Protein Kinases Fpk1p and Fpk2p are Novel Regulators of Phospholipid Asymmetry

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Type 4 P-type ATPases (flippases) are implicated in the generation of phospholipid asymmetry in membranes by the inward translocation of phospholipids. In budding yeast, the DRS2/DNF family members Lem3p-Dnf1p/Dnf2p and Cdc50p-Drs2p are putative flippases that are localized, respectively, to the plasma membrane and endosomal/trans-Golgi network (TGN) compartments. Herein, we identified a protein kinase gene, FPK1, as a mutation that exhibited synthetic lethality with the cdc50Δ mutation. The kinase domain of Fpk1p exhibits high homology to plant phototropins and the fungus Neurospora crassa NRC-2, both of which have membrane-associated functions. Simultaneous disruption of FPK1 and its homolog FPK2 phenocopied the lem3Δ/dnf1Δ dnf2Δ mutants, exhibiting the impaired NBD-labeled phospholipid uptake, defects in the early endosome-to-TGN pathway in the absence of CDC50, and hyperpolarized bud growth after exposure of phosphatidylethanolamine at the bud tip. The fpk1Δ fpk2Δ mutation did not affect the subcellular localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Further, the purified glutathione S-transferase (GST)-fused kinase domain of Fpk1p phosphorylated immunoprecipitated Dnf1p and Dnf2p to a greater extent than Drs2p. We propose that Fpk1p/Fpk2p are upstream activating protein kinases for Lem3p-Dnf1p/Dnf2p.

INTRODUCTION

Despite experimental uncertainties concerning the distribution of lipids in organelle membranes, it is widely accepted that eukaryotic cell membranes have asymmetric lipid distribution. This phospholipid asymmetry has been most well characterized in the plasma membrane. In general, the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are enriched in the inner leaflet facing the cytoplasm, whereas phosphatidylcholine (PC), sphingomyelin, and glycolipids are predominantly found in the outer leaflet of the plasma membrane (Devaux, 1991; Zachowski, 1993; Pomorski et al., 2001). Phospholipid asymmetry is generated and maintained by ATP-driven lipid transporters or translocases. The type 4 subfamily of P-type ATPases (also called “flippases”) is implicated in the translocation of phospholipids from the external to the cytosolic leaflet (Graham, 2004; Pomorski et al., 2004; Holhuis and Levine, 2005; Devaux et al., 2006; Daleke, 2007). Five members of this subfamily (Drs2p, Neo1p, Dnf1p, Dnf2p, and Dnf3p) are encoded in the genome of the yeast Saccharomyces cerevisiae (Catty et al., 1997).

Though no type 4 P-type ATPase has been shown to exhibit phospholipid translocase activity in reconstitution experiments with purified enzymes and chemically defined vesicles, accumulating evidence suggests that the yeast ATPases possess this activity in membranes. Dnf1p and Dnf2p are localized to the plasma membrane, and loss of Dnf1p and Dnf2p abolishes ATP-dependent transport of fluorescent 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled analogues of PE, PS, and PC from the outer to the inner plasma membrane leaflet (Pomorski et al., 2003). Chemical labeling of outer-leaflet phospholipids and staining with a PE-specific probe showed that PE is exposed on the outer leaflet in dnf1Δ dnf2Δ mutant cells (Pomorski et al., 2003; Iwamoto et al., 2004). Drs2p is localized to endosomes and the trans-Golgi network (TGN), and Drs2p-dependent NBD-phospholipid translocase activity has been detected in Golgi membranes (Natarajan et al., 2004) and post-Golgi secretory vesicles (Alder-Baerens et al., 2006). In such vesicles, Drs2p was required for the asymmetric arrangement of PE (Alder-Baerens et al., 2006).

Our recent results suggest that the Drs2p and Dnf1p/Dnf2p/Dnf3p proteins form complexes with members of the conserved Lem3p-Cdc50p family of membrane proteins. Cdc50p, Lem3p, and Crf1p were communoprecipitated with Drs2p, Dnf1p and Dnf2p, and Dnf3p, respectively, and these proteins were required for the endoplasmic reticulum (ER) exit of the P-type ATPases (Saito et al., 2004; Furuta et al., 2007). Although we should await purification of the native proteins to conclude that these putative phospholipid translocases except for Neo1p are composed of noncatalytic and catalytic subunits, their heteromeric nature is reminis-
Our collection of mutations that exhibited synthetic lethality because Lem3p-Dnf1p/Dnf2p are involved in the endocytic pathway. However, neither Dnf1p nor Dnf2p interacts with Dnf2p are regulated in a different manner.

That the Ypt31p/32p-Rcy1p pathway regulates Cdc50p-Drs2p and exploration of the upstream signals that they receive. We have recently shown that Cdc50p-Drs2p and Lem3p-Dnf1p are recycled from the plasma membrane through early endosomes to the TGN and back to the plasma membrane (Saito et al., 2004; Liu et al., 2007). This overlapping localization may underlie the functional redundancy between Cdc50p-Drs2p and Lem3p-Dnf1p/Dnf2p. Combined mutation of these genes compromises vesicle transport pathways, including endocytic internalization at low temperatures (dnf1Δ dnf2A drs2Δ; Pomsorksi et al., 2003), vacuolar transport of alkaline phosphatase from the TGN (dnf1Δ dnf2A; Hua et al., 2002), and transport from early endosomes to the TGN (cdc50 lem3Δ cf r1Δ; Furuta et al., 2007).

One important approach toward understanding the physiological significance of phospholipid asymmetry would be identification of the regulatory pathways signaling to fli ppases and exploration of the upstream signals that they receive. We have recently shown that Cdc50p-Drs2p interacts with Rcy1p, a potential effector of the Rab family small GTPases Ypt31p/32p (Chen et al., 2005), and have proposed that the Ypt31p/32p-Rcy1p pathway regulates Cdc50p-Drs2p to promote the formation of transport vesicles destined for the TGN from early endosomes (Furuta et al., 2007). Because Lem3p-Dnf1p/Dnf2p are involved in the endocytic recycling pathway in conjunction with Cdc50p-Drs2p (Furuta et al., 2007), it might be expected that Lem3p-Dnf1p and Lem3p-Dnf2p would be regulated by the Ypt31p/32p-Rcy1p pathway. However, neither Dnf1p nor Dnf2p interacts with Rcy1p (Furuta et al., 2007), suggesting that Lem3p-Dnf1p/Dnf2p are regulated in a different manner.

In this study, we identify a protein kinase gene, FPK1, in our collection of mutations that exhibited synthetic lethality with cdc50A (Kishimoto et al., 2005). Our results suggest that Fpk1p and its homologue Fpk2p are upstream regulatory protein kinases for Lem3p-Dnf1p and Lem3p-Dnf2p.

MATERIALS AND METHODS

Media and Genetic Methods

Strains were cultured in YPDA rich medium (1% yeast extract [Difco Laboratories, Detroit, MI], 2% bacto-peptone [Difco], 2% glucose, and 0.01% adenine). Strains carrying plasmids were selected in synthetic medium (SD) containing the required nutritional supplements (Rose et al., 1990). Synthetic complete medium (SC) was SD medium containing all required nutritional supplements. When appropriate, 0.5% casamino acids (Difco) were added to SC medium without uracil (SDA-Ura). For induction of the GAL1 promoter, 3% galactose and 0.2% sucrose were used as carbon sources instead of glucose (YPGA and SGA-Ura). Growth sensitivity to duramycin was examined on YPDA plates containing 5 μg ml–1 duramycin (Sigma, St. Louis, MO). Standard genetic manipulations of yeast were performed as described previously (Guthrie and Fink, 1991). Escherichia coli strains DH5α and XLI-Blue were used for construction and amplification of plasmids. The lithium acetate method was used to introduce plasmids into yeast cells (Ebbe, 1992; Gietz and Woods, 2002).

Strains and Plasmids

Yeast strains used in this study are listed in Table 1. Yeast strains carrying complete gene deletions (f pk1Δ and f pk2Δ), green fluorescent protein (GFP)-tagged genes (KEX2, MYO2, DNF1, and DNF2), monomeric red fluorescent protein 1 (mRFP1)-tagged SECl, three tandem repeats of the influenza virus hemagglutinin epitope (3HA)-tagged genes (DNFI and DNFD2), and 13 × myc-tagged genes (DNFI, DNF2, DNF3, DRS2, and NEO1) were constructed by PCR-based procedures as described previously (Noji et al., 1998; Goldshmidt and McCusker, 1999). The GAL1 promoter-inducible C-terminal FPK1 fragment (amino acids 445-893) tagged with glutathione S-transferase (pGAL-GST-FPK1ΔN) was similarly constructed. All strains constructed by PCR-based procedures were verified by colony-PCR amplification to confirm that the replacement had occurred at the expected location.

The plasmids used in this study are listed in Table 2. The FPK1(K525S) allele was generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with pkT631 (Ypal11-FPK1). We sequenced the entire gene and reading frame of FPK1(K525S) to verify that only the desired substitutions were introduced. Schemes detailing the construction of plasmids and DNA sequences of nucleotide primers are available on request.

Isolation of New Mutants Synthetic Lethally with the cdc50Δ Mutation

Mutants synthetically lethal with cdc50Δ were newly isolated according to procedures described previously (Kishimoto et al., 2005). Single recessive mutations were identified by genetic analyses, and the corresponding wild-type genes were cloned. For nonessential genes, null mutations were confirmed to be synthetically lethal with cdc50Δ. For essential genes, identity was confirmed by tetrad analysis with marker-tagged wild-type or temperature-sensitive alleles (our unpublished results).

Microscopic Observations

Most GFP- or mRFP1-tagged proteins were observed in living cells, which were grown to early-mitochondrial phase, harvested, and resuspended in SC medium. Cells were mounted on microscope glass and immediately fixed in 37% formaldehyde stock (Wako Pure Chemicals, Osaka, Japan) into the medium to a final concentration of 0.5%, followed by a 10-min incubation at 30°C. After fixation, cells were washed twice with phosphate-buffered saline (PBS) and examined.

Endosomal structures were visualized by brief labeling with the lipophilic styryl dye FM4-64 (Invitrogen, Carlsbad, CA). Cells were grown to early-mitochondrial phase in YPDA medium at 30°C for 3 h. Four OD600 units of cells were labeled with 32 μM FM4-64 in 100 μl of YPDA medium for 30 min on ice. Cells were harvested by centrifugation, resuspended in 200 μl of fresh YPDA medium, and incubated at 30°C for 1 min. Cells were washed twice with 100 μl of ice-cold SC medium and immediately observed using a G-2A filter set. The vacuole lumens was visualized using Cell Tracker Blue CMAC (Invitrogen) according to the manufacturer’s protocol.

Cells were observed using a Nikon Eclipse E800 microscope (Nikon Insect, Tokyo, Japan) equipped with an HB-10103 AF super high pressure mercury lamp and a 1.4 NA 100X Plan Apo oil immersion objective (Nikon Insect) with the appropriate fluorescence filter sets (Nikon Insect) and differential interference contrast (DIC) optics. Images were acquired with a digital cooled charge-coupled device camera (C4742-95-12NR; Hamamatsu Photonics, Hamamatsu, Japan) using AQUACOSMOS software (Hamamatsu Photonics). Observations are compiled from the examination of at least 100 cells.

Ultrastructural observations of cells by conventional electron microscopy was performed using the glutaraldehyde-osmium fixation technique according to procedures described previously (Sakane et al., 2006).

Staining of Phosphatidyethanolamine with Biotinylated Ro09-0198 Peptide

PE staining in the outer leaflet of the plasma membrane was performed using biotinylated Ro09-0198 peptide (Bio-Ro) as described (Iwamoto et al., 1994) with the following modifications. Bio-Ro was prepared essentially as described (Aoki et al., 1994). A 1-ml culture of cells (mid-mitochondrial phase, generally at a cell density of 0.4–0.6 OD600/ml) was harvested in 20 μl of YPDA containing 100 μM Bio-Ro (for wild type), 30 μM (for lem3Δ), or 15 μM (for fpk1Δ fpk2Δ), and incubated for 3 h at 30°C (for f pk1Δ f pk2Δ) or 15 min (for lem3Δ) on ice. The cells were washed once with PBS and fixed with 5% formaldehyde in PBS for 1 h at room temperature. After two washes with spheroplast buffer (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.4), cells were resuspended in 100 μl of spheroplast buffer containing 100 μg/ml zymolyase 100T (Seikagaku Kogyo, Tokyo, Japan) and 2.2 mg/ml β-mercaptoethanol (Wako Pure Chemicals) and incubated for 10 min at 30°C. After two washes with spheroplast buffer, spheroplast cells were attached to poly-l-lysine–coated multwell slides, fixed in methanol and acetone, and incubated in PBS containing 0.1% bovine serum albumin (Sigma) for 20 min at room temperature. To visualize Bio-Ro, cells were washed three times with PBS and incubated in PBS containing 5 μg/ml fluorescein streptavidin (Vector Labs, Burlingame, CA) for 1 h at room temperature. After five washes with PBS, cells were stained with 4’-6-diamidino-2-phenylindole (DAPI), suspended in 90% glycerol containing n-propyl gallate, and observed using an IT/C (for fluorescein) bandpass or a UV-1A filter set.

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Fluorescently labeled phospholipid internalization experiments were performed as described by Hanson and Nichols (2001). Cells were labeled with NBD-PS, the fpk1Δ fpk2Δ mutant was incubated at 2.5 μM, whereas the wild type and the fpk1Δ fpk2Δ mutant were incubated at 2.5 and 3 μM, respectively. For NBD-PE, the fkp1Δ fkp2Δ mutant was incubated at 50 μM whereas the wild type and the fkp1Δ fkp2Δ mutant were incubated at 5 and 2 μM, respectively. After labeling, the cells were washed three times with ice-cold SC medium, resuspended in SC medium, and incubated at 30°C. At the given time

**Table 1. Yeast strains used in this study**

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*a KKT strains are isogenic derivatives of BY4743. YKT strains are isogenic derivatives of YEF473.

**Internalization of Fluorescence-labeled Phospholipids into Yeast Cells**

Large unilamellar vesicles containing NBD-phospholipids were prepared as described (Saito et al., 2004). 1-Palmityloyl-2-(6-NBD-aminocaproyl)-PE (NBD-PE), 1-palmityloyl-2-(6-NBD-aminocaproyl)-PC (NBD-PC), 1-palmityloyl-2-(6-NBD-aminocaproyl)-PS (NBD-PS), and dioleoylphosphatidicholines (DOPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Fluorescently labeled phospholipid internalization experiments were performed as described previously (Kato et al., 2002; Saito et al., 2004). Briefly, cells were grown to early-midlogarithmic phase in YPDA media at 30°C. After dilution to 0.35 OD600/ml in SC medium, cells were shaken for 30 min at 30°C with vesicles containing 40% NBD-phospholipids and 60% DOPC at a final concentration of 50 μM. Cells were then suspended in SC medium containing 0.1% NaN3, and 2.5 μg/ml propidium iodide (PI) to allow the exclusion of PI-positive dead cells in flow cytometric analysis. Flow cytometry of NBD-labeled cells was performed on a FACS Calibur cytometer using the CellQuest software (BD Biosciences, San Jose, CA). NBD green fluorescence was plotted on a histogram to allow calculation of the mean fluorescence intensity.

**Eflux of Phospholipids from Yeast Cells**

Fluorescently labeled phospholipid efflux experiments were performed as described by Hanson and Nichols (2001). Cells were labeled with NBD-phospholipids for 60 min at 30°C as described above. To achieve equal levels of fluorescence in the strains to be compared, the concentration of an NBD-lipid for loading was adjusted according to the capability of each strain for the uptake of NBD-phospholipid. For the uptake of NBD-PE, the lem3Δ mutant was incubated at 50 μM, whereas the wild type and the fpk1Δ fpk2Δ mutant were incubated at 2.5 and 3 μM, respectively. For NBD-PE, the fkp1Δ fkp2Δ mutant was incubated at 50 μM, whereas the wild type and the fkp1Δ fkp2Δ mutant were incubated at 5 and 2 μM, respectively. After labeling, the cells were washed three times with ice-cold SC medium, resuspended in SC medium, and incubated at 30°C. At the given time...
The collected cells were resuspended in 1.2 l of YPGA (for KKT345) or SGA-U (for KKT268) and incubated at 30°C to early-logarithmic phase in YPDA (for fpk1/H9004/motor. KKT345 cells or KKT268 (pKT1631/H20851, pKT1566/H20851, pKT1563/H20851) were grown at 30°C to a cell density of 0.5 OD600/ml and incubated at 37°C for 30 min before loading.

Expression and Purification of the GST-Fpk1p-kinase Domain (GST-Fpk1ΔNp)

The KKT345 (pCFL-GST-FPK1ΔNp) strain and the pKT1700 (pKO10-GST-FPK1ΔN-


d to a cell density of 0.5 OD600/ml and incubated at 30°C. The collected cells were resuspended in 1.2 l of YPGA (for KKT345) or SGA-U (for KKT268) medium to a cell density of 0.5 OD600/ml and incubated at 30°C. The newly isolated genes were used in the phosphorylation assays.

Immunoprecipitation of P-type ATPases and Phosphorylation Assays with GST-Fpk1ΔNp

Immunoprecipitation of P-type ATPases tagged with 13 × mc was performed as described previously (Saito et al., 2004) with the following modifications. Briefly, cells were grown at 30°C to a cell density of 0.5 OD600/ml in YPDA medium. Cells collected from 300 ml (Dnf1p-13myc, Dnf2p-13myc, Dnp2p-13myc, and Nop1p-13myc) or 1.2 l (Dnp3p-13myc) of culture were washed twice with ice-cold water and resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 300 mM sorbitol, 100 mM NaCl, and 5 mM MgCl2) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). The cells were lysed with glass beads using Multi-beads shocker. Cell lysates were centrifuged at 400,000 g for 30 min, and the resulting supernatant was centrifuged at 20,000 g for 5 min. The resulting supernatant was centrifuged at 20,000 g for 5 min, and the resulting supernatant was centrifuged at 20,000 g for 5 min. The cleared lysates were incubated with 7.5 μg of anti-myc antibody for 1 h at 4°C. The samples were rotated with 30 μl of protein G-Sepharose 4 Fast Flow (GE Healthcare) for 1 h at 4°C. The protein G-Sepharose beads were pelleted, washed twice with IP buffer, and washed twice with kinase buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) containing protease inhibitors. Insoluble material was removed by centrifugation at 20,000 g for 5 min at 4°C. The cleared lysates were incubated with 7.5 μg of anti-myc antibody for 1 h at 4°C. The samples were rotated with 30 μl of protein G-Sepharose 4 Fast Flow (GE Healthcare) for 1 h at 4°C. The protein G-Sepharose beads were pelleted, washed twice with IP buffer, and washed twice with kinase buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1 mM EGTA). The beads were eluted from the three tubes. One was subjected to immunoblot analysis with anti-myc antibody (1:1000 dilution), and the others were used for kinase assays. Relative amounts of immunoprecipitated P-type ATPases were estimated by chemiluminescence with a FLA3000 fluorescence image analyzer (Fuji Photo Film). For kinase assays, beads were suspended in 45 μl of kinase buffer with or without 100 nM purified GST-Fpk1ΔNp. Reactions were started by adding 5 μl of 1 mM [γ-32P]-ATP (2000 cpm/pmol; Perkin Elmer-Cetus, Norwalk, CT; 222 Kbp/ml), followed by incubation at 30°C for 10 min. Reactions were stopped by adding 16.7 μl of 4× SDS-PAGE sample buffer, followed by incubation at 37°C for 30 min. Samples were resolved by SDS-PAGE, and phosphorylated proteins were visualized and 32P incorporation into each P-type ATPase was quantified with a FLA3000 fluorescent image analyzer (Fuji Photo Film).

RESULTS

A Mutation in a Novel Protein Kinase Gene FPK1 Exhibits a Synthetic Lethal Genetic Interaction with the cdc50Δ Mutation

To identify genes involved in CDC50 and DRS2 function, we previously screened for mutations that are synthetically lethal with cdc50Δ, resulting in isolation of four mutants, each of which harbored an allele of erg3, rgp1, vps1, or sro2 (Kishimoto et al., 2005). We expanded this screen and isolated allelic sets of seven genes: ypt6, gog1/tes5, pep6, ibp3, neo1, sec3,

Antibodies

Mouse anti-HA (HA.11) mAb was purchased from BabCO (Richmond, CA). Mouse anti-myc (9E10) mAb was purchased from Sigma. Rabbit anti-Keq2p and anti-Pma1p polyclonal antibodies were gifts from S. Nothwehr (University of Missouri, Columbia, MO) and R. Serrano (Polytechnic University of Valencia, Valencia, Spain), respectively. Mouse anti-Fpk1p a monoclonal antibody was a gift from Y. Ohsumi (National Institute for Basic Biology, Okazaki, Japan). For immunoblot analysis, these antibodies were used at the following dilution: anti-HA and anti-myc, 1:1000; anti-Pma1p, 1:5000; anti-Kex2p and anti-Pep12p polyclonal antibodies were gifts from S. Nothwehr (University of Missouri, Columbia, MO) and R. Serrano (Polytechnic University of Valencia, Valencia, Spain), respectively. Mouse anti-Pep12p mAb was a gift from R. Valencia, USA. Rabbit anti-Kex2p and anti-Pma1p mAbs were gifts from Lewis et al. (2000). For kinase assays, beads were suspended in 45 μl of kinase buffer with or without 100 nM purified GST-Fpk1ΔNp. Reactions were started by adding 5 μl of 1 mM [γ-32P]-ATP (2000 cpm/pmol; Perkin Elmer-Cetus, Norwalk, CT; 222 Kbp/ml), followed by incubation at 30°C for 10 min. Reactions were stopped by adding 16.7 μl of 4× SDS-PAGE sample buffer, followed by incubation at 37°C for 30 min. Samples were resolved by SDS-PAGE, and phosphorylated proteins were visualized and 32P incorporation into each P-type ATPase was quantified with a FLA3000 fluorescent image analyzer (Fuji Photo Film).
and ynr047w. Ypt6p is a Rab family small GTPase that promotes fusion of recycling endocytic vesicles with the TGN membrane, and Rgp1p is a GDP/GTP exchange factor (GEF) for Ypt6p (Siniossoglou et al., 2000; Siniossoglou and Pelham, 2001). The genetic interaction with ypt6 and rgp1 seems to reflect the involvement of Cdc50p-Drs2p in the endocytic recycling pathway (Hua et al., 2002; Saito et al., 2004; Kishimoto et al., 2005; Furuta et al., 2007). Gsg1p/Trs85p is a nonessential subunit of the transport protein complex (TRAPP II), which is proposed to be a GEF for Ypt31/32p (Furuta et al., 2007), suggest that TRAPP II is an upstream regulator of Cdc50p-Drs2p. Synthetic lethal interactions with drs2Δ were also reported for mutations in other subunits of the TRAPP II complex, TRS33 and KRE11 (Sciorra et al., 2005). Pep5p (Vps26p) is a subunit of the retromer, the sorting machinery required for retrieval from the late endosome to the TGN (Seaman et al., 1998; Reddy and Seaman, 2001). Null mutations of VPS29 and VPS35, encoding other retromer subunits, also exhibited synthetic lethality with cdc50Δ (our unpublished data), implicating Cdc50p in the late endosome-to-TGN pathway. NEO1 is the one essential gene among five putative flippases in yeast. Synthetic growth defects of drs2Δ and the neo1-1 temperature-sensitive allele have been reported (Hua and Graham, 2003). Genetic interactions with ubp3 and sec3 implicate Cdc50p in unknown functions; Ubp3p is a ubiquitin-specific protease that regulates transport between the ER and Golgi compartments (Cohen et al., 2003), and Sec3p is a spatial landmark that defines sites for polarized secretion (Finger and Novick, 1998). YNR047W encodes a novel protein kinase and the results reported herein suggest that Ynr047wp is a regulatory protein kinase for Lem3p-Dnf1p and Lem3p-Dnf2p. We have renamed YNR047W FPK1 (flipase kinase 1). The synthetic growth defect of the cdc50Δ fpk1Δ mutant was shown in Figure 1A by tetrad dissection of a sporulated diploid heterozygous for cdc50Δ and fpk1Δ.

The 893-amino acid Fpk1p contains a C-terminal kinase domain that belongs to the S6 kinase family of serine/threonine kinases (Hunter and Plowman, 1997). Fpk1p has a homolog Kin82p (Ycr091wp). Because the name Kin82 does not relate to the function of the encoded protein and because we find that Kin82p is functionally related to Fpk1p, we have renamed KIN82 FPK2. The kinase domain of Fpk1p is 82% identical and 91% homologous to that of Fpk2p (Figure 1B), whereas the N-terminal domain is 29% identical and 39% homologous (our unpublished results). The kinase domain of Fpk1p is also homologous to proteins from other organisms: NRC-2 (72% identical and 84% homologous) is a negative regulator of the condensation program in Neurospora crassa (Kothe and Free, 1998); phototropin-1 (47% identical and 64% homologous) and phototropin-2 (48% identical and 65% homologous) are blue light–activated protein kinases in Arabidopsis thaliana (Briggs and Christie, 2002; Figure 1B).

Single or double knockout of FPK1 and FPK2 did not affect cell growth at 18, 30, or 37°C (Figure 1C and our unpublished results). To perform phenotypic analyses of the cdc50Δ fpk1Δ mutant, we first constructed a conditional mutant in the fpk1Δ background in which CDC50 is expressed under the control of the glucose-repressible GAL1 promoter. As shown in Figure 1C, the P_GAL1-HA-CDC50 fpk1Δ mutant grew normally in galactose-containing medium (YPGA), but exhibited only residual growth in glucose-containing medium (YPD) at 30°C. Deletion of FPK2 in this strain resulted in a more severe growth defect, whereas the Cdc50p-depleted fpk2Δ mutant grew normally (Figure 1C). In addition, the growth defect of the Cdc50p-depleted fpk1Δ mutant was suppressed by overexpression of FPK2 (our unpublished results). These results suggest that Fpk1p and Fpk2p are functionally redundant, but that Fpk1p plays a major role. The FPK1(K525R) allele carries an amino acid substitution in the conserved ATP-binding site that results in a kinase-negative mutant protein (Hanks et al., 1988). The Cdc50p-depleted FPK1(K525R) mutant also exhibited a growth defect (Figure 1D), suggesting that Fpk1p performs its Cdc50p-related function by phosphorylating target proteins.

The Cdc50p-depleted fpk1Δ fpk2Δ Mutant Exhibits Defects in the Retrieval Pathway from Early Endosomes to the TGN

The lem3Δ mutation also exhibits synthetic lethality with the cdc50Δ and rcy1Δ mutations (Saito et al., 2004; Furuta et al., 2007; Supplementary Figure S1). Similarly, the fpk1Δ mutation exhibited synthetic lethality with the drs2Δ and rcy1Δ mutations (our unpublished results); Drs2p is a potential catalytic subunit of Cdc50p (Saito et al., 2004), and Rcy1p is a regulatory factor for the Cdc50p-Drs2p complex (Furuta et al., 2007). These results suggest that Fpk1p/Fpk2p may be functionally related to Lem3p-Dnf1p/Dnf2p. We have recently reported that a temperature-sensitive cdc50-ts lem3Δ cry1Δ mutant exhibits severe defects in the retrieval pathway from early endosomes to the TGN (Furuta et al., 2007). Thus, we examined this pathway in the Cdc50p-depleted fpk1Δ fpk2Δ mutant. Tlg1p is a target-SNARE that is recycled between the TGN and early endosomes; GFP-Tlg1p thus shows a punctate pattern reminiscent of endosomal/TGN membranes (Holthuis et al., 1998; Siniossoglou and Pelham, 2001). Snc1p is an exocytic vesicle-SNARE that is recycled from the plasma membrane via early endosomes to the TGN; GFP-Snc1p is primarily localized to the plasma membrane at polarized sites where exocytosis is actively occurring, such as buds (Lewis et al., 2000). Because Tlg1p and Snc1p are both recycled via the retrieval pathway from early endosomes to the TGN, these two proteins accumulate in the same aberrant intracellular structures when this pathway is blocked (Furuta et al., 2007).

Cells expressing mRFP1-Snc1p and GFP-Tlg1p were grown in glucose medium for 6 h. Under these conditions, Cdc50p-depleted cells (P_GAL1-HA-CDC50) did not exhibit intracellular accumulation of mRFP1-Snc1p, unlike cdc50Δ cells (Saito et al., 2004), probably because 6-h incubation was insufficient to completely deplete Cdc50p as described previously (Sakane et al., 2006). Both mRFP1-Snc1p and GFP-Tlg1p were normally localized in wild-type, lem3Δ, fpk1Δ, and P_GAL1-HA-CDC50 cells (Figure 2A). In contrast, mRFP1-Snc1p and GFP-Tlg1p were colocalized in abnormal membranous structures in the Cdc50p-depleted fpk1Δ fpk2Δ mutant as well as in the Cdc50p-depleted lem3Δ mutant; 92% (n = 105) of the mRFP1-Snc1p–positive structures were also labeled with GFP-Tlg1p in the Cdc50p-depleted fpk1Δ fpk2Δ mutant (Figure 2A).

The GFP-Snc1p–pm mutant is transported to the plasma membrane by the exocytic pathway but is not internalized by endocytosis (Lewis et al., 2000). GFP-Snc1p–pm was exclusively localized to the plasma membrane in the Cdc50p-depleted fpk1Δ fpk2Δ mutant as well as in the Cdc50p-depleted lem3Δ mutant (Figure 2B). These results are consistent with the notion that intracellular accumulation of mRFP1–Snc1p in the Cdc50p-depleted fpk1Δ fpk2Δ mutant was due to defects in the retrieval pathway from early endosomes to the TGN, but not due to defects in the exocytic pathway from the TGN.
Furuta et al. (2007) also reported the mislocalization of C-terminally GFP-tagged Kex2p (Kex2p-GFP) in the cdc50-ts lem3Δ mutant. Kex2p is a TGN resident furin-like protease that is localized due to constant retrieval from late and early endosomes (Brickner and Fuller, 1997; Lewis et al., 2000). When cycling between endosomes and the TGN is impaired, Kex2p mislocalizes to the vacuole (Wilcox et al., 1992; Spelbrink and Nothwehr, 1999). We examined the localization of Kex2p-GFP in the Cdc50p-depleted fpk1(K525R) fpk2Δ mutant and compared it with vacuoles visualized by staining with CellTracker Blue CMAC. In wild-type cells and control mutants, Kex2p-GFP exhibited a punctate pattern reminiscent of TGN membranes, whereas in the Cdc50p-depleted fpk1(K525R) fpk2Δ and Cdc50p-depleted lem3Δ mutants, Kex2p-GFP was primarily localized to vacuoles with concomitant decrease of the punctate localization (Figure 2C).

In the cdc50-11 lem3Δ crf1Δ mutant, double membrane structures with ring-, horseshoe-, or crescent-like morphology were accumulated (Furuta et al., 2007). Because Snc1p was localized to these structures by immunoelectron microscopy (Furuta et al., 2007), they seemed to represent the intracellular membranes visualized by light microscopy. We examined membranous structures that accumulated in the Cdc50p-depleted fpk1(K525R) fpk2Δ mutant by electron microscopy. When grown in YPDA medium at 30°C for 8 h to deplete Cdc50p, the PGAL1-HA-CDC50 fpk1(K525R) fpk2Δ mutant cells accumulated double membrane structures (approximately nine structures (~200 nm in diameter)/10 μm²; n = 31 sections) to an extent similar to that in the PGAL1-HA-CDC50 lem3Δ mutant (~10 structures/10 μm²; n = 35 sections; Figure 3). The PGAL1-HA-CDC50 mutant cells accumulated smaller numbers of these structures (approximately four structures/10 μm², n = 34 sections; Figure 3). By light microscopy, however, the accumulation of these structures was not apparent (Figure 2A); this difference may be due to longer depletion of Cdc50p (8 h) in the cells used for electron microscopy than in those for light microscopy (6-h depletion).
The fpk1Δ fpk2Δ mutant is defective in vesicle transport from early endosomes to the TGN. (A) mRFP1-Snc1p and GFP-Tlg1p colocalized in abnormal intracellular structures in the Cdc50p-depleted fpk1Δ fpk2Δ mutant. Cells carrying plK1566 (YEplac181-GFP-TLG1) and plK1563 (pRS416-mRFP1-SNC1) were grown at 30°C for 6 h in YPDA medium, followed by microscopic examination after fixation with 0.5% formaldehyde. Strains are those described in the legend for Figure 1C, KKT102 (lem3Δ), and KKT293 (P_{CASL}::CDC50 lem3Δ). Images were merged to compare the two signal patterns. (B) GFP-Snc1p-pm localized to the plasma membrane in the Cdc50p-depleted fpk1Δ fpk2Δ mutant. Cells harboring pRS416-GFP-SNC1 pm were grown and examined as described in A. (C) Kex2p-GFP mislocalized to the vacuole in the Cdc50p-depleted fpk1Δ fpk2Δ mutant. KKT58 (WT), KKT346 (lem3Δ), KKT350 (fpk1Δ fpk2Δ), KKT347 (P_{CASL}::CDC50), KKT348 (P_{CASL}::CDC50 lem3Δ), and KKT349 (P_{CASL}::CDC50 fpk1Δ fpk2Δ) cells expressing Kex2p-GFP were grown in YPDA medium at 30°C for 6 h, labeled with CellTracker Blue CMAC at 30°C for 30 min, and observed by fluorescence microscope. Bars, 5 μm.

Abnormal membrane structures were not observed in wild-type, lem3Δ, or fpk1Δ fpk2Δ cells. Taken together, the Cdc50p-depleted fpk1Δ fpk2Δ and Cdc50p-depleted lem3Δ mutants exhibited similar defects in the early endosome-to-TGN transport pathway.

The fpk1Δ fpk2Δ Mutant Is Defective in the Inward Translocation of Phospholipids

Dnf1p and Dnf2p are localized to internal punctate structures reminiscent of endosomal/Golgi compartments and to the plasma membrane at polarized sites (Hua et al., 2002; Pomorski et al., 2003). Lem3p-Dnf1p/Dnf2p and Cdc50p-Drs2p seem to be involved in endocytic recycling as described above (Furuta et al., 2007), whereas Lem3p-Dnf1p/Dnf2p localized at the plasma membrane has been implicated in flipping phospholipids (Kato et al., 2002; Hanson et al., 2003; Pomorski et al., 2003). If Fpk1p and Fpk2p are involved in the function or regulation of Lem3p-Dnf1p/Dnf2p, the fpk1Δ fpk2Δ mutant might exhibit defects in phospholipid translocation across the plasma membrane.

We thus examined whether the fpk1Δ fpk2Δ mutations affect the inward translocation of phospholipids at the plasma membrane. If the fpk1Δ fpk2Δ mutant is defective in flipping phospholipids, a phospholipid enriched in the inner leaflet, such as PE, would be exposed on the outer leaflet of the plasma membrane. Ro09-0198 is a 19-amino acid tetra-cyclic polypeptide that forms a tight complex with PE in biological membranes (Choung et al., 1988). The lem3Δ/ro3Δ and dnf1Δ dnf2Δ mutants exhibited hypersensitivity to duramycin, an analogue of Ro09-0198 (Noji et al., 2006). Wild-type, lem3Δ, fpk1Δ, fpk2Δ, and fpk1Δ fpk2Δ strains were grown in YPDA medium containing 5 μM duramycin at 30°C. The fpk1Δ fpk2Δ mutant showed sensitivity to duramycin, but not to the same extent as the lem3Δ mutant (Figure 4A). The kinase-negative FPK1(K525R) fpk2Δ mutant also exhibited duramycin sensitivity (our unpublished results). In contrast, neither cdc50A nor drs2Δ mutant exhibited sensitivity to 5 μM duramycin (Supplementary Figure S2).

PE exposed on the outer leaflet of the plasma membrane can be visualized in live cells by staining with biotinylated PE (Figure 4B, WT). In contrast, in lem3Δ cells, PE remained exposed at the bud tip throughout the budding process (77% of medium-budded cells, n = 106; 49% of large-budded cells, n = 102), probably due to defective inward translocation of PE (Figure 4B, lem3, arrows). Similarly, PE remained exposed in the fpk1Δ fpk2Δ strain until late stages of budding (40% of medium-budded cells, n = 103; 7% of large-budded cells, n = 107; Figure 4B, fpk1 fpk2, arrows). These results suggest that PE is exposed on the outer plasma membrane leaflet in the fpk1Δ fpk2Δ mutant. Hua et al. (2002) reported that the dnf1Δ dnf2Δ mutant exhibited elongated bud morphology. We confirmed this phenotype in the lem3Δ mutant; elongated bud morphology was most prominent in large-budded cells (Figure 4B, lem3, large-budded). Interestingly, the fpk1Δ fpk2Δ mutant also exhibited elongated bud mor-
morphology (Figure 4B, fpk1 fpk2, large-budded), implying that both Lem3p-Dnf1p/Dnf2p and Fpk1p/Fpk2p are involved in the regulation of bud morphology.

Very recently, we have shown that the elongated bud morphology of the lem3Δ mutant is due to defects in the switch from apical to isotropic bud growth (apical-isotropic switch), which is activated by G2 cyclins-Cdc28p (Saito et al., 2007). Lem3p-Dnf1p/Dnf2p-mediated translocation of phospholipids into the inner leaflet of the apical plasma membrane seems to activate GTPase-activating protein (GAP) activities of Rga1/2 toward the small GTPase Cdc42p, resulting in the dispersal of Cdc42p from the bud tip. In the lem3Δ mutant, polarity proteins including a type V myosin Myo2p remained polarized at the bud tip even at a late mitotic phase. We thus examined this phenotype in the fpk1Δ fpk2Δ mutant. The fpk1Δ fpk2Δ cells expressing Myo2p-GFP were grown asynchronously, and the localization of Myo2p-GFP was examined in late mitotic cells recognized by DAPI staining. In this analysis, cells with Myo2p-GFP at the bud neck were excluded, because the ratio of the bud neck localization pattern in late mitotic cells was not very different among the stains examined (see the legend to Figure 4C).

As shown in Figure 4C, most of the fpk1Δ fpk2Δ and lem3Δ cells exhibited polarization of Myo2p-GFP to the bud tip (68.6 and 75.7%, respectively), whereas only 10.9% of the wild-type cells did. These results indi-
Figure 5. Defective inward translocation of NBD-labeled phospholipids in the fpk1Δ fpk2Δ mutant. (A) Uptake of NBD-labeled phospholipids. Cells were preincubated in SC medium for 3 h at 30°C, and labeled with NBD-PE, -PC, or -PS for 30 min at 30°C, and internalized NBD-phospholipids were quantitated by flow cytometry as described in Materials and Methods. Strains used were as follows: top panel, KT75 (WT), KT75 (lem3Δ), KT77 (fpk1Δ), KT75 (fpk2Δ), KT267 (fpk1Δ fpk2Δ), and KT268 (fpk1Δ lem3Δ fpk2Δ); bottom panel, the wild-type (WT) strain transformed with YCplac111 (vector) and the fpk1Δ fpk2Δ mutant transformed with YCplac111 (vector), YCplac111-FPK1 (pFPK1), or YCplac111-FPK1 (K525R) [pFPK1 (K525R)]. Data are presented as average percentage of accumulation relative to wild-type cells ± SD of three independent experiments. (B) Efflux of NBD-PE and NBD-PS was not affected in the fpk1Δ fpk2Δ mutant. Phospholipid efflux in wild-type (KT75, ○), lem3Δ (KT75, □), and fpk1Δ fpk2Δ (KT268, △) cells was measured at the indicated times as described in Materials and Methods. Relative values for the initial fluorescence in the lem3Δ mutant were 91.6 ± 1.3 and 90.5 ± 16.5% of the wild type for NBD-PE and -PS, respectively, and those in the fpk1Δ fpk2Δ mutant were 96.3 ± 12.4 and 91.2 ± 3.0%, respectively. Data are presented as means ± SD of at least three independent experiments. (C) Simultaneous overexpression of DNF1 and LEM3 suppressed the duramycin-sensitive growth of the fpk1Δ fpk2Δ mutant. fpk1Δ fpk2Δ cells (KT268) harboring YEpplac181 and YEplac195 (vector), YEpplac181 and YEplac195-FPK1 (pFPK1), or YEpplac181-LEM3 and YEplac195-DNF1 (pDNF1/LEM3) were tested for duramycin sensitivity as described in the legend of Figure 4A. (D) Simultaneous overexpression of DNF1 and LEM3 restored the uptake of NBD-labeled phospholipids in the fpk1Δ fpk2Δ mutant. Wild-type (WT) and fpk1Δ fpk2Δ cells harboring YEpplac181 and YEplac195 (vector) or YEpplac181-LEM3 and YEplac195-DNF1 (pDNF1/LEM3) were assayed for NBD-phospholipid internalization. Results are presented as described in A.

icate that the fpk1Δ fpk2Δ mutant is also defective in the apical-isotropic growth switch.

We next examined whether the uptake of NBD-labeled phospholipids (NBD-PE, NBD-PC, and NBD-PS) across the plasma membrane was affected in our strains. As described previously, the lem3Δ mutant exhibited clear defects in the uptake of NBD-PE (34 ± 1% of wild type) and NBD-PC (16 ± 1% of wild type; Kato et al., 2002; Hanson et al., 2003). In contrast, the uptake of NBD-PS was minimally affected (84 ± 7% of wild type; Figure 5A; Kato et al., 2002; Hanson et al., 2003). Pomorski et al. (2003) reported that NBD-PS uptake in dnf1Δ dnf2Δ cells was ~50% of uptake in wild-type cells; this was also mild compared with the pronounced defects in NBD-PE and -PC uptake. Internalization of NBD-phospholipids was not affected in the fpk1Δ or fpk2Δ strains, but uptake was significantly decreased in the fpk1Δ fpk2Δ double deletion strain (62 ± 3% for NBD-PE and 66 ± 5% for NBD-PC relative to wild type; Figure 5A). These results are consistent with weaker sensitivity of the fpk1Δ fpk2Δ mutant to duramycin. Surprisingly, the uptake of NBD-PS was much more impaired in the fpk1Δ fpk2Δ mutant (34 ± 4% of wild type) than in the lem3Δ mutant. These results raise the interesting possibility that the fpk1Δ fpk2Δ mutations affect multiple phospholipid translocases. We confirmed that expression of wild-type FPK1, but not kinase-negative FPK1 (K525R), restored NBD-phospholipid internalization in the fpk1Δ fpk2Δ mutant (Figure 5A, bottom panel).

Reduced accumulation of NBD-phospholipids in the fpk1Δ fpk2Δ mutant could be due to increased efflux of NBD-phospholipids rather than decreased internalization. However, the NBD-phospholipid efflux rates measured in the fpk1Δ fpk2Δ mutant were indistinguishable from those in the wild type for NBD-PE and -PS (Figure 5B). The lem3Δ mutant exhibited a little slow efflux of NBD-PE, but the fpk1Δ fpk2Δ mutant effluxed NBD-PE with a wild-type rate.

Taken together, these results suggest that the reduction in accumulation of NBD phospholipids in the fpk1Δ fpk2Δ cells is due to a defect in the inward transport of these lipids across the plasma membrane.

One plausible function of Fpk1p/Fpk2p would be the upstream regulation of Lem3p-Dnf1p/Dnf2p. If this were the case, we would expect suppression of the fpk1Δ fpk2Δ mutations by overexpression of LEM3 and DNF1. The fpk1Δ fpk2Δ mutant was cotransformed with two multicopy plasmids encoding either LEM3 or DNF1. Simultaneous overexpression of LEM3 and DNF1 suppressed the duramycin-sensitive growth of the fpk1Δ fpk2Δ mutant to an extent similar to that of expression of FPK1. The uptake of NBD-PE, -PC, and -PS was also increased in these cells (Figure 5, C and D). Overexpression of LEM3 and DNF1 also suppressed the growth defects observed in the Cdc50p-depleted fpk1Δ fpk2Δ mutant (our unpublished results). In contrast, overexpression of FPK1 did not suppress the duramycin sensitivity of the lem3Δ mutant (our unpublished results). These results are consistent with the hypothesis that Fpk1p/Fpk2p are upstream regulatory kinases of Lem3p-Dnf1p.
Expressing C-terminal GFP- or HA-tagged Dnf1p and Dnf2p membrane. To examine these possibilities, we used strains Dnf1p/Dnf2p at endosomal compartments or the plasma membrane. Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p or Lem3p-Dnf2p.

The Localization of Dnf1p and Dnf2p Is Unaltered in the fpk1Δ fpk2Δ Mutant

Defective inward phospholipid movement across the plasma membrane in the fpk1Δ fpk2Δ mutant could be due to altered expression or localization of Lem3p-Dnf1p or Lem3p-Dnf2p. Given that Lem3p-Dnf1p is recycled through the endocytic recycling pathway (Saito et al., 2004; Liu et al., 2007), Fpk1p/Fpk2p might regulate localization of Lem3p-Dnf1p/Dnf2p at endosomal compartments or the plasma membrane. To examine these possibilities, we used strains expressing C-terminal GFP- or HA-tagged Dnf1p and Dnf2p from their endogenous loci (Pomorski et al., 2003; Noji et al., 2006). The expression levels of Dnf1p-HA and Dnf2p-HA, as estimated by immunoblotting, were similar in wild-type and mutants (Figure 6A). The fractionation profiles of Dnf1p-HA and Dnf2p-HA were similar in Dnf1p-HA and Dnf2p-HA in the fpk1Δ fpk2Δ strain (our unpublished results).

Localization of Dnf1p-HA and Dnf2p-HA was examined by subcellular fractionation on sucrose density gradients (Figure 6A). In wild-type cells, a plasma membrane marker, Pma1p (a proton ATPase; Serrano et al., 1986; Bagnat et al., 2001), and a late endosome marker, Pep12p (t-SNARE; Becherer et al., 1996; Lewis et al., 2000), were recovered in high- and low-density fractions, respectively. Kex2p, which is localized to endosomal/TGN compartments (Brickner and Fuller, 1997; Lewis et al., 2000), peaked at an intermediate density. Dnf1p-HA peaked at a density similar to that of Kex2p and was distributed into higher densities at which Pma1p fractionated. These results suggest that Dnf1p-HA is primarily localized to endosomal/TGN compartments and is partially localized to the plasma membrane. In contrast, Dnf2p-HA cofractionated with Pma1p, suggesting that it is localized to the plasma membrane. As shown in Figure 6A, the fractionation profiles of Dnf1p-HA and Dnf2p-HA were similar in Dnf1p-HA and Dnf2p-HA in the fpk1Δ fpk2Δ mutant. Wild-type (WT) and fpk1Δ fpk2Δ cells expressing either Dnf1p-GFP (left) or Dnf2p-GFP (right) were grown to early-mid-logarithmic phase in YPD medium at 30°C, followed by fluorescence microscopic observation. Strains used were KKT33 (DNF1-HA), KKT34 (DNF2-HA), KKT30 (fpk1Δ fpk2Δ DNF1-HA), and KKT348 (fpk1Δ fpk2Δ DNF2-HA). (B) Localization of Dnf1p-GFP and Dnf2p-GFP in the fpk1Δ fpk2Δ mutant. Wild-type (WT) and fpk1Δ fpk2Δ cells expressing either Dnf1p-GFP (left) or Dnf2p-GFP (right) were grown to early-mid-logarithmic phase in YPD medium at 30°C, followed by fluorescence microscopic observation. (D) Localization of GFP-Fpk1p. GFP-Fpk1p was expressed by transforming cells with the pKT1634 plasmid (pRS416-PFPK1). Cells were grown to early-mid-logarithmic phase in SDA-U medium at 30°C, followed by fluorescence microscopic observation. Top, KKT72 (fpk1Δ) cells expressing GFP-Fpk1p. Bottom, KKT62 (fpk1Δ) cells expressing GFP-Fpk1p were examined for colocalization of GFP-Fpk1p and FM4-64. Arrowhead indicates the localization of GFP-Fpk1p at the plasma membrane. Bar, 5 μm.

Figure 6. Localization of Dnf1p and Dnf2p is not affected by the fpk1Δ fpk2Δ mutations. (A) Sucrose gradient centrifugation analysis of Dnf1p-HA and Dnf2p-HA in fpk1Δ fpk2Δ cells. Cell lysates were prepared from wild-type (WT) and fpk1Δ fpk2Δ cells expressing either Dnf1p-HA or Dnf2p-HA and fractionated in 18–65% sucrose step density gradients as described in Materials and Methods. Equivalent volumes from each fraction were subjected to SDS-PAGE, and proteins were detected by immunoblotting. Strains used were KKT33 (DNF1-HA), KKT342 (DNF2-HA), KKT340 (fpk1Δ fpk2Δ DNF1-HA), and KKT348 (fpk1Δ fpk2Δ DNF2-HA). (B) Localization of Dnf1p-GFP and Dnf2p-GFP in the fpk1Δ fpk2Δ mutant. Wild-type (WT) and fpk1Δ fpk2Δ cells expressing either Dnf1p-GFP (left) or Dnf2p-GFP (right) were grown to early-mid-logarithmic phase in YPD medium at 30°C, followed by fluorescence microscopic observation. (D) Localization of GFP-Fpk1p. GFP-Fpk1p was expressed by transforming cells with the pKT1634 plasmid (pRS416-PFPK1). Cells were grown to early-mid-logarithmic phase in SDA-U medium at 30°C, followed by fluorescence microscopic observation. mRFP1-Fpk1p was expressed by transforming cells with the pKT1634 plasmid. Bars, 5 μm.
Localization of N-terminal GFP-tagged Fpk1p was examined by fluorescence microscopy. GFP-FPK1 was fully functional, because the Cdc50p-depleted GFP-FPK1 fpk1Δ mutant grew normally (our unpublished results). GFP-Fpk1p fluorescence appeared to be distributed throughout the cytoplasm. GFP-Fpk1p was also observed in punctate structures reminiscent of endosomal/TGN compartments in 37.7% of the observed cells (n = 154). In fact, 67.5% of these structures stained with FM4–64 after brief incubation (n = 114) and 62.5% colocalized with the TGN marker Sec7p-mRFP1 (n = 112; Franzusoff et al., 1991; Figure 6D). Furthermore, 64.9% of the mRFP1-Fpk1p punctate structures colocalized with Dnf1p-GFP dots (n = 111; Figure 6E). GFP-Fpk1p was also observed in the plasma membrane, albeit at low frequency (<10% of observed cells; Figure 6D, arrowhead). These results suggest that Fpk1p colocalizes with Dnf1p and Dnf2p at early endosomal/TGN compartments and at the plasma membrane, consistent with the notion that Fpk1p may be a regulatory protein kinase for Dnf1p and Dnf2p.

**Phosphorylation of Dnf1p and Dnf2p by GST-Fpk1p In Vitro**

We next determined whether purified Fpk1p would phosphorylate immunoprecipitated Dnf1p or Dnf2p in vitro. For this purpose, we expressed the C-terminal fragment of Fpk1p (amino acid residues 445–893) fused to the C-terminus of GST (GST-Fpk1ΔNp) in yeast. GST-Fpk1ΔNp was functional, because ΔC GST-FPK1ΔN drs2Δ mutant cells grew normally in galactose-containing medium, but not in glucose medium (our unpublished results).

GST-Fpk1ΔNp was purified from yeast cell lysates by glutathione-Sepharose affinity column to apparent homogeneity, as assessed by SDS-PAGE (our unpublished results). Because Fpk1p has been grouped into the S6 kinase family (Hunter and Phowman, 1997), we first assayed GST-Fpk1ΔNp for phosphorylation of the S6 peptide (AKRRRLLSSRA). GST-Fpk1ΔNp phosphorylated the S6 peptide in a dose- and time-dependent manner at 30°C (our unpublished results). We next carried out kinase reactions on immunoprecipitated myc-tagged Dnf1p. Phosphorylated proteins were fractionated by SDS-PAGE, followed by autoradiography. GST-Fpk1ΔNp phosphorylated Dnf1p-myc in a dose-dependent manner (our unpublished results). Immunoblot analysis showed that the immunoprecipitates also contained Lem3p, as previously described (Saito et al., 2004); however, no phosphorylation of Lem3p was observed (our unpublished results), suggesting specific phosphorylation of Dnf1p-myc.

We next determined whether GST-Fpk1ΔNp would phosphorylate Dnf2p, Dnf3p, Drs2p, and Neolp. The myc-tagged ATPases, including Dnf1p-myc, were immunoprecipitated from cells lacking FPK1 and FPK2, to eliminate possible FPK1/2-dependent endogenous phosphorylation. As shown in Figure 7A, in addition to Dnf1p, some extent of phosphorylation was observed for Dnf2p, Dnf3p, and Drs2p, but not for Neolp. The results also showed that GST-Fpk1ΔNp was autophosphorylated, reminiscent of phototropin autophosphorylation (Briggs et al., 2001). Phosphorylation of the potential noncatalytic subunits of these ATPases, Lem3p (for Dnf2p), Crf1p (for Dnf3p), and Cdc50p (for Drs2p), was not observed (our unpublished results). Because the amount of immunoprecipitated P-type ATPases varied, probably due to differences in expression level and/or protein degradation in cell lysates (Hua et al., 2002; Figure 7B), the extent of 32P incorporation into the P-type ATPases was quantified and normalized to P-type ATPase levels. The results of three
(Dnf2p, Dnf3p, and Neo1p) or six (Dnf1p and Drs2p) independent experiments are shown in Figure 7C. These results indicate that Dnf1p and Dnf2p were most efficiently phosphorylated by GST-Fpk1ΔNp, that Dnf3p and Drs2p were phosphorylated to a lesser extent, and that Neo1p was not phosphorylated.

To exclude the possibility that P-type ATPases were phosphorylated by a nonspecific kinase by which the GST-Fpk1ΔNp preparation was contaminated, we performed the kinase reaction with the kinase-negative GST-Fpk1(K525R)ΔNp. As shown in Figure 7D, Dnf1p and Drs2p were phosphorylated by GST-Fpk1ΔNp, but not by GST-Fpk1(K525R)ΔNp. A minor phosphorylation of Dnf1p observed in the absence of GST-Fpk1ΔNp might be due to a kinase that was present in Dnf1p immunoprecipitates. Autophosphorylation was still observed in GST-Fpk1(K525R)ΔNp, suggesting that the kinase activity of GST-Fpk1(K525R)ΔNp was not completely impaired, possibly due to the substitution to a chemically similar amino acid (Spitaler et al., 2000). In fact, GST-Fpk1(K525R)ΔNp retained 30–40% of the wild-type kinase activity when assayed for the S6 peptide (our unpublished results). Functional deficiency of FPK1(K525R) (Figure 1D) seems to be consistent with a notion that the inability of GST-Fpk1(K525R)ΔNp to phosphorylate Dnf1p is physiologically significant. We also wanted to exclude a possibility that GST-Fpk1ΔNp indirectly stimulated phosphorylation of the Aspartate 667, which occurs in the catalytic cycle of P-type ATPases (Bramkamp et al., 2007). The kinase reaction was performed on an immunoprecipitated ATPase-defective mutant Dnf1p667Np. Dnf1p667Np as well as Dnf1p was phosphorylated by GST-Fpk1ΔNp, but not by GST-Fpk1(K525R)ΔNp (Supplementary Figure S3).

DISCUSSION

The importance of the type 4 subfamily of P-type ATPases (flippases) in cellular function is becoming increasingly appreciated, yet is much to learn about their regulation. We have recently proposed that Cdc50p-Drs2p is regulated in these two membranes, early endosomal membranes and the plasma membrane. Are Lem3p-Dnf1p/Dnf2p differently regulated in these two membranes? Because Cdc50p-Drs2p has been implicated in clathrin function at the TGN (Chen et al., 1999) and in endocytic internalization at lower temperatures in conjunction with Dnf1p and Dnf2p (Pomorski et al., 2003).

Defects in NBD-PE and -PC uptake as well as growth sensitivity to duramycin were milder in the fpk1Δ fpk2Δ mutant than the lem3Δ mutant, suggesting that the lipid translocase activity of Lem3p-Dnf1p/Dnf2p is completely abolished in the fpk1Δ fpk2Δ mutant. In contrast, NBD-PS uptake was severely impaired in the fpk1Δ fpk2Δ mutant, whereas it was minimally affected in the lem3Δ mutant (Figure 5). Because Cdc50p-Drs2p has been implicated in PS translocation (Natarajan et al., 2004) and is recycled through the endocytic-recycling pathway (Saito et al., 2004), it might be that Cdc50p-Drs2p becomes localized to the plasma membrane in the lem3Δ mutant to compensate for the loss of PS-translocating activity. However, Cdc50p-GFP was not detected at the plasma membrane in the lem3Δ mutant (Saito et al., 2004). Thus, there seems to be an unknown phospholipid translocase that inwardly transports NBD-PS in the lem3Δ mutant, and it is an intriguing possibility that Fpk1p/Fpk2p also regulate this lipid translocase.

It should be noted that Elvington et al. (2005) showed that there are multiple transport pathways for acyl chain--labeled PC analogs; deletion of LEM3 reduced uptake of NBD-PC and Bodipy FL-PC but had no effect on uptake of Bodipy 581-PC or Bodipy 530-PC. Interestingly, the observation that the fpk1Δ fpk2Δ mutants exhibit synthetic growth defects with the lem3Δ mutant (Supplementary Figure S4) suggests another function of Fpk1p/Fpk2p in addition to the activation of Dnf1p and Dnf2p. In fact, mRF1p-Snc1p was mainly localized to the mother cell plasma membrane in the fpk1Δ fpk2Δ lem3Δ mutant, suggesting that this mutant is defective in endocytosis of Snc1p (our unpublished observations). It is an interesting question whether this defect in endocytosis of Snc1p is related to defects responsible for the loss of NBD-PS uptake in the fpk1Δ fpk2Δ mutant.

Fpk1p/Fpk2p are involved in endocytic recycling and NBD-phospholipid internalization, indicating that Fpk1p/Fpk2p regulate transbilayer phospholipid movement in two different membranes, early endosomal membranes and the plasma membrane. Are Lem3p-Dnf1p/Dnf2p differently regulated in these two membranes? Because Lem3p-Dnf1p/Dnf2p are recycled through the endocytic recycling pathway (Saito et al., 2004; Liu et al., 2007), complexes activated by Fpk1p/Fpk2p at early endosomes may be still active after they have been transported to the plasma membrane or vice versa. However, Alder-Baeren et al. (2006) reported that depletion of Dnf1p and Dnf2p did not affect NBD-phospholipid transport activity on post-Golgi secretory vesicles, suggesting that Lem3p-Dnf1p/Dnf2p on these vesicles are not active. Taken together with our results that GFP-Fpk1p...
localized to both early endosomal/TGN membranes and the plasma membrane, it seems that Fpk1p/Fpk2p independently regulate Lem3p-Dnf1p/Dnf2p on these membranes.

Upstream signals that are transmitted via Fpk1p/Fpk2p need to be elucidated in future investigations. Dnf1p and Dnf2p localized at the plasma membrane have been implicated in endocytic uptake of FM4-64 at low temperature (Pomorski et al., 2003), but other functions need to be explored. We have shown that hyperpolarized bud growth in the fpk1Δfpk2Δ mutant as well as the lem3Δ mutant is caused by defects in a switch from apical to isotropic bud growth that underlies the formation of an ellipsoidal bud shape in budding yeast (Saito et al., 2007; this study). Here, Lem3p-Dnf1p/Dnf2p–mediated transbilayer phospholipid movement at the polarized plasma membrane site seems to trigger down-regulation of Cdc42p through activation of Rga1/2p GAPs, resulting in dispersal of Cdc42p and polarity regulators from the bud tip. Because the apical-isotropic switch is activated by Clb/Cdc28p (Lew and Reed, 1995), Fpk1p/Fpk2p may transduce this signal to Lem3p-Dnf1p/Dnf2p.

An activating signal to Lem3p-Dnf1p/Dnf2p localized at early endosomes may promote endocytic recycling by stimulating vesicle budding from early endosomes. The regulatory signal to Cdc45p-Drs2p may be transmitted via Ypt31/32 and Rcy1p, which interacts with Cdc50p and Drs2p. In contrast, Lem3p-Dnf1p/Dnf2p seem to be differently regulated because neither Dnf1p nor Dnf2p interacts with Rcy1p (Furuta et al., 2007). Efficient endocytic recycling has been implicated in polarized bud growth that is restricted at S and G2 cell cycle phases (Valdez-Taubas and Pelham, 2003). Lem3p-Dnf1p/Dnf2p at early endosomes might also be regulated in a cell cycle–dependent manner.

The kinase domains of NRC-2 and phototropins exhibit sequence homology to Fpk1p, suggesting that these kinases might recognize similar target proteins. NRC-2 was identified as a mutation that constitutively caused conidiation, an asexual developmental program that accompanies changes in cell morphology in the fungus N. crassa (Kothe and Free, 1998). Phototropin-1 and -2 are blue-light receptors controlling a range of responses, including phototropism, light-induced stomatal opening, and chloroplast movements, that optimize the photosynthetic efficiency of plants (Christie, 2007). Thus, NRC-2 and phototropins are implicated in membrane-associated functions, and, interestingly, both phototropin-1 and -2 are localized to the plasma membrane (Sakamoto and Briggs, 2002). Downstream targets of NRC-2 and phototropins remain to be identified, but it is noteworthy that phosphorylation of a plasma membrane H+-ATPase (a type 2 P-type ATPase) is increased when phototropin-1 is autophosphorylated (Kinoshita et al., 2003). It is an interesting possibility that transbilayer changes in phospholipid asymmetry by type 4 P-type ATPases in internal and/or plasma membranes might be involved in downstream functions of NRC-2 and phototropins.

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