A Novel Membrane Protein, Ros3p, Is Required for Phospholipid Translocation across the Plasma Membrane in Saccharomyces cerevisiae*

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Utako Kato‡‡, Kazuo Emoto‡‡, Charlotte Fredriksson‡‡, Hidemitsu Nakamura††, Akinori Ohta††, Toshihide Kobayashi, Kimiko Murakami-Murofushi, Tetsuyuki Kobayashi, and Masato Umeda‡‡‡

From the ‡Department of Molecular Biodynamics, the Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, the §Department of Biology, Faculty of Science, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, ¶Supra-Biomolecular System Research Group, RIKEN (Institute of Physical and Chemical Research), Frontier Research System, 2-1 Hiroswa, Wako-shi, Saitama 351-0198, and the ‡Department of Biotechnology, the University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Ro09-0198 (Ro) is a tetracyclic peptide antibiotic that binds specifically to phosphatidylethanolamine (PE) and causes cytolysis. To investigate the molecular basis of transbilayer movement of PE in biological membranes, we have isolated a series of budding yeast mutants that are hypersensitive to the Ro peptide. One of the most sensitive mutants, designated ros3 (Ro-sensitive 3), showed no significant change in the cellular phospholipid composition or in the sensitivity to amphotericin B, a sterol-binding polycyclic macrolide antibiotic. These results suggest that the mutation of ros3 affects the PE organization on the plasma membrane, rather than PE synthesis or overall organization of the membrane structures. By functional complementation screening, we identified the gene ROS3 affected in the mutant, and we showed that the hypersensitive phenotype was caused by the defective expression of the ROS3 gene product, Ros3p, an evolutionarily conserved protein with two putative transmembrane domains. Disruption of the ROS3 gene resulted in a marked decrease in the internalization of fluorescence-labeled analogs of PE and phosphatidylcholine, whereas the uptake of fluorescence-labeled phosphatidylinerse and endocytotic markers was not affected. Neither expression levels nor activities of ATP-binding cassette transporters of the ros3Δ cells differed from those of wild type cells, suggesting that Ros3p is not related to the multidrug resistance activities. Immunocytochemical analyses of the structure and subcellular localization showed that Ros3p was a glycosylated membrane protein localized in both the plasma membrane and the endoplasmic reticulum, and that a part of Ros3p was associated with the detergent-insoluble glycolipid-enriched complexes. These results indicate that Ros3p is a membrane glycoprotein that plays an important role in the phospholipid translocation across the plasma membrane.

Phospholipids in most biological membranes are arranged asymmetrically between the two leaflets of the bilayer. In eukaryotic plasma membrane, aminophospholipids such as phosphatidylethanolamine (PE)1 and phosphatidylinerse (PS) reside predominantly in the inner leaflet, whereas phosphatidylethanolamine (PC) and sphingolipids are enriched in the outer leaflet (1–3). This transbilayer distribution of membrane lipids is not a static situation but is likely to be a result of the balance between the inward and outward translocation of phospholipids across the bilayer membranes (4). The rapid translocation of phospholipids between the outer and the inner leaflets of plasma membranes has been detected in various mammalian and yeast cells by fluorescence-labeled or short-chain analogs of phospholipids (5–7).

In budding yeast, it is shown that the fluorescence-labeled analogs of phospholipids, including PC, PE, and PS, are translocated across the plasma membrane and that this translocation is not significantly prevented in the end and cts yeast mutants in which intracellular vesicular transport, including endocytosis, is impaired (8–11). Because the translocation reaction requires ATP and is sensitive to sulfhydryl-modifying reagents (8, 9), the movements of fluorescence-labeled phospholipids are likely to be facilitated by proteins. Several molecules have been suggested to mediate the transbilayer movements of phospholipids in yeast cells. Some members of yeast ABC (ATP-binding cassette) transporters, such as Ste6p (12), Pdr5p, and Yor1p (13), are shown to facilitate the ATP-dependent outward movement of phospholipid analogs as well as amipathic drugs. For the inward-directed translocation, an integral membrane P-type ATPase, called Drs2p, was proposed to function as an aminophospholipid translocase, because disruption of yeast DRS2 gene resulted in a significant decrease of the internalization of fluorescence-labeled PS across the plasma membrane (14). However, several groups have failed to detect a difference between wild type and drs2Δ cells in the translocation of fluorescence-labeled phospholipids across the plasma membrane (10, 15). Thus, at present, the molecular identity of the proteins involved in the inward-directed translocation of

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** To whom correspondence should be addressed: Dept. of Molecular Biodynamics, the Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan. Tel.: 81-3-3823-2105, ext.5430; Fax: 81-3-3823-2130; E-mail: umeda@rinshoken.or.jp.

1 The abbreviations used are: PE, phosphatidylethanolamine; Ro, Ro09-0198; PC, phosphatidylcholine; PS, phosphatidylserine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; C6-NBD-PE, 1-myristoyl-2-NBD-phosphatidylethanolamine; C6-NBD-PC, 1-myristoyl-2-NBD-phosphatidylcholine; C6-NBD-aminocaproic acid; C6-NBD-PC, 1-myristoyl-2-(6-NBD-aminocaproic acid)-PC; C6-NBD-PS, 1-myristoyl-2-(6-NBD-aminocaproic acid)-PS; DOPC, dioleoylphosphatidylcholine; FM4-64, N-(3-triethylammoniumpropyl)4-(6-(4-diethyaminophenyl)hexatrienyl)pyridinium dibromide; ABC transporter, ATP-binding cassette transporter; EGFP, the red-shifted green fluorescence protein variant; LY-CH, Lucifer yellow carboxyhydrate; DIGs, detergent-insoluble glycolipid-enriched complexes; ORF, open reading frame; ER, endoplasmic reticulum.
phospholipids still remains unknown.

Ro is a 19-amino acid tetracyclic polypeptide that strictly recognizes the structure of PE and forms a tight equimolar complex with PE in biological membranes (16, 17). The Ro peptide has become a useful tool in monitoring the transbilayer movement of PE in biological membranes (18, 19) and in studying the functional role of PE in cytokinesis (20, 21) and membrane protein folding (22). Because Ro peptide specifically binds the cell surface PE and subsequently induces cytolyis (18), the peptide is also useful for isolation of mutants with defective PE synthesis as variants that are resistant to the cytolytic activity of the peptide. We have previously isolated a peptide-resistant CHO-K1 cell mutant with specific decrease in cellular PE content, and we have shown that the mutant is defective in intramitochondrial transport of phosphatidylserine (23).

In this study, we have isolated a yeast mutant, designated as ros3, that shows hypersensitivity to the Ro peptide. We found that the ros3 mutation was caused by defective expression of the ROS3 gene product, Ro3p, an evolutionarily conserved protein with two putative transmembrane domains. We have performed a detailed analysis of the structure, function, and subcellular localization of Ros3p, and we have shown that Ros3p is a unique transmembrane protein present in lipid rafts. Evidence that Ros3p plays an important role in regulating phospholipid translocation across the bilayer membranes is presented in this paper.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Culture—Saccharomyces cerevisiae** strain MY501 (MATa his3 leu2 lys2 trpl uro3) was used for the construction of strains deleted for the ROS3 gene. The media used are as follows: YPD, the master YPD plates: 1% yeast extract, 2% peptone, and 2% glucose; SD, medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, and the required amino acids; SG, SD lacking glucose but containing 2% galactose; SC, SD lacking glucose but containing 2% sorbitol; SCNaN3, SC containing 20 mM sodium azide. Unless otherwise noted, the indicated strains were grown to early to mid-log phase at 30 °C. Yeast transformations were performed by the lithium acetate method (24).

**Isolation of Ro-sensitive (ros) Mutant—** Mutagenesis with ethyl methanesulfonate was carried out as described previously (25). Mutagenized plates were plated for single colonies at 23 °C. Colonies were replicated onto YPD plates containing 10 μM Ro peptide and cultivated for 3 days at 30 °C. The negative colonies on the ro-containing plates were isolated and grown as single clones to isolate the ro allele or other small subclones. For assimilation experiments, cells were grown to stationary phase in SC media containing required amino acids and incubated for 30 min at 30 °C before addition of vesicles. After washing the cells three times with ice-cold SCNaN3 on ice, they were observed in a Zeiss Axioplan microscope equipped with 100× Planneofluar oil immersion objective (Carl Zeiss Co., Ltd., Oberkochen, Germany). Micrographs were taken with a FACScan cytomter and CellQuest software (BD PharMingen) as described previously (29).

**Vital Dyes Uptake Studies—FM4-64 and Lucifer yellow carboxyhydrazide (LY-CH) were obtained from Molecular Probes Inc. (Eugene, OR). Flow cytometric analyses of the internalization of C6-NBD-phospholipid into cells was performed with a FACScan cytomter and CellQuest software (BD PharMingen) as described previously (29).

**Production of Anti-Ros3p Antibody—** Polyclonal antibodies against Ros3p were raised in New Zealand White female rabbits against the synthetic peptides corresponding to the amino-terminal sequence MVNFDLGQVGEVFRRKDKGC, according to the method described previously (27). Antibodies were isolated from the immune sera of the rabbits by affinity chromatography on a synthetic peptide-conjugated SulfoLink column (Pierce).

**Deletion of the ROS3 Gene—** The Saccharomyces cerevisiae strain from ps3 was subcloned into a pUC119 vector. The HphI fragment between nucleotides 210 and 740 was replaced with the HIS3 gene, which was digested with BamHI from pYAC3 vector and the end blunt with T4 polymerase. The resulting plasmid was cut with AccIII and NcoI, and then the vector was digested into MHY501. The deletion of chromosomal ROS3 gene was confirmed by PCR analysis using oligonucleotides specific to C1- and 5'-ends of the open reading frame within ROS3 gene. The DNA amplification product from the ROS3-deleted strain (ros3Δ strain) was ~1.2-kb pair larger than the product obtained from the parental strain. The PCR product from the ros3Δ strain was digested by HindIII into three fragments, whereas the product from the wild type was not digested (Fig. 4A), confirming that the ROS3:HIS3 fragment was integrated into the predicted site of chromosome.

**Clone Preparation—** 1-Myristoyl-2-(6-NBD-aminocaproyl) phosphatidylethanolamine (C6-NBD-PE), 1-Myristoyl-2-(6-NBD-aminocaproyl) phosphatidylcholine (C6-NBD-PC), and dioleoylphosphatidylethanolamine (DOPC) were from Avanti Polar Lipids Inc. (Alabaster, AL). 1-Myristoyl-2-(6-NBD-aminocaproyl)phosphatidylserine (C6-NBD-PS) was synthesized from C6-NBD-PC by phospholipase D (Seikagaku Corp., Tokyo, Japan)-catalyzed transphosphatidylation (28) and purified. To prepare vesicles, lipids were mixed in desired proportions (46 mol % C6-NBD-PE, PC, or PS, 60 mol % DOPC), and chloroform was removed by evaporation followe by vacuum desiccation. The resulting lipid film was solubilized in SC medium, and the mixture was passed seven times through a LiposoFast-Base Stabilizer (Avestin, Inc., Ottawa, Canada) equipped with 0.1-μm filters to produce evenly sized vesicles. Total lipids from the stock solution was also used.

**Internalization of Fluorescence-labeled Phospholipids into Yeast Cells—** Fluorescence-labeled phospholipids internalization experiments were performed as described by Keen et al. (9). In brief, cultures in SD (a405 = 0.3–0.8) were diluted to 108 cells/ml in fresh SD medium. Cells were then incubated with vesicles containing 40% C6-NBD-phospholipids and 60% DOPC (65 μM total lipid concentration) with shaking for 30 min at 30 °C. For ATP depletion experiments, cells were collected in SCNaN3 containing required amino acids and incubated for 30 min at 30 °C before addition of vesicles. After washing the cells three times with ice-cold SCNaN3 on ice, they were observed in a Zeiss Axioplan microscope equipped with 100× Planneofluar oil immersion objective (Carl Zeiss Co., Ltd., Oberkochen, Germany). Micrographs were taken with a FACScan cytomter and CellQuest software (BD PharMingen) as described previously (29).

**Activities of ABC Transporters—** Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Firststrand cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA) enzyme. cDNA Synthesis (Invitrogen) was used to prepare cDNA template (1 μg cDNA) with the supplied oligo(dT) 12–18 primer. 25 ng of cDNA was used for each subsequent PCR. For drug resistance assay, cycloheximide, 4-nitroquinoline-N-oxide, miconazole, and ketoconazole were from Sigma. 10 mg/ml stock solutions were made by dissolving the drugs in dimethyl sulfoxide or ethanol. In the case of limited medium, drug sensitivity was determined as described above.
Ros3p, a Regulator of Phospholipid Transbilayer Movement

Protein Extraction and Endoglycosidase H Treatment—Harvested cells were washed in phosphate-buffered saline and resuspended in SDS buffer containing 1/20th volume of 2-mercaptoethanol, and glass beads were added. Disruption of cells was carried out with vortex mixer 7 times for 30 s, and solutions were kept on ice. Lysates were boiled for 5 min, centrifuged to clear the cell debris, and analyzed in 10% SDS-PAGE and Western blotting analysis. Protein concentration was determined by BCA protein assay reagent (Pierce). For endoglycosidase H treatment, the total cell lysates were added to and equal volume of 0.15 M citrate/phosphate buffer (pH 7.5) and 5 mM EDTA (Seikagaku Corp.). The reaction mixtures were incubated for 20 h at 37 °C.

Construction of EGFP-tagged ROS3—To create a fusion protein of Ros3p with EGFP, EGFP was digested with BamHI and NotI from pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA). The 3′-uncoding region of the ROS3 gene was amplified from pS3 plasmid by PCR using an upper primer added NotI site (5′-TAATCTGATGGGC-GCAGAAAAAGGTGATGTTTTTC-TATG-3′) and a lower primer T3 (5′-AATTAACCTGAGTAAAGG-3′). The NotI/PstI-digested 3′-uncoding region was ligated with BamHI/NotI-digested EGFP fragment and subcloned to pRS416 vector (EGFP-3′-pRS416). The 5′ promoter region of ROS3 and the coding region, lacking stop codon (5′-RO3S), was amplified from pS3 plasmid using an upper primer T7 (5′-GTATACGACTCAGTTAGG-3′) and a lower primer added BamHI site (5′-GCGTATGGATGCTTTATCGATTAGCACAAGTTG-3′). The NotI/BamHI-digested 5′-RO3S fragment was inserted into the NotI/BamHI site of the EGFP-3′-pRS416 plasmid. The resulting plasmid (5′-RO3S-EGFP-3′ in pRS416) contained EGFP-fused Ros3p at its carboxy terminus under the control of the ROS3 promoter, and it was transformed into the ros3a strain. For microscopy, Ros3p-EGFP strain was cultured to early log phase at 20 or 30 °C and observed with Zeiss confocal laser scanning microscope using a 488-nm laser line. Images were processed with LSM510 version 3.2 (Carl Zeiss).

Subcellular Fractionation—The plasma membrane was isolated as described (32). The isolation of detergent-insoluble glycolipid-enriched complexes (DIGs) was performed according to the method described by Bagnat et al. (33) with some modifications. In brief, mid-log phase cultures (equal to ~1 × 10^8 cells) were disrupted with glass beads. The lysates were mixed with an equal volume of TNE buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA) containing 2% Triton X-100 and incubated at 4 °C for 7.5 h at 4 °C in a Beckman SW55 Ti rotor. Nine fractions of equal volume were collected from the SW55Ti rotor. Nine fractions of equal volume were collected from the top, precipitated by adding trichloroacetic acid, and resuspended in SDS buffer containing 1/20th volume of 2-mercaptoethanol, and glass beads were added. Disruption of cells was carried out with vortex mixer 7 times for 30 s, and solutions were kept on ice. Lysates were boiled for 5 min, centrifuged to clear the cell debris, and analyzed in 10% SDS-PAGE and Western blotting analysis. Protein concentration was determined by BCA protein assay reagent (Pierce). For endoglycosidase H treatment, the total cell lysates were added to and equal volume of 0.15 M citrate/phosphate buffer (pH 5.0) and 0.1 unit/ml endoglycosidase (Seikagaku Corp.). The reaction mixtures were incubated for 20 h at 37 °C.

Isolation of the ros3 Mutant in a Screen for Mutants Hyper-sensitive to the Ro09-0198—In the course of searching for mutants that were hypersensitive to the peptide—Ro09-0198 is a tetracyclic peptide that binds specifically to PE in biological membranes and subse-

RESULTS

Isolation of the ros3 Mutant in a Screen for Mutants Hyper-sensitive to Ro09-0198—Ro09-0198 is a tetracyclic peptide that binds specifically to PE in biological membranes and subsequently induces cytolyis (18, 23). To isolate mutants with an altered PE organization on the plasma membrane, we selected a series of S. cerevisiae mutants that were hypersensitive to the peptide-induced cytolyis. We screened ~55,000 mutagenized colonies and identified 17 ros (Ro sensitive) mutants. One of the mutants, designated ros3, was most sensitive to the peptide-induced cytolyis, and the L50 of the peptide for the ros3 mutant was approximately one-tenth compared with wild type cells (Fig. 1A). There was no significant difference between the mutant and wild type cells in their cellular phospholipid compositions (Table I) or in the sensitivities to various agents such as amphotericin B, a polyene macrolide antibiotic that binds to membrane ergosterol and induces cellular leakage (34) (Fig. 1B), detergents (SDS and Triton X-100), and ethanol (data not shown). These results suggest that the hypersensitivity of the ros3 mutant to the peptide resulted from an altered organization of PE on the plasma membrane and not from an increased cellular PE content nor from cell wall leakage caused by impaired cell wall integrity.

Cloning of the ROS3 Gene—The gene affected in the ros3 mutant was cloned by complementation of the peptide hypersensitive phenotype after transformation with a genomic library (35). The DNA fragment that complemented the ros3 mutant contains four ORFs (Fig. 2A). Subcloning experiments indicated that the short fragment derived from KpnI digestion was able to complement, but the other long fragment was not (Fig. 2A). These results demonstrate that YNL323W (GenBank accession number, ZT1599), which is also named as LEM3 (36) and BRE3 (37), is the gene that complements the ros3 mutant. In this study, we refer to the gene as ROS3. ROS3 encodes a protein of 414 amino acids that contains two putative transmembrane domains (Fig. 2B). Data base search revealed two genes with high similarity to ROS3, CDC50 (GenBank accession number, X59720, 40% identity in deduced amino acid sequence) and YNR048W (GenBank accession number, ZT1663, 38% identity) in yeast genome (Fig. 2C). In addition, the ROS3 genes are conserved in various organisms including worm (GenBank accession number, U61953, 30% identity in deduced amino acid sequence), fly (GenBank accession number, AE003502, 33% identity), and mammals (GenBank accession number, AA238841, 27% identity), suggesting that ROS3 homologs constitute a large gene family that has been
well conserved during evolution.

To examine further whether the 
ros3 mutant shows impaired
expression of the 
ROS3 gene product, we produced a polyclonal
antibody against a synthetic peptide corresponding to the ami-
no-terminal amino acids 1–19 of Ros3p. In immunoblotting, the
affinity-purified anti-Ros3p antibody bound specifically to a
62-kDa protein in the wild type cells, whereas no protein band
was detected in the 
ros3 mutant (Fig. 3A). The observed rela-
tive molecular mass was larger than that predicted by the
primary structure (47.4-kDa), but the treatment of cell lysate
with endoglycosidase H resulted in the reduction of molecular
mass to 47.5-kDa which is fully compatible with the predicted
mass (Fig. 3B). Because the 62-kDa protein was not present in
the lysate of the 
ROS3-deleted strain (see below) (Fig. 3A),
these results clearly demonstrate that the 62-kDa protein is
Ros3p and that the mutation of 
ros3 is caused by the defective
expression of Ros3p. Ros3p was not extracted with 1 M NaCl, 2
M urea, and 0.1 M NaCO3 (pH 11.0) but was partially extracted
with 1% (w/v) Triton X-100 (Fig. 3C). These results indicate
that Ros3p is a glycosylated transmembrane protein, which is
consistent with the predicted structure of the protein that has
two putative membrane-spanning domains and six possible
glycosylation sites (Fig. 2B).

Ros3p Is Essential for Internalization of C6-NBD-PE and C6-NBD-PC via a Non-Endocytic Pathway—To examine the
 cellular function of Ros3p, we disrupted the
ROS3 gene in 
S. cerevisiae by the replacement of a 531-bp internal
HpaI fragment with 1,768-kb fragment encoding the yeast
HIS3 gene as shown in Fig. 4A. The deletion of chromosomal
ROS3 gene was confirmed by PCR analysis using oligonucleotides
specific to the 5/-H11032- and 3/-H11032-ends of the open reading frame
within
ROS3. The
ROS3-deleted strain (ros3/H9004 strain) could not grow
on the YPD plate containing 10 M Ro peptide, and the
ROS3 gene on a single copy plasmid suppressed this sensitivity (Fig.
4B). The
ros3/H9004 strain also showed similar phospholipid compo-
sition (Table I) and amphotericin B sensitivity with those ob-
served with the 
ros3 mutant (data not shown).

To understand the role of Ros3p in PE organization in the
plasma membrane, we examined the transport and localization of
C6-NBD-PE, a fluorescence-labeled analog of PE molecule, in
the
ros3 strain. It was shown previously that yeast cells
incubated with lipid vesicles containing C6-NBD-PE internal-
Ros3p, a Regulator of Phospholipid Transbilayer Movement

Ros3p is responsible for the ATP-dependent uptake of the fluorescence-labeled phospholipids. The uptake and intracellular distribution of the endocytic markers, Lucifer yellow (31) and FM4-64 (30), was not affected in the ros3Δ strain (Fig. 5A, panels d and f), indicating that the ros3Δ strain was not defective in endocytosis of soluble and amphiphatic molecules. Because the expression of the ROS3 gene by using centromeric plasmid restored the uptake of both C6-NBD-PE and C6-NBD-PC of the ros3Δ strain (Fig. 5B), these results suggest that Ros3p is required for the translocation of both C6-NBD-PE and C6-NBD-PC across the plasma membrane through the non-endocytic pathway.

Ros3p Is Unrelated to Multidrug Resistance Activity—Overexpression of the yeast ABC transporters gives the yeast cells a multidrug resistance phenotype, which results from the accelerated efflux of various amphiphatic drugs (38, 39). It was shown recently (9, 13) that some of the yeast ABC transporters, such as Pdr5p and Yor1p, exhibit outward-directed phospholipid translocase activity. To examine whether the reduced uptake of the fluorescence-labeled phospholipids in the ros3Δ strain resulted from the enhanced outward-directed translocation of the phospholipid analogs through ABC transporters, we investigated the mRNA levels of yeast ABC transporters as well as the sensitivity of the ros3Δ strain against various cytotoxic compounds that are shown to be substrates of the yeast ABC transporters (40, 41). Reverse transcriptase-PCR analyses showed no significant difference in the mRNA levels of both Pdr5p and Yor1p between the ros3Δ strain and wild type cells (Fig. 6A). In addition, there was no significant difference between the ros3Δ strain and wild type cells in the sensitivities against various amphiphatic cytotoxic drugs, such as miconazole, ketoconazole, 4-nitroquinoline-N-oxide, and cycloheximide (Fig. 6B–E). These results suggest that the uptake deficiency of the fluorescence-labeled phospholipids in the ros3Δ strain is not caused by the enhanced outward movement of the lipids mediated by the multidrug resistance activity of the transporters.

Subcellular Localization of Ros3p—To assess the subcellular localization of Ros3p, Ros3p tagged at the COOH terminus with EGFP was introduced into the ros3Δ strain. Chimeric protein Ros3p-EGFP complemented the Ro peptide sensitivity phenotype of the ros3Δ strain on the centromeric plasmid (data not shown), indicating that Ros3p-EGFP is fully functional. Logarithmically growing cells expressing Ros3p-EGFP by its own promoter showed the fluorescence staining depicted in Fig. 7A, panel a. Ros3p-EGFP was uniformly localized in the nuclear periphery and in discrete patches associated with the cytoplasmic membrane. This pattern coincides with the typical ER markers such as Sec63p (42). In addition to ER, a part of Ros3p-EGFP appeared to be localized at the plasma membrane (Fig. 7A, panel b). To examine further Ros3p localization, a fraction enriched in plasma membranes was prepared by sucrose density gradient fractionation. Ros3p was concentrated in the plasma membrane fraction, which was enriched for the plasma membrane marker protein Pma1p. Other organelle marker proteins including Whi5p (ER), Emp47p (Golgi), and Por1p (mitochondria) distributed differentially in the sucrose gradient (Fig. 7B). The results indicate that Ros3p is localized in both the plasma membrane and the ER membrane.

Bagnat et al. (33) recently isolated the detergent-insoluble glycolipid-enriched complexes (DIGs) from yeast cells and showed that DIGs were composed of phosphoinositide-based sphingolipids, ergosterol, and glycosylphosphatidylinositol-anchored proteins, suggesting that this yeast DIGs is identical to the lipid rafts of mammalian cells. Because Ros3p was partially resistant to Triton X-100 extraction (Fig. 3C), we examined

**Fig. 3. Defective expression of Ros3p in the ros3 mutant and biochemical characterization of Ros3p.** A, total cellular proteins from wild type (WT) cells, the ros3 mutant, and ROS3-deleted cells (ros3Δ strain; see "Experimental Procedures") were subjected to SDSPAGE and analyzed by immunoblotting with affinity-purified rabbit anti-Ros3p polyclonal antibody. B, the cellular proteins were treated with endoglycosidase H (Endo H) at 37 °C for 20 h and analyzed by immunoblotting using the anti-Ros3p antibody. C, the total cellular homogenates were treated with various chemical reagents as indicated at top and centrifuged at 100,000 × g for 30 min. Soluble (S) and particulate (P) fractions were derived from the same amount of the homogenates. TX-100, Triton X-100. Ros3p was detected by immunoblotting.

**Fig. 4. Sensitivity of the ros3Δ strain to the Ro peptide.** A, the disruption of ROS3 with HIS3 gene. The protein-coding regions are shown by open arrows. A, AccIH site; Hp, His1 site; N, Neo1 site; B, BanHI site; H, HindIII site. B, single colonies of the wild type (WT), the ros3 mutant, ros3Δ strain, and ros3Δ strain transformed with a single copy of the ROS3 gene (ros3Δ + ROS3) were transferred to YPD plates with or without 10 μM Ro peptide and cultured at 30 for ~2 days.
whether Ros3p was localized in the yeast lipid rafts using the Opti-Prep density gradient (33, 44). As shown in Fig. 7a, a significant portion of Ros3p was present in the low density DIGs fraction (fraction numbers 2 and 3) that was enriched with Gas1p and Pma1p, the major components of the yeast lipid rafts (33, 44), whereas the non-DIG-associated protein Wbp1p was absent from

**FIG. 5. Internalization of fluorescence-labeled analogs of phospholipids.** A, internalization of C<sub>6</sub>-NBD-PE and endocytic markers was examined as described under “Experimental Procedures.” The cells were incubated with C<sub>6</sub>-NBD-PE (panels a and b), FM4-64 (panels c and d), or Lucifer yellow (LY-CH) (panels e and f) at 30 °C. Left panels a, c, and e, wild type (WT) cell; right panels b, d, and f, ros3<sup>A</sup> strain. B, cells were incubated with C<sub>6</sub>-NBD-PE, -PC, or -PS, and the intensity of fluorescence-labeled phospholipids within the cells was analyzed with a FACScan cytometer. Top, wild type cell; middle, ros3<sup>A</sup> strain; bottom, ros3<sup>A</sup> strain transformed with a single copy plasmid containing ROS3 gene.

**FIG. 6. Expression of ABC transporters and multidrug resistance activities in the ros3<sup>A</sup> strain.** A, cells were grown in SD medium. mRNA levels of ROS3, PDR5, and YOR1 in wild type cells (WT, left) and the ros3<sup>A</sup> strain (right) were examined by reverse transcriptase-PCR analyses as described under “Experimental Procedures.” B–E, cells grown in YPD medium were diluted to 1 × 10<sup>6</sup> cells/ml in YPD medium containing cycloheximide (B), 4-nitroquinoline-N-oxide (C), miconazole (D), or ketoconazole (E) at indicated concentrations and incubated for 24 h. Viabilities of the cells were determined by measuring A<sub>600</sub> values.

**FIG. 7. Subcellular localization of Ros3p.** A, the ros3<sup>A</sup> strain expressing Ros3p-EGFP was grown to early log phase at 20 °C, and observed by confocal fluorescence microscopy. Arrow (panel a) and arrowhead (panel b) indicate the ER and the plasma membrane staining, respectively. B, the plasma membrane fraction was purified as described under “Experimental Procedures.” The total cellular lysate (L) and plasma membrane fraction (PM) were subjected to SDS-PAGE, followed by immunoblotting analyses with anti-Ros3p, anti-Pma1p (plasma membrane marker), anti-Wbp1 (ER marker), anti-Emp47p (Golgi marker), and anti-Por1p (mitochondria marker). C, detergent-insoluble glycolipid-enriched complexes (DIGs) were isolated as described under “Experimental Procedures.” The presence of Ros3p and marker proteins Gas1p and Pma1p, for the major DIG-associated proteins, and Wbp1p for non-DIG-associated protein in each fraction collected from the top of the Opti-Prep density gradient was examined by immunoblotting. These results suggest that a part of Ros3p is associated with the yeast lipid rafts and raise the possibility that Ros3p is involved in phospholipid translocation across the bilayer membranes in the plasma membrane and the ER.
DISCUSSION

In the present study, we have screened yeast mutants that showed hypersensitivity to the PE-binding antibiotic peptide, Ro09-0198. We have isolated 17 clones of mutants and present our initial observation of a mutant, named ros3, which showed the most significant increase in the sensitivity to the peptide. The ros3 mutant did not show any significant change in cellular phospholipid composition or sensitivity to various agents such as amphoterin B, a sterol-binding polypeptide, and detergents. These data suggest that the ros3 mutation affects the PE organization in the plasma membrane, rather than PE synthesis or overall organization of the membrane structures. We first assumed that the hypersensitivity of the ros3 mutant to the peptide resulted from cell surface exposure of PE, which is normally localized in the inner leaflet of the plasma membrane. Hence, we determined the bilayer distribution of PE by using the membrane-impermeable reagent, 2,4,6-trinitrobenzenesulfonic acid, that chemically modifies the polar head group of PE (45). We could, however, not detect more than a slight increase in the 2,4,6-trinitrobenzenesulfonic acid-reactive PE in the ros3 mutant compared with that observed with wild type cells (data not shown). This could not account for the 10 times increase in sensitivity to the peptide. The results suggested that the asymmetric distribution of PE in the plasma membrane was not totally disrupted in the ros3 mutant. Instead a disturbance in either the dynamic movement or surface distribution of PE in the plasma membrane of the ros3 mutated cells could cause the Ro09-0198 hypersensitivity. These observations have prompted us to undertake a detailed analysis of the ros3 mutant. We identified the gene defective in the ros3 mutant and studied the role of Ros3p in the regulation of dynamic movement of PE in the plasma membrane.

Role of Ros3p in Transbilayer Movements of Phospholipids—The gene ROS3 was determined as a defective gene in the ros3 mutant by the functional complementation screening of the genomic library. The ROS3 gene product, Ros3p, was not expressed in the ros3 mutant, and disruption of the ROS3 gene caused the same hypersensitive phenotype as observed in the ros3 mutant. These data further support that the mutation of ros3 was caused by a defective expression of the ROS3 gene product, Ros3p. We then demonstrated that Ros3p is an essential component in the phospholipid translocation at the plasma membrane. The following lines of evidence support our conclusion. First, disruption of the ROS3 structural gene resulted in a marked decrease in the uptake of the fluorescence-labeled PE analog, C6-NBD-PE (Fig. 5). Second, the uptake of both soluble and lipophilic endocytic markers, Lucifer yellow and FM4-64, was not affected in the ros3 mutant (Fig. 5). This indicates that Ros3p is not involved in the lipid internalization via the conventional endocytic pathway. Finally, a significant portion of Ros3p is localized in the plasma membrane (Fig. 7). It has been shown that ATP depletion treatment at low temperature impairs the C6-NBD-phospholipid internalization from the outer leaflet of the yeast plasma membrane but not insertion into the outer leaflet after transport from the donor vesicles (9, 11). In the present study, we also confirmed that the C6-NBD-PE internalization was ATP-dependent. As the disruption of the ROS3 gene greatly reduced the uptake of C6-NBD-PE, it is likely that Ros3p is responsible for the ATP-dependent internalization of the lipid analogs from the outer leaflet of plasma membrane, rather than the ATP-independent insertion of lipid analogs into the outer leaflet of plasma membrane. Taken together, Ros3p is likely to play a role in the phospholipid translocation across the plasma membrane, probably by the transbilayer transport.

Disruption of the ROS3 gene also caused total inhibition of the uptake of C6-NBD-PC but showed no significant effect on the uptake of C6-NBD-PS, suggesting that Ros3p is involved in the transmembrane movement of PE and PC but not PS. Based on the following discussions, we conclude that Ros3p is a novel type of phospholipid transporter or a regulator of phospholipid translocation, which is distinct from ABC transporters and P-type ATPase.

Two protein families have been implicated in phospholipid transport across the yeast plasma membrane so far. The S. cerevisiae genome encodes 15 full-size putative ABC transporters, of which Pdr5p, Yor1p, and Ste6p are suggested to exhibit outward-directed phospholipid translocate activity as well as multidrug resistance activity (12, 13). On the other hand, a novel P-type ATPase Drs2p is suggested to mediate the inward-directed translocation of C6-NBD-PS in yeast (14). Ros3p exhibits no significant homology with ABC transporters and P-type ATPase (Fig. 2). Neither the expression levels nor the cellular activities of ABC transporters were changed in the ros3Δ strain (Fig. 6), suggesting that Ros3p is unrelated to ABC transporters. Furthermore, C6-NBD-PS internalization was not affected by the loss of Ros3p function (Fig. 5), and Ros3p function is thereby not related to Drs2p.

The transbilayer movements of the aminophospholipids such as PE and PS across the plasma membrane is well established and appears to be a common feature in eukaryotic cells. In contrast, an inward-directed translocation of PC at the plasma membrane has only been observed in few cell types, such as SV40-transformed WI-38 (46) and MDCK-II (47). Transbilayer transport of C6-NBD-PC across the yeast plasma membrane was also demonstrated previously (8–11). The translocation of C6-NBD-PC, as well as C6-NBD-PE and C6-NBD-PS, in the yeast plasma membrane requires ATP and is sensitive to sulfhydryl-modifying reagents. Ros3p is likely to mediate translocation of both C6-NBD-PE and C6-NBD-PC, but not C6-NBD-PS (Fig. 5), strongly suggesting that in budding yeast C6-NBD-PE and C6-NBD-PC are internalized by the same mechanism in which Ros3p is involved and that C6-NBD-PS internalization is mediated by another transporter, such as Drs2p.

Intracellular Localization of Ros3p and Its Homolog—Intracellular localization of EGFP-tagged Ros3p suggests that Ros3p is localized in the ER membrane as well as in the plasma membrane (Fig. 7). There are several reports of protein-mediated phospholipid transport across yeast microsomal membrane (48) and mammalian ER membrane (49–52). These translocase activities are less specific for head group structure and do not require energy. Although the molecular nature of ER phospholipid translocate has not been well defined, it is possible that Ros3p may contribute to the transilayer movements of phospholipids in the ER membrane. Subcellular fractionation studies indicate that a part of Ros3p is associated with the yeast lipid rafts (Fig. 7). Because the yeast lipid rafts are involved in protein sorting from ER to plasma membrane (33, 43), Ros3p may be transported from ER to cell surface through the lipid rafts on demand, though most of Ros3p is retained in the ER membrane during its biosynthesis. Alternatively, Ros3p may be involved in phospholipid translocation in the yeast lipid rafts.

Recent genetic analyses of yeast mutants suggest that the ROS3 gene is involved in the glucocorticoid signal transduction pathway (36) and in the brefeldin A sensitivity (37). Although no obvious explanation for the correlations between these functions of Ros3p has been obtained, these studies imply that the mutation of the ROS3 gene affects diverse cellular functions. The ROS3 gene shows a significant homology with two other yeast genes, CDC50 and YNR048W (Fig. 2). Although it has
been reported (53) that the CDC50 mutation gives a cold-sensitive phenotype, the functions of these Ros3p homologs are unknown. Despite such remarkable similarities (~60% similarity in amino acid level), deletion of either CDC50 or YNR048W had little effect on the internalization of the C₆-NBD phospholipids from the plasma membrane (data not shown). It was shown recently that Cde50p appears to be predominantly localized in late endosome membranes rather than in the ER or the plasma membrane, and the cdc50 mutant shows defects in the uptake of C₆-NBD-PS, -PC, and -PE at low temperature.² Thus, it is possible that the members of Ros3p protein family have distinct characteristics of cellular localization and ability to regulate phospholipid transport in various intracellular organelle membranes.

In conclusion, we have identified a novel membrane protein Ros3p involved in the phospholipid translocation on the yeast plasma membrane. In addition, we have shown that Ros3p is localized in multiple organelles including the plasma membrane and the ER. Further analyses of Ros3p and the ros3Δ strain will help us understand the regulation of the phospholipid translocation across the bilayer membranes as well as its role in the regulation of various cellular functions.

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