular Fractionation and Preparation of Clathrin-coated Vesicles

Subcellular fractionation was performed as previously described (Phan et al., 1994), except that cells were converted to spheroplasts and washed twice in buffer containing 0.8 M sorbitol before lysing with a Dounce homogenizer in buffer A containing 0.1 mg/ml RNase A. The cells were prep- incubated for 1 h at 15° or 30°C, lysed, and subjected to centrifugation at 13,000 g and the resulting supernatant was then centrifuged at 100,000 g. Immunoblots bearing 20 μg of protein from each sample were probed with antibodies to Chc1p, Mnn1p (Graham et al., 1988), Aps1 and Aps2 (Phan et al., 1994), and A RF1 (Kahn et al., 1995). Clathrin-coated vesicles (CCVs) were prepared from SEY6210 and 6210 drs2Δ as previously described (Muller and Branton, 1984). For 15°C-treated samples, cells from a 3-liter culture grown to 6–8 OD were collected, resuspended into 1 liter of 3-liter culture grown to 6–8 OD were collected, resuspended into 1 liter of fresh 3-liter culture grown to 6–8 OD were collected, resuspended into 1 liter of Laemmli sample buffer, and one third of each sample was subjected to im-

Results

drs2Δ is Synthetically Lethal with arf1Δ and chc1-ts Alleles

To clone the SWA3 gene, a yeast genomic library (CEN, LEU2) was used to transform the swa3-1 mutant, and the transformants were screened for complementation of the cold-sensitive growth and arf1Δ synthetic lethal phenotypes. A single library plasmid was isolated that was able to complement both mutant phenotypes and further subcloning analyses revealed that a 5-kb SpeI-SnaBI genomic fragment containing the full-length DRS2 gene (and no other open reading frame, ORF) retained the complementing activity (see Materials and Methods).

A drs2 null strain was generated by replacing most of the DRS2 coding sequences with TRP1. Consistent with the phenotype previously reported for drs2Δ strains (Ripmaster et al., 1993) and that exhibited by the swa3 mutants (Chen and Graham, 1998), the drs2Δ null mutant was unable to grow at 20°C or below, but grew well at temperatures above 23°C. Linkage analysis indicated that SWA3 is allelic to DRS2, and synthetic lethality between arf1Δ and drs2Δ was confirmed by crossing the single mutants and characterizing the progeny by tetrad analysis (Fig. 1 A and data not shown). This genetic interaction suggested that Drs2p may be involved in an ARF-dependent vesi-
cle-mediated protein transport event(s).

To determine if drs2Δ would exhibit synthetic lethality with other mutations that perturb transport vesicle formation, we crossed drs2Δ with strains harboring ts mutations in subunits of COPI (ret1-1, sec21-1, and sec27-1 encoding α, γ, β′-COP, respectively), the ER transport vesicle coat gene (and no other open reading frame, ORF) retained the complementing activity (see Materials and Methods). The band from the preparative gel was excised and eluted using an Elutrap (Schleicher & Schuell, Inc.). Polyclonal rabbit anti-
munoblotting and probed with antibodies to Chc1p, Mnn1p (Graham et al., 1994), and Pep12p (Seaman et al., 1998).

Electron Microscopy

Spheroplasts were prepared for electron microscopy as previously de-
scribed (Rieder et al., 1996) except that the initial glutaraldehyde fixation was overnight at 4°C. 50-60-nm sections were viewed on an electron mi-
croscope (CM 12; Philips). Vesicle fractions were similarly prepared, but included the following modifications: 5-ml fractions were fixed overnight at 4°C by addition of 700 μl of 25% glutaraldehyde (3% final concentra-
tion). Fixed vesicles were pelleted at 100,000 g for 70 min. The pellicle wash was in 100 mM sodium cacodylate, pH 6.8, 5 mM CaCl2, and then stained identically to the cell blocks, with one exception. A 30-min room temperature incubation with 1% tannic acid in 100 mM sodium cacodylate, pH 7.4, was included before en bloc staining with uranyl acetate. Dehydration, embedding, and section poststaining were performed as for the cell samples.

Preparation of Antiserum Against Drs2p

A TrpE-DRS2 fusion construct was prepared by ligating the BglII/HindIII

digested pATH2 vector (Koerner et al., 1991). The resulting plasmid con-
tained an in-frame fusion between TrpE and a region of Drs2p (amino ac-
cids 528–920) carrying ATPase motifs and predicted to be a cytoplasmic

ty. The band from the preparative gel was excised and eluted using an Elutrap (Schleicher & Schuell, Inc.). Polyclonal rabbit anti-
Figure 1. drs2Δ is synthetically lethal with arf1Δ, chc1, and pan1 alleles. (A) Genetic analyses between drs2Δ and mutations that perturb the secretory pathway. Strains carrying the indicated mu-
tations (see Materials and Methods) were crossed with a drs2Δ mutant (6210 drs2Δ or PRY6222) to generate diploids. A tetra-
ad analyses of the progeny, the viable double mutants were streaked at 20°, 26.5°, and 37°C to compare the growth relative to parental strains carrying single mutations. *Double mutants of the alleles and drs2 that were able to grow at 20°C, where single drs2 mutants could not. †Double mutants that grew more slowly than either single mutant at 26.5°C. (B) Tetrad analysis of progeny derived from crossing PRY6222 (drs2Δ) with 6210 chc1-5 (chc1-5). Spores that failed to grow were predicted to be drs2Δ chc1-5 double mutants.

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COPII (sec12-1 and sec23-1), and the clathrin heavy chain (chc1-5, chc1-Δ57, chc1-521), and analyzed the progeny by tetrad dissection. A II of the sec drs2Δ and the ret1 drs2Δ double mutants were obtained at the expected frequency (approximately one fourth of the progeny) and most grew well at 26.5°C (Fig. 1 A). The exception was sec21-1 drs2Δ mutants, which grew somewhat more slowly at this temperature than either single mutant. A mong those growing well at 26.5°C, double mutants harboring drs2Δ and sec12-1 or sec23-1 also grew at 20°C, which is a nonpermissive temperature for drs2Δ single mutants. This suggests that sec12 and sec23 are able to partially suppress the cold-sensitive growth defect of drs2Δ, and that COPII may act upstream of Drs2p.

In stark contrast, chc1-ts drs2Δ double mutants were nearly always inviable. A II of the spores that failed to grow in the tetrad analysis shown in Fig. 1 B were predicted to be chc1-5 drs2Δ double mutants based on the genotype of the viable spores from each tetrad. The drs2Δ and chc1-5 single mutants grew nearly as well as wild-type progeny at 26.5°C, double mutants harboring drs2Δ and chc1-5, and wild-type, respectively. Microscopic examination of the plates indicated that the drs2Δ chc1-5 spores germinated and divided a few times to form microcolonies, indicating that mitotic growth, rather than germination, was impaired. In addition, this genetic interaction was not allele-specific since all three of the chc1 ts alleles tested were synthetically lethal with drs2Δ (Fig. 1 A).

To further address the specificity of the synthetic lethal interaction between drs2Δ and chc1-ts alleles, we crossed drs2Δ with several mutants that exhibit a defect in protein transport through the secretory or endocytic pathways. Double mutants combining drs2Δ with sec1-1, sec7-1, sec14-1, sec18-1, vps21Δ, vps33Δ, vps35Δ, vpt7Δ, or end4-1 were obtained at the expected frequency, although the end4-1 drs2Δ, vps33Δ drs2Δ, and sec18-1 drs2Δ mutants exhibited a slow growth phenotype. Interestingly, a mutant allele of the yeast Eps15 homologue, pan1-20, was also synthetically lethal with drs2Δ (Fig. 1 A). Eps15 is a cytoplasmic protein that localizes to clathrin-coated pits and may have an adaptor-like function in clathrin coated vesicle formation (van Delft et al., 1997; Wendland and Emr, 1998). Of particular note was the lack of genetic interactions with sec7 and sec14, which both perturb protein transport through the yeast Golgi complex.

The drs2Δ Mutant Secretes Pro–α-factor at the Nonpermissive Temperature

One of the more striking phenotypes of yeast clathrin mutants is the mislocalization of several late Golgi (TGN) proteins that are required for pro-α-factor proteolytic processing. This results in the secretion of fully glycosylated pro-α-factor rather than the mature peptide (Payne and Schekman, 1989). If clathrin function at the TGN is perturbed in the drs2Δ mutant, we would expect this mutant to secrete pro-α-factor. To test this, wild-type, drs2Δ, and clc1Δ (clathrin light chain null) strains were grown at 30°C, and after shifting to 20°C for 1 h, were metabolically labeled and chased at this nonpermissive temperature for drs2Δ. A aliquots of cells were removed at the chase times indicated in Fig. 2, converted to spheroplasts, and then centrifuged to separate intracellular (I) from extracellular (E) fractions. α-Factor was then recovered from each sample by immunoprecipitation.

At the beginning of the chase, labeled α-factor was present throughout the secretory pathway of the cells, as indicated by the presence of the core glycosylated ER proform, Golgi-modified hyperglycosylated proforms and the mature form (Fig. 2, lanes 1, 7, and 13). In wild-type cells, complete proteolytic processing of pro-α-factor occurred within 15 min, and most of the mature α-factor was secreted and degraded in the extracellular space. However, the clc1Δ cells were clearly deficient in the processing of pro-α-factor, and most of the hyperglycosylated precursor was secreted into the extracellular space within 15 min, as previously reported (Fig. 2, lane 18; Chu et al., 1996). Similarly, the drs2Δ mutant secreted the hyperglycosylated pro-α-factor and partially processed α-factor forms into the extracellular space (Fig. 2, lanes 10 and 12). The drs2Δ mutant also exhibited a modest defect in Golgi-specific glycosylation since the hyperglycosylated pro-α-factor secreted from drs2Δ cells showed a slightly faster mobility within the SDS-polyacrylamide gel relative to that from the clc1Δ strains. The kinetics of pro-α-factor secretion at this temperature (20°C) was nearly equivalent to that of clc1Δ cells. These results indicate that late Golgi function is specifically perturbed in the drs2Δ cells and suggest a loss of clathrin function at the TGN at the nonpermissive temperature. In pulse-chase experiments performed at 30°C, most of pro-α-factor was processed and secreted as the mature form from drs2Δ cells (data not shown), indicating that this defect was temperature conditional. Moreover, the pro-α-factor processing defect was observed within 15 min of preincubation at 20°C, suggesting a rapid onset of the temperature conditional phenotype (data not shown).
**The drs2Δ Mutant Exhibits an Endosomal Defect**

To examine protein transport through the endocytic pathway of drs2Δ cells, we followed the turnover of the α-factor receptor, Ste3p, which is constitutively endocytosed and delivered to the yeast vacuole where it is degraded (Davis et al., 1993). A c-myc tagged Ste3p expressed from a galactose-regulated promoter (Davis et al., 1993) was used so new Ste3p synthesis can be shut off by shifting the cells to glucose. Wild-type, drs2Δ, and arf1Δ cells were grown in galactose at 30°C to induce expression of the construct and populate the plasma membrane with the Ste3-myc protein. Glucose was added to the culture, which was then shifted to 15°C, and the disappearance of Ste3-myc was followed over time by immunoblotting. Even though the arf1Δ mutant displays an abnormal endosome morphology (Gaynor et al., 1998), the kinetics of Ste3p transport to the vacuole as measured by Ste3-myc degradation was very similar to that of wild-type cells (Fig. 3 A, arf1Δ). In contrast, the rate of Ste3-myc turnover was three- to fivefold slower in the drs2Δ cells (Fig. 3 A, drs2Δ) compared with wild type, suggesting a defect in endocytosis. To control for recovery of protein in each sample, the same blots were probed for carboxypeptidase Y (CPY), which was recovered equally in each sample (data not shown). drs2Δ cells cultured in glucose did not express Ste3p-myc, indicating that glucose repression of the GAL promoter was not perturbed (data not shown). A gain, this phenotype was temperature conditional since the rate of Ste3-myc turnover at 30°C in the drs2Δ strain was only slightly (1.4-fold) slower than the wild-type strain (data not shown).

Clathrin mutants display a similar defect in Ste3p turnover caused by inefficient internalization of the receptor from the plasma membrane (Tan et al., 1993). However, examination of drs2Δ cells by immunofluorescence localization of Ste3p-myc at each time point after glucose was added indicated an accumulation of Ste3-myc in intracellular structures with very little plasma membrane staining (data not shown). This result suggested that transport of Ste3p from the endosome to the vacuole, rather than internalization from the plasma membrane, was perturbed.

To more specifically test whether uptake from the plasma membrane or transport from endosomes to the vacuole was defective in drs2Δ cells at the nonpermissive temperature, we stained cells with the fluorescent endocytic marker FM 4-64 (Vida and Emr, 1995) on ice, and then shifted the cultures to 15°C to initiate endocytosis of the dye (Fig. 3 B). The FM 4-64 was rapidly cleared from the plasma membrane of both wild-type and drs2Δ cells (data not shown), and in wild-type cells it was delivered to the vacuole membrane in 30–60 min (WT, 1 h). In contrast, the FM 4-64 accumulated in endosomes of drs2Δ cells, which appear as punctate fluorescent spots in Fig. 3 B (dr{s2Δ}, 1 h). Even after 4 h at 15°C, most of the FM 4-64 was not in the vacuoles and stained what appeared to be clusters of smaller structures (dr{s2Δ}, 4 h). As the drs2Δ cells warmed up on the microscope stage, the FM 4-64 was rapidly delivered to the vacuoles at all times tested, indicating that the defect was fully reversible (data not shown). In addition, vacuoles in drs2Δ cells that were stained with FM 4-64 at 30°C did not appreciably fragment when the cells were shifted to 15°C (data not shown), so the structures stained at 15°C were indeed endosomes and not fragmented vacuoles.

These studies indicated that the endocytic defect observed in drs2Δ cells was attributable to a defect in the endosome-to-vacuole pathway rather than clathrin-dependent endocytosis from the plasma membrane. If so, CPY transport should be affected as well since this protein follows a TGN to endosome to vacuole delivery route. To test this, we examined the transport of CPY to the vacuole in the drs2Δ mutant. CPY is synthesized in the ER as the p1 precursor form and is modified on N-linked oligosaccharides by the Golgi α1,3 mannosyltransferase (Mnn1p) to form the p2 precursor. p2 CPY is sorted from secreted proteins in the TGN and ultimately processed to the mature form in the vacuole (Stevens et al., 1982). Wild-type, drs2Δ, and arf1Δ cells were pulse-labeled and chased at either the permissive or nonpermissive temperature of drs2Δ. A aliquots of cells were removed at the chase times indicated and CPY was recovered by immunoprecipitation. As shown in Fig. 4, drs2Δ cells displayed near wild-

![Figure 3](https://jcb.rupress.org/issue/15)/1227.png)
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...CPY transport kinetics at 30°C, while at 15°C the transport of CPY in drs2Δ mutants was significantly delayed relative to that in the wild-type cells, and was similar to the defect observed in arf1Δ cells at either temperature (approximately threefold slower transport kinetics). The partial glycosylation defect observed in drs2Δ (and arf1Δ cells) prevented the formation of a p2 CPY form that could be resolved from the p1 form in SDS-polyacrylamide gels. Thus, the kinetics of ER-to-Golgi transport could not be assessed for CPY. However, the ER-to-Golgi transport kinetics for α-factor and invertase in drs2Δ cells was found to be nearly wild type at the nonpermissive temperature, as scored by disappearance of the ER core form (Fig. 2, and data not shown). Therefore, it is unlikely that ER-to-Golgi transport for CPY is disturbed in drs2Δ cells. These data are most consistent with the interpretation that protein transport from the late Golgi or endosomes to the vacuole is perturbed by the drs2Δ mutation.

Interestingly, the chc1-5 allele isolated in the arf1Δ synthetic lethal screen also exhibits a partial glycosylation defect and an approximately threefold slower transport kinetics for CPY (Chen and Graham, 1998).

The drs2Δ Mutant Accumulates Abnormal Membrane-bound Structures Similar to Berkeley Bodies

Most of the sec mutants that exhibit a temperature-conditional block in protein transport also accumulate an organelle or vesicular intermediate of the secretory pathway at the nonpermissive temperature. For example, the sec7

Figure 4. The drs2Δ mutant exhibits a cold-sensitive kinetic defect in CPY transport to the vacuole. Wild-type, drs2Δ, and arf1Δ (all isogenic to SEY6210) strains were labeled for 10 min at 15°C or 30°C, and then chased for the times indicated. CPY was recovered from each sample by immunoprecipitation and subjected to SDS-PAGE.

Figure 5. The drs2Δ mutant accumulates abnormal membrane structures that are similar to Berkeley bodies. Electron micrographs of drs2Δ cells incubated for 2 h at 15°C (A) or kept at 30°C (B) showing numerous double-membrane ring and crescent-shaped structures. Plasma membrane (PM) and vacuoles (V) are labeled. The black arrowhead in A denotes a modestly fenestrated ring structure. The arrowhead in B shows a ring structure with a stacked crescent membrane. For comparison, an electron micrograph of similar structures observed in a chc1Δ cell (C, GPY1103) is shown. Narrower, more fenestrated, and typically incomplete ring structures were also found in wild-type (WT) cells grown at 30°C (D, arrowhead). These wild-type ring structures did not stain as darkly as rings found in the drs2Δ mutant (compare to double arrowhead in A). Bars, 0.2 μm. E, drs2Δ and wild-type cells grown at the indicated temperatures were visualized by transmission electron microscopy. Membrane bound structures from 23–25 randomly selected cells were counted and expressed as an average number of structures per cell section.
and sec14 mutations block protein transport out of the Golgi complex and accumulate Golgi structures called Berkeley bodies (Novick et al., 1980). The Golgi cisternae in these mutants are sometimes stacked and appear to adopt a deep, cup-shaped morphology that in electron micrographs of thin sections present double-membrane ring or crescent shaped structures depending on the section plane. Clathrin mutants also exhibit aberrant membrane structures similar to Berkeley bodies, although these structures are rarely stacked (Payne et al., 1987).

Morphological studies by electron microscopy revealed that drs2Δ cells accumulated aberrant double-membrane ring and crescent-shaped structures at both 15° and 30°C (Fig. 5 A and B). The double-membrane rings in the drs2Δ cells measured 200–250 nm in diameter (average, 240 nm) and often presented a significant gap between the concentric membranes, which should be equivalent to the luminal space of the cisternae. Representative double-membrane ring structures are marked with white arrowheads in Fig. 5 A. Very similar ring structures also accumulated in the chc1Δ mutant (Fig. 5 C; Payne et al., 1987). These types of structures were never observed in wild-type cells; however, ring structures could be found that were more highly fenestrated and appeared to be breaking down into tubules or vesicles (Fig. 5 D, arrowhead). In fact, similar fenestrated ring structures accumulate dramatically in arf1Δ cells (Gaynor et al., 1998). Some of the double-membrane rings in the drs2Δ cells were also modestly fenestrated (Fig. 5 A, black arrow).

To quantitate the effect of temperature on the accumulation of ring structures in wild-type and drs2Δ cells, these cells were grown at 26.5°C and shifted to the temperature indicated in Fig. 5 E for 2 h before fixation. Regions containing 23–25 cell sections were randomly selected and the number of crescent-shaped structures, double-membrane rings, and vesicles (50–100-nm spheres) were counted and expressed as the number of structures per cell section. Relative to the wild-type strain, the drs2Δ mutant accumulated four- to eightfold more crescent and ring structures at the temperatures examined (Fig. 5 E). In this analysis, structures similar to that shown in Fig. 5 D were counted as rings in the wild-type cells. There was also a greater accumulation of these structures in the drs2Δ cells at the colder temperatures, which was particularly evident for the double-membrane rings. The number of vesicles in the drs2Δ cells dropped modestly at the colder temperatures, although within the range of values observed for the wild-type cells.

Golgi membranes that accumulate at the nonpermissive temperature in the sec7 mutant have been reported to become more extensively stacked in low glucose medium (Novick et al., 1980). Some structures appeared stacked in the drs2Δ mutant grown in 2% glucose (Fig. 5 B, arrow), but no significant difference was observed in the morphology of the membranes accumulating in drs2Δ cells incubated in low glucose (0.1%) medium for 2 h at the nonpermissive temperature. However, the effect of low glucose on Berkeley body structure in a sec7 mutant in our strain background was modest (data not shown).

Plasma membrane invaginations have been proposed to represent endocytic intermediates in yeast (Mulholland et al., 1999) and are extended in some mutants that exhibit a defect in endocytosis [for example, sjl1 sjl2 (Srinivasan et al., 1997) and pan1 (Wendland et al., 1996)]. However, no difference in the depth of plasma membrane invaginations between wild-type and drs2Δ cells was observed under any of the growth conditions described above.

Reduction of Intact Clathrin-coated Vesicles that Can Be Isolated from the drs2Δ Mutant

A mammalian cytosolic ARF guanine nucleotide exchange factor requires both PS and phosphatidylinositol-4,5-bisphosphate for optimal membrane binding and subsequent activation of ARF (Chardin et al., 1996). This observation suggested a requirement for Drs2p (a potential PS translocase) to recruit ARF and clathrin to membranes. To test whether Drs2p plays a role in regulating ARF activation and therefore association of ARF and clathrin with membranes, we fractionated both wild-type and drs2Δ cellular lysates by differential centrifugation to compare the amounts of ARF, clathrin, and adaptin subunits recovered in the 13,000-g pellets, and the 100,000-g pellets and supernatants. A small increase in the amount of clathrin heavy chain found in the 100,000-g supernatant fraction was occasionally observed in the mutant samples relative to the wild-type samples at both 15° and 30°C; otherwise, the fractionation pattern for clathrin was very similar between the two strains (data not shown). In addition, the fractionation pattern for ARF and the two adaptin small subunits (Aps1p for AP-1 and Aps2p for AP-2) was nearly identical between drs2Δ and wild-type samples. Therefore, the association of ARF and clathrin with bulk cellular membranes seemed to be unaffected in the drs2Δ mutant, although we cannot rule out the possibility that the distribution of ARF between specific organelles is perturbed.

To further assess clathrin function in the drs2Δ mutants, we asked whether CCVs could be purified from this mutant. CCV preparations were generated from drs2Δ and wild-type cells with or without a 1-h shift to 15°C (see Materials and Methods). Cell lysates were centrifuged at 21,000 g for 30 min to pellet large organellar membranes (e.g., plasma membrane, vacuolar membrane, ER, and mitochondria) and the resulting supernatant was centrifuged at 100,000 g to pellet vesicles. The 100,000-g pellet was then applied to a Sephacryl S-1000 gel filtration column (Mueller and Branton, 1984), and the fractions were probed for the clathrin heavy chain by immunoblotting. As shown in Fig. 6 A, the clathrin heavy chain was highly enriched in fractions 23 to 27 for both wild-type and drs2Δ samples from cells shifted to 15°C (Fig. 6 A) or 30°C (Fig. 6 D, and data not shown). In addition, from Coomassie blue-stained gels, we estimated that the recovery of clathrin heavy chain in these fractions was comparable for both strains at both temperatures (data not shown).

However, a clear difference between wild-type and mutant samples was evident when fractions highly enriched for the clathrin heavy chain were examined by EM. Peak clathrin fractions obtained from wild-type cells were substantially enriched in CCVs with a clear lipid bilayer present in a substantial number of the vesicle profiles (Fig. 6 C, arrowheads). In contrast, most of the vesicle profiles in the drs2Δ peak clathrin fractions contained an electron
dense center, but no lipid bilayer within the clathrin basket (Fig. 6 D, open arrowheads). In addition, the drs2Δ fractions contained what appeared to be partially assembled clathrin lattices (Fig. 6 D, arrow) that were rarely observed in wild-type fractions. CCVs with a lipid bilayer were occasionally observed in the drs2Δ fractions, but were found at 1/10 of the frequency per section as compared with the wild-type samples (multiple sections from three different preparations were compared). This was a temperature-conditional phenomenon as CCV preparations from drs2Δ cells maintained at a permissive temperature were indistinguishable from wild-type samples (data not shown).

It is well established that clathrin triskelions can self-assemble into baskets in slightly acidic, low ionic strength buffers (Schmid, 1997, and references therein). The method we used for isolating CCVs from yeast applies such buffer conditions to retain assembled coats on vesicles. In addition, the lysate is incubated for 30 min at 30°C with RNase to reduce the amount of ribonucleoprotein complexes in the P100 sample. These conditions are likely to induce assembly of free clathrin triskelions into baskets and lattices within the drs2Δ cell lysate. It appears that these clathrin structures pellet at 100,000 g and elute from the S-1000 column in similar fractions as bona fide CCVs. The simplest interpretation of these data is that the drs2Δ cells are deficient in producing CCVs at the nonpermissive temperature. It is also possible that clathrin dissociates more readily from CCVs produced in drs2Δ cells and reassembles into baskets during purification, thus preventing the isolation of bona fide CCVs. In either case, there is a marked difference between drs2Δ and wild-type cells either in the ability to produce CCVs or in the physical properties of CCVs from these strains.

Even though most large membranes pellet at 21,000 g, we had noticed by EM that the 100,000-g pellet from drs2Δ cells (that was applied to the S1000 column) contained a significant number of aberrant membrane structures that were similar in appearance and size to those observed in cell sections (Fig. 5 B). Because drs2Δ cells exhibited both Golgi and endosome-associated defects, it was possible that both organelles would accumulate in the drs2Δ mutant. Therefore, we examined the S1000 column fractions from the CCV preparations for membranes containing a late Golgi protein, Mnn1p, and an endosomal t-SNARE Pep12p. (B–D) Membranes in fraction 16 from the drs2Δ sample where Mnn1p was found (B), and fraction 26 from wild-type (C) and drs2Δ (D) samples where Chc1p was enriched were fixed, pelleted, and examined by EM. Bars, 50 nm.

Figure 6. The drs2Δ mutant accumulates aberrant Golgi membranes and exhibits a deficiency of clathrin-coated vesicles. (A) Wild-type and drs2Δ cells were grown at 30°C and shifted to 15°C for 1 h, and then lysed and subjected to centrifugation at 21,000 g for 30 min. The 21,000-g supernatant was centrifuged at 100,000 g for 80 min to generate a 100,000-g pellet. This pellet was resuspended and further resolved on a Sephacryl S-1000 column. Samples of every other fraction from fraction 15 to 35 were assayed by immunoblotting for the clathrin heavy chain (Chc1p), a late Golgi protein Mnn1p, and an endosomal t-SNARE Pep12p. (B–D) Membranes in fraction 16 from the drs2Δ sample where Mnn1p was found (B), and fraction 26 from wild-type (C) and drs2Δ (D) samples where Chc1p was enriched were fixed, pelleted, and examined by EM. Bars, 50 nm.
Drp2p Localizes to Late Golgi Membranes Containing Kex2p and Mnn1p

To analyze the intracellular localization of Drp2p, we prepared a rabbit polyclonal antiserum against a bacterially expressed fragment of Drp2p (amino acid residues 528–920). A affinity-purified anti–Drp2p antibodies recognized an ~150-kD protein on an immunoblot of a wild-type cell lysate (Fig. 7 A, Drp2p), which is in close agreement to the predicted mass of 154 kD. This protein was not present in a drp2Δ cell lysate and was found in greater abundance in wild-type strain carrying Drp2p on a multicopy plasmid (Fig. 7 A, drp2Δ and 2μ Drp2p). Thus, the 150-kD protein is equivalent to Drp2p.

Drp2p was recently suggested to localize to the plasma membrane based on cofractionation of an epitope-tagged Drp2p with the plasma membrane ATPase, Pma1p, in sucrose gradient fractions from a crude cell lysate (Siegmand et al., 1998). However, we found that membranes containing Drp2p could be quantitatively separated from membranes containing Pma1p by differential centrifugation as described above for the CCV preparations. Equal amounts of protein from the lysate (L), pellet (P21), and supernatant (S21) of 21,000 g and pellet (P100) and supernatant (S100) of 100,000 g were probed for Drp2p and Pma1p. Nearly all of Drp2p remained in the supernatant after centrifuging at 21,000 g (S21) while most of Pma1p was found in the pellet (P21) (Fig. 7 B). All of the Drp2p in the S21 was pelleted during the subsequent 100,000 g centrifugation step and was found in the P100 fraction.

The fractionation profile for Drp2p shown in Fig. 7 B is very similar to how CCV and late Golgi proteins such as Kex2p fractionate. To further characterize the localization of Drp2p, a P100 fraction was applied to the bottom of a sucrose gradient and centrifuged to equilibrium (Reynolds et al., 1998). Fractions were collected from the top and probed for Drp2p and Mnn1p and assayed for GDPase and Kex2p (Fig. 7 C). GDPase marks early Golgi compartments, Kex2p the late Golgi (or TGN), and Mnn1p is typically split between these two regions of the gradient. The fractionation pattern for Drp2p clearly matches that of Kex2p (Fig. 7 C). In similar gradients, we found that the endosomal marker Pep12p was broadly distributed throughout the gradient (data not shown), although others have observed a defined peak in fractions significantly lighter than that of Kex2p (Becherer et al., 1996). In no case did the Pep12p fractionation pattern match with that of Drp2p. In addition, the S1000 column elution profile of membranes containing Drp2p (from wild-type cells grown at 30°C) matched that of membranes containing Mnn1p (Fig. 7 D). The Drp2p elution profile overlapped that of CCV’s (Fig. 7 D), raising the possibility that some Drp2p is present within the CCVs. This possibility is currently being tested; however, it appeared that the bulk of Drp2p resides in Golgi membranes.

To determine whether Drp2p and Kex2p reside in the same membrane structures, wild-type cells overexpressing a hemagglutinin (HA)-tagged Kex2p were stained simultaneously with antibodies to Drp2p and HA. Localization of Drp2p by immunofluorescence produced a punctate staining pattern that is typical for the yeast Golgi complex (Fig. 8, WT 2μ KEX2HA, α–Drp2p). Although the sig-

different between the wild-type and drp2Δ fractions (Fig. 6 A). These data suggest that the abnormal membrane structures that accumulate in the drp2Δ cells are Golgi membranes and not endosomal membranes.
nal was weak, it was clearly above the background staining observed for the 

\( \text{drs2}^D \) strain (Fig. 8, \( \text{drs2}^D \)). The HA antibody also produced a punctate staining pattern (Fig. 8, \( \text{WT}^12 \text{m} \text{KEX2HA}, \alpha \text{-HA} \)) and, importantly, most of the structures that were positive for Drs2p also stained for Kex2-HA. As expected, very little overlap was observed between Drs2p and the early Golgi marker Och1-HA (data not shown).

We could not detect plasma membrane staining with the Drs2p antibodies, which is inconsistent with a previous report suggesting that Drs2p resides in the plasma membrane (Siegmund et al., 1998). Overexpression of Drs2p from a multicopy plasmid resulted in an ER staining pattern (Siegmund et al., 1998, and data not shown), so this method could not be used to enhance the signal. To determine whether the lack of plasma membrane staining with Drs2p antibodies was due to a technical limitation, we tested whether we could detect plasma membrane staining for an antigen that is present in the Golgi, endosomes, and plasma membrane. Wild-type spheroplasts were stained with antibodies to \( \alpha 1,3 \)-linked mannose residues that are found on many glycoproteins. For these cells, staining of the plasma membrane is clearly observed as a stained rim around the cells (Fig. 8, anti-\( \alpha 1,3 \)). Thus, by both immunofluorescence and subcellular fractionation studies, it appears that most, if not all, of Drs2p is found in late Golgi membranes.

**Mutation of Aspartic Acid 560 Abolishes Drs2p Function In Vivo**

Drs2p is predicted to be a P-type ATPase based on the presence of five well-conserved ATPase motifs involved in the binding and hydrolysis of ATP (Fig. 9 A). For P-type ATPases, an aspartic acid within the second motif forms an aspartyl-phosphate catalytic intermediate that is essential for ATP hydrolysis (Allen and Green, 1976; Fagan and Saier, 1994). Sequence alignments between Drs2p and other P-type ATPases predict that aspartic acid 560 (D560) in Drs2p would form the aspartyl-phosphate intermediate (Fig. 9 A). To test whether this amino acid is critical for Drs2p function, we mutated D560 to either an asparagine (D560N) or a glutamic acid (D560E) residue and examined the ability of the mutants to complement the cold-sensitive growth defect of the \( \text{drs2}^D \) strain. These mutations would be expected to cause minimal structural...
changes in Drs2p, but should abolish the presumed ATPase activity of this protein. The drs2Δ strain carrying either the wild-type DR52 gene (Wild-type), an empty vector (drs2Δ), or two independent isolates of each mutant (D560N and D560E) were tested for growth at 30°C and 20°C (Fig. 9 B). As previously reported, the drs2Δ strain failed to grow at 20°C, but grew well at 30°C. None of the strains carrying the D560 point mutations were able to grow at 20°C (Fig. 9 B), even though each of the strains expressed a wild-type level of Drs2p (data not shown). These data support the assignment of Drs2p as a P-type ATPase and suggest that the ATPase activity of Drs2p is essential for its function in vivo.

Discussion

In this report, we present several lines of evidence that strongly implicate the integral membrane P-type ATPase Drs2p in late Golgi function, and suggest a link between Drs2p and CCV formation from Golgi membranes. (a) A specific synthetic lethal interaction was found between drs2Δ, arf1Δ, and chc1-ts alleles. (b) The drs2Δ mutant exhibits a cold-sensitive defect in the proteolytic processing of pro-α-factor in the yeast TGN that is comparable with the defect shown by clathrin mutants. (c) The drs2Δ mutant accumulates aberrant Golgi structures that are morphologically comparable to the membrane structures that accumulate in clathrin mutants. (d) We observed a substantial decrease in the yield of CCVs that could be isolated from the drs2Δ mutant preincubated at the nonpermissive temperature. (e) Drs2p localizes to late Golgi membranes containing Kex2p.

Specific Genetic Interactions between drs2Δ, arf1Δ, and chc1-ts Alleles

Our rationale for performing the arf1Δ synthetic lethal screen was that it could provide an unbiased, in vivo approach to identify proteins that regulate ARF function, or participate with ARF in CCV or COPI vesicle formation. This was the first yeast genetic screen we are aware of that specifically screen. However, from crosses with 19 different mutants that perturb the secretory or endocytic pathways, we found a specific interaction between pairwise combinations of arf1Δ, drs2Δ, and chc1-ts alleles. The drs2Δ allele was also synthetically lethal with pan1-20, an eps15-related protein that interacts with Y4 P180 (yeast homologue of clathrin assembly protein A P180) in a yeast two-hybrid assay (Wendland and Emr, 1998). Importantly, there was no genetic interaction between drs2Δ and mutant ts alleles encoding three different COPI subunits, even though combination of arf1Δ with these COPI alleles produces a synthetic growth defect (Gaynor et al., 1998). These results suggest that Drs2p is specifically involved in clathrin, but not COPI, function. This interpretation is supported by the finding that membranes containing Drs2p could be separated from early Golgi membranes where COPI would be expected to function, and fractionated with late Golgi membranes where clathrin function is required.

Phenotypes Exhibited by the drs2Δ Mutant

These genetic analyses prompted us to examine the drs2Δ mutant for defects in the secretory and endocytic pathways. The secretion kinetics and Golgi-specific glyco-sylation of pro-α-factor and invertase were only modestly perturbed. However, a substantial cold-sensitive defect in the Kex2p-dependent processing of pro-α-factor was observed. This resulted in secretion of the pro-α-factor precursor, a phenotype also exhibited by clathrin mutants (Payne and Schekman, 1989). Kinetic defects were also observed for CPY transport to the vacuole and the turnover of the Ste3p pheromone receptor within the vacuole. These phenotypes are likely caused by a delay in endosome-to-vacuole transport that was clearly observed using the fluorescent endocytic tracer FM 4-64. Thus, the drs2Δ mutation seems to specifically perturb late Golgi (TGN) and endosome function.

As visualized by EM, the drs2Δ mutant accumulates abnormal membrane-bound structures that were morphologically equivalent to structures that accumulate in clathrin mutants. The abnormal membrane structures that were isolated from drs2Δ cells by differential centrifugation and gel exclusion chromatography were enriched for a late Golgi protein, but not for an endosomal marker protein. Since the localization studies suggest that Drs2p is a late Golgi (TGN) resident, these data strongly suggest that Drs2p acts in the late Golgi to maintain normal structure and function of this compartment. It is possible that Drs2p has some function in the endosome as well, or the effect on endosome function could be a secondary consequence of perturbing late Golgi function.

Perhaps the most compelling evidence that Drs2p plays a role in clathrin function is the striking difference in the appearance of CCV preparations between drs2Δ and wild-type cells. Very few bona fide CCVs could be isolated from drs2Δ cells preincubated at the nonpermissive temperature. These preparations contained clathrin baskets and lattices with no associated membrane. In contrast, CCV preparations from drs2Δ cells maintained at 30°C were indistinguishable from wild-type samples. These results suggest that Drs2p is required to form clathrin-coated vesicles from Golgi membranes at temperatures below 23°C. However, it is also possible that the association of clathrin coats with vesicle membranes from drs2Δ cells is less stable and the coat dissociates during cell lysis. In either case, loss of Drs2p clearly perturbs CCVs.

At this time, it is not possible to distinguish whether the effect of Drs2p on clathrin function is direct or a secondary consequence of the abnormal TGN structure. However, this effect is specific since the TGN of drs2Δ cells
functions normally in the ability to sort vacuolar proteins and in protein secretion, despite the abnormal morphology. This suggests that late secretory vesicles bud normally from the TGN in drs2Δ cells. The genetic interactions also showed a high degree of specificity between drs2Δ and clathrin mutations. Therefore, there is not a wholesale loss of Golgi function in drs2Δ cells at the nonpermissive temperature. Indeed, most of the specific defects observed can be explained by a loss of clathrin function. Even the accumulation of abnormal membrane at the permissive temperature could be the result of inefficient CCV budding.

The temperature-conditional defects observed for a strain carrying a complete loss of function allele is somewhat unusual. This suggests that Drs2p is required to overcome an inherently cold-sensitive process in the cell (perhaps CCV budding), such that the growth defect is caused by the combination of low temperature and loss of Drs2p. However, this cold-sensitive process is not at the extreme of the normal growth range for yeast; drs2Δ cells fail to grow at room temperature (20°C) and the mutant phenotypes described here are observed at this temperature. In addition, some of the drs2Δ phenotypes, such as arf1Δ synthetic lethality and abnormal Golgi morphology, are also observed at 30°C. Thus, it appears that Drs2p plays a role in Golgi function at all temperatures examined but is only essential below 23°C. Alternatively, it is possible that Drs2p function is essential at all temperatures, but loss of Drs2p is compensated by one or more of the Drs2p-related P-type ATPases at higher temperatures. In either case, Drs2p clearly plays a critical role for organisms such as yeast since the ability to adapt to daily fluctuations in temperature is essential for their survival.

Potential Function of Drs2p as an Aminophospholipid Translocase

What is the biochemical function of Drs2p? Many P-type ATPases use the energy of ATP hydrolysis to pump cations such as Ca2+, H+, Na+, or heavy metals across a membrane against their electrochemical gradient. These transporting ATPases contain signature motifs that allowed the identification of 16 P-type ATPases in the yeast genome that can be phylogenetically grouped into six distinct families (Catty et al., 1997). Four of the families correspond to the cation transporters just described, one family contains two ORFs of unknown function, and the last family are potential aminophospholipid transporters, which includes Drs2p and four other uncharacterized ORFs. Drs2p is 28–35% identical to its four other yeast family members, but is 47% identical to bovine or murine ATPase II from chromaffin granules (FA STA comparisons), arguing that Drs2p and ATPase II are orthologues. The closest related P-type ATPase to the Drs2 family are the Ca2+ transporters (21% identity between Drs2p and Pmc1p). However, Drs2p and ATPase II are missing negatively charged amino acids in transmembrane domains 4 and 6 that are essential for cation transport and have hydrophobic residues in their place. This is consistent with the proposed role of ATPase II and Drs2p in transporting aminophospholipids and makes it less likely that Drs2p transports cations.

The drs2Δ mutant has been reported to exhibit a defect in the translocation of a fluorescent PS derivative across the plasma membrane (Tang et al., 1996). However, Siegmund et al. (1998) have recently challenged this observation and have failed to detect a difference between wild-type and drs2Δ cells in the translocation of fluorescent lipid derivatives across the plasma membrane. This group also found no difference in the amount of PE exposed on the outer leaflet of the plasma membrane as detected by trinitrobenzene sulfonic acid labeling. These latter observations could suggest that Drs2p is not an aminophospholipid translocase, but it is more likely that the Drs2p protein is simply not present at the plasma membrane and therefore is not required to maintain an asymmetric distribution of aminophospholipids in this membrane. In fact, we cannot detect Drs2p at the plasma membrane by immunofluorescence localization or subcellular fractionation (Figs. 7 and 8). Measurement of translocase activity with Golgi membrane fractions is more complicated because the direction of flip is expected to be from the luminal to the cytoplasmic leaflet and thus requires incorporation of the probe into the luminal leaflet. Thus, further work is required to determine if Drs2p is an aminophospholipid translocase, as suggested by its homology to ATPase II.

Since an aminophospholipid translocase activity is the only biochemical function attributed to ATPase II and Drs2p in the literature, it is relevant to speculate on how membrane asymmetry may affect Golgi structure and perhaps clathrin function. The bilayer couple hypothesis of Sheetz and Singer (1974) proposes that asymmetric changes in the two leaflets of a bilayer should induce conformational changes in the membrane. In fact, induced bilayer asymmetry can cause conversion of spherical liposomes into tubular and interconnected vesicular structures (Farge and Devaux, 1992). In this regard, it is possible that changes in the asymmetric distribution of PS or PE could influence the formation of tubules or fenestrated regions of Golgi cisternae to produce a structure on which clathrin can assemble more productively. This is consistent with the morphological defect observed for Golgi membranes in the drs2Δ mutant, which are notable for their lack of fenestration or tubular regions. Particularly in comparison to the arf1Δ mutant in which the Golgi is highly fenestrated or tubular in appearance (Gaynor et al., 1998).

Others have proposed that the transbilayer movement of lipid could induce the budding of membranes to facilitate vesicle budding (Devaux, 1991). Zha et al. (1998) have reported that changes in the composition of the plasma membrane outer leaflet caused by sphingomyelinase treatment can induce an energy-independent budding of functional endocytic vesicles. In addition, Farge et al. (1999) have recently reported that exogenous PS incorporated into the external leaflet of the plasma membrane was pumped to the inner leaflet and markedly enhanced the level of bulk endocytosis. Thus, it is feasible that Drs2p actively participates in CCV budding from Golgi membranes, and becomes essential for this process as the temperature drops below a specific threshold where a decreased fluidity of the membrane may prevent clathrin from performing this function alone.

A third possibility is that an increase in the PE or PS concentration of the cytoplasmic leaflet may influence the...
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