Phosphatidylethanolamine Is Required for Normal Cell Morphology and Cytokinesis in the Fission Yeast *Schizosaccharomyces pombe*

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Received 21 January 2009/Accepted 6 March 2009

To investigate the contributions of phosphatidylethanolamine to the growth and morphogenesis of the fission yeast *Schizosaccharomyces pombe*, we have characterized three predicted genes in this organism, designated *psd1*, *psd2*, and *psd3*, encoding phosphatidyletherine decarboxylases, which catalyze the conversion of phosphatidyletherine to phosphatidylethanolamine in both eukaryotic and prokaryotic organisms. *S. pombe* mutants carrying deletions in any one or two *psd* genes are viable in complex rich medium and synthetic defined minimal medium. However, mutants carrying deletions in all three *psd* genes (*psd1-3Δ*) grow slowly in rich medium and are inviable in minimal medium, indicating that the *psd1* to *psd3* gene products share overlapping essential cellular functions. Supplementation of growth media with ethanolamine, which can be converted to phosphatidylethanolamine by the Kennedy pathway, restores growth to *psd1-3Δ* cells in minimal medium, indicating that phosphatidylethanolamine is essential for *S. pombe* cell growth. *psd1-3Δ* cells produce lower levels of phosphatidylethanolamine than wild-type cells, even in medium supplemented with ethanolamine, indicating that the Kennedy pathway can only partially compensate for the loss of phosphatidyletherine decarboxylase activity in *S. pombe*. *psd1-3Δ* cells appear morphologically indistinguishable from wild-type *S. pombe* cells in medium supplemented with ethanolamine, but when cultured in nonsupplemented medium, they produce high frequencies of abnormally shaped cells as well as cells exhibiting severe septation defects, including multiple, mispositioned, deformed, and misoriented septa. Our results demonstrate that phosphatidylethanolamine is essential for cell growth and for normal cytokinesis and cellular morphogenesis in *S. pombe*, and they illustrate the usefulness of this model eukaryote for investigating potentially conserved biological and molecular functions of phosphatidylethanolamine.

Phosphatidylethanolamine (PE) is a major phospholipid component of cell membranes in both prokaryotic and eukaryotic organisms (34, 35). There are three distinct pathways for PE synthesis in eukaryotic cells: (i) decarboxylation of phosphatidyletherine (PS) via reactions catalyzed by PS decarboxylase (PSD) enzymes; (ii) the CDP-ethanolamine branch of the Kennedy pathway, which converts ethanolamine to PE (34); and (iii) acylation of lysophosphatidylethanolamine (21, 29), a second PSD enzyme in *S. pombe* catalyzed by the enzyme Ale1 (22). Genetic studies have demonstrated that PE is essential for cell viability in *S. cerevisiae*, although the minimal threshold of PE required for cell growth in this organism can apparently be provided by any of the routes of PE synthesis listed above (22). In contrast, the results of mouse knockout experiments indicate that both PSD- and Kennedy pathway-catalyzed pathways for PE synthesis are essential for embryonic development (9, 28, 35).

While PE is present in most, if not all, eukaryotic cell membranes, it is particularly enriched in the membranes of mitochondria (32, 35, 37). Indeed, *S. cerevisiae* mutants carrying a null mutation in the *PSD1* gene, which encodes a mitochondrial localized PSD, exhibit phenotypes indicative of mitochondrial dysfunction, as do cells derived from mouse embryos carrying a disruption of the *Psid* gene, which encodes a protein highly homologous in structure to *S. cerevisiae* Psd1 (28, 32). A second PSD enzyme in *S. cerevisiae*, encoded by the *PSD2* gene, is localized to Golgi and vacuolar membranes (33, 37). Consistent with a role in vacuolar function, PE has been implicated in the process of autophagy by genetic studies utilizing *S. cerevisiae* vacuolar targeting mutants and by studies showing that Atg8, a ubiquitin-like protein required for yeast autophagy, is conjugated to PE, as are several related mammalian proteins (19, 20, 27).

Interestingly, studies utilizing a streptavidin-conjugated form of the PE-binding peptide cinnamycin demonstrated that PE is enriched at cell division sites in *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe*, and mammalian cells (6, 11). Moreover, streptavidin-conjugated cinnamycin was shown to inhibit the disassembly of the contractile ring and the completion of cytokinesis in cultured Chinese hamster ovary cells, and a PE-deficient cell line from the same species was found to arrest growth in cytokinesis with an intact contractile ring (7). PE has also been shown to be enriched at the growing ends of interphase *S. pombe* cells and at the emerging bud cortex in dividing cells of *S. cerevisiae*, findings that implicate PE in processes controlling polarized cell growth (11).

Although *S. pombe* mutants defective in enzymes that directly catalyze PE synthesis have not been described previously, we recently showed that mutants carrying a null mutation in the PS synthase gene *pps1* are ethanolamine auxotrophs that exhibit severe morphology- and cytokinesis-defective phe-
notypes under ethanolamine-limited growth conditions (17). These findings implicated PE in the regulation of cellular morphogenesis and cytokinesis in S. pombe. To investigate the biological functions of PE in S. pombe, in particular its contributions to the control of cell morphology and cytokinesis, we have in the present study generated and characterized mutants carrying null mutations in three open reading frames predicted to encode PSD enzymes in this organism. In this paper, we describe the phenotypes of S. pombe PSD-null mutants, which demonstrate central roles for PE in the regulation of cell morphology and cytokinesis in this model eukaryote.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The S. pombe strains used in this study are listed in Table 1. The recombinant PCR gene knockout method described by Krawchuk and Wahl (14) was used to generate the psd1::kanMX6, psd2::hphMX6, and psd3::ura4 deletion cassettes, in which the psd1, psd2, and psd3 open reading frames are replaced by the kanMX6 (kanamycin) and G418 resistance), hphMX6 (hygromycin resistance), and ura4 markers, respectively. PCRs used plasmid pAtu-kanMX5 (2), pAtu-hphMX6, or pAtu-ura4 as the template DNA together with the oligonucleotide primer sets psd1-F1 (5’-GGCA GTATGCTTACGCAAG-3’)–psd1-R1 (5’-GGGAGGCTGCTAGCAGTGCCAGTAGCTTACGCTGTTTTATTTTATTTAAGCAG-3’)-psd1-R2 (5’-GTTTAAACGAGCTCGAATTCATCGATCTTG-3’)–psd1-R2, psd2-F1 (5’-GTAGGAAGAGAGAAATACACG TAGA-3’)–psd2-R1 (5’-GGGAGGCTGCTAGCAGTGCCAGTAGCTTACGCTGTTTTATTTTATTTAAGCAG-3’)–psd2-R2 (5’-GTTTAAACGAGCTCGAATTCATCGATCTTG-3’)–psd2-R2, psd3-F1 (5’-GTAGGAAGAGAGAAATACACG TAGA-3’)–psd3-R1 (5’-GGGAGGCTGCTAGCAGTAGCTTACGCTGTTTTATTTTATTTAAGCAG-3’)–psd3-R2 (5’-GTTTAAACGAGCTCGAATTCATCGATCTTG-3’)–psd3-R2, and psd1-F1 (5’-GTTTAAACGAGCTCGAATTCATCGATCTTG-3’)–psd2-F1 (5’-GTAGGAAGAGAGAAATACACG TAGA-3’)–psd2-R1 (5’-GGGAGGCTGCTAGCAGTAGCTTACGCTGTTTTATTTTATTTAAGCAG-3’)–psd3-F1 (5’-GTAGGAAGAGAGAAATACACG TAGA-3’)–psd3-R2 (5’-GGGAGGCTGCTAGCAGTAGCTTACGCTGTTTTATTTTATTTAAGCAG-3’)-psd3-R2 (5’-CATCATTCAATCTTGTGGAGAATTATAC-3’). The S. pombe haploid strain Sp870 was transformed with the resulting psd1::kanMX6, psd2::hphMX6, or psd3::ura4 cassette, and transformants were isolated on selective Edinburgh minimal medium (EMM) (for psd3::ura4 transformants) or on YEAU (0.5% yeast extract, 3% dextrose, 225 mg/liter adenine, 225 mg/liter uracil) containing either G418 (for psd1::kanMX6 transformants) or hygromycin (for psd2::hphMX6 transformants). Transformants carrying psdΔ gene deletions were identified by colony PCR (17). S. pombe psdΔ double and triple deletion mutants were constructed by genetic crosses using standard yeast genetic methods (1, 24). S. pombe cultures were grown in either YEAU or synthetic minimal medium (EMM) with appropriate auxotrophic supplements (1). Where indicated, media were supplemented with 1 mM ethanolamine.

Radiolabeling and phospholipid analysis. For phospholipid composition analysis of S. pombe strains cultured in rich medium, cells were cultured in YEAU containing 20 μCi of 32P/ml at 30°C with shaking to a density of 6 × 108 cells/ml and were harvested by centrifugation for subsequent lipid extraction (see below). Where indicated, YEAU was supplemented with 1 mM ethanolamine. For analysis of strains cultured in minimal medium, cells were grown in YEAU containing 1 mM ethanolamine to mid-log phase, washed three times with EMM containing 1 mM ethanolamine, resuspended at a density of 7.5 × 107 cells/ml in EMM containing 1 mM ethanolamine and 20 μCi/ml 32P, and cultured to a density of 6 × 109 cells/ml. A portion of each culture was harvested by centrifugation for lipid extraction, and the remainder was washed with EMM containing 20 μCi/ml 32P, resuspended at 3 × 106 cells/ml in EMM containing 20 μCi/ml 32P, and incubated for 8 h. For lipid extraction, 1 ml of each labeled cell culture was harvested by centrifugation. The cells were washed with 1 ml sterile water, resuspended in 1 ml 5% trichloroacetic acid, and incubated on ice for 15 min. Cells were washed twice with 1 ml of sterile water and resuspended in 1 ml of extraction solvent I (43.7% water, 39.5% ethanol, 13.9% diethyl ether, 2.8% pyridine, and 0.1% ammonium hydroxide) for 1 h at 60°C with occasional swirling. Samples were centrifuged at 1,800 × g for 3 min, and 1 ml of the resulting supernatant volume was added to 0.25 ml of extraction solvent II (water–chloroform–methanol, 9:60:30). After centrifugation at 200 × g for 2 min, the lower chloroform phase was transferred to the new tube and dried down under nitrogen. The extracted lipids were resuspended in chloroform-methanol (2:1), counted by liquid scintillation, and spotted onto Partisil 25 (60-Å silica gel) thin-layer chromatography plates (Whatman), which were then developed in chloroform–ethanol–water–triethylamine (30:35:7:35). Phospholipids were detected by autoradiography and were quantified using the Quantity One program (Bio-Rad).

Fluorescence staining and microscopy of S. pombe cells. For visualization of S. pombe cell walls by fluorescence microscopy, 1 ml of mid-log-phase culture was pelleted by centrifugation, and the majority of the culture supernatant was removed, leaving a volume of approximately 10 μl. Two microliters of the concentrated cell suspension was mixed on a microscope slide with 2 μl of a calcefluor white stock solution (1 mg/ml H2O) for a final concentration of 0.5 mg/ml, and the stained cells were imaged by epifluorescence microscopy. To visualize F-actin, rhodamine-phalloidin staining was performed using the method of Swion and Nurse (25). For time lapse microscopy of cells with deletions in all three psd genes (psd1-2A cells), petri dishes containing a thin layer (8 ml) of YEAG agar with 1 mM ethanolamine were prepared. About 105 cells were pipetted onto the agar, overlaid with a coverslip, and imaged at regular intervals by differential interference contrast (DIC) photomicroscopy. DIC and epifluorescence microscopic analyses were carried out using a Nikon 90i automated DIC/fluorescence microscope system operated using Nikon NIS-Elements software. Images were captured using a CoolSNAP HQ2 monochrome charge-coupled device camera (Photometrics), and the level was adjusted using Adobe Photoshop CS3 software (Adobe Systems, Inc.).

RESULTS

Identification of fission yeast PSD genes. To identify S. pombe open reading frames encoding proteins structurally homologous to eukaryotic PSD enzymes, we conducted TBLASTN and BLASTP searches of the S. pombe nucleic acid and protein sequence databases, respectively, using the S. cerevisiae Psd1 and Psd2 proteins as the query sequences. Two predicted S. pombe genes were identified, which we have designated psd1 (GenBank locus tag SPBC16E9.18) and psd2 (GenBank locus tag SPAC25B5.03), encoding proteins highly homologous in structure to S. cerevisiae Psd1 (E values, 10−6 to 10−85 and 5 × 10−51, respectively). A third predicted gene, which we have designated psd3 (GenBank locus tag SPAC31G5.15), was identified; it encodes a protein highly homologous in structure to S. cerevisiae Psd2 (E value, 9 × 10−96). Like S. cerevisiae Psd1, the S. pombe psd1 and psd2 gene products share high degrees of structural homology with mammalian PSD enzymes (E values, 10−71 and 10−41, respectively) and moderate homologies with various bacterial PSDs (E values as high as 3 × 10−25). In contrast, the S. pombe psd3 gene product as well as its counterpart in S. cerevisiae, encoded by the PSD2 gene, share relatively little sequence similarity with mammalian PSDs (E values, <10−16 and 10−9, respectively) but exhibit substantial homology with PSDs found in a variety of bacterial organisms (E values as high as 4 × 10−17).
in structural organization to mammalian PSDs, represented in
Fig. 1 by the human Psd protein (GenBank accession no.
CR456540). In both Psd2-Sp and S. cerevisiae Psd1 (Psd1-Sc),
the predicted PSD superfamily domain is disrupted by a non-
conserved sequence, which is quite extensive in the case of
Psd2-Sp (Fig. 1A). Interestingly, both Psd3-Sp and Psd2-Sc
contain substantial noncatalytic N-terminal sequences that
account for more than two-thirds of the length of each protein
(Fig. 1A). Psd3-Sp and Psd2-Sc share relatively little sequence
homology within their respective noncatalytic N-terminal do-
main, except for a predicted protein kinase C region 2 (C2)
Ca2+-binding domain, which spans amino acid residues 281 to 387 in
Psd3-Sp and residues 495 to 602 in
Psd2-Sc (Fig. 1A). The C2 domain is a relatively common motif
present in a variety of eukaryotic proteins, including protein

kinase C, a number of different phospholipases, and synap-
togamins (23, 26). Particular C2 domains have been shown to
bind to phospholipids, inositol polyphosphates, and other pro-
teins. While some C2 domains have been shown to bind Ca2+,
others appear to lack Ca2+-binding sites. However, it is note-
worthy that the predicted C2 domains of both Psd3-Sp (Fig.
1B) and Psd2-Sc (data not shown) contain all four amino acid
residues that constitute the conserved metal binding pocket of
the archetypal Ca2+-binding C2 domain.

The phylogenetic relationships of S. pombe, S. cerevisiae,
human (Psd), and representative bacterial PSD proteins were
determined using the CLUSTAL W program (31). A PSD
from the marine bacterium Alcanivorax sp. was identified by a
BLASTP search as the bacterial PSD exhibiting the greatest
degree of sequence homology with Psd1-Sp (E value, 10−20),
while a PSD from the bacterium Fusobacterium nucleatum
showed the greatest degree of homology to Psd3-Sp (E value,
4 × 10−25). As shown in Fig. 1C, the yeast, human, and bac-
terial PSDs used for predicting phylogenetic relationships lie in
three branches, two with multiple nodes, derived from a hypo-
thesised common ancestor. Psd1-Sp and Psd2-Sc are predicted
to share a common phylogenetic lineage with mammalian
PSDs. Psd3-Sp and Psd2-Sc occupy nodes on a separate branch
shared with bacterial PSDs. Interestingly, Psd1-Sc lies in a
branch that is phylogenetically separate from those occupied
by the other PSDs included in this comparative analysis.

The psd1, psd2, and psd3 gene products share overlapping
functions essential for the normal growth of S. pombe cells. To
determine the phenotypes resulting from deletion of the psd1-3
genes, we used PCR to construct the psd1::kanMX6, psd2::
IhphMX6, and psd3::ura4 gene knockout cassettes, in which the
psd1 to psd3 protein-coding sequences are each replaced by
genes encoding different selectable markers (see Materials and
Methods). The psd1-3 knockout cassettes were transformed
separately into wild-type S. pombe strain SP870. Transformants
were plated onto media selective for the growth of the respec-
tive knockout mutants, which were subsequently identified by
colony PCR (see Materials and Methods). The resulting
psd1Δ, psd2Δ, and psd3Δ deletion mutants were tested for
growth on complex rich medium (YEAU) and synthetic de-
fining minimal medium (EMM). As shown in Fig. 2A, the
psd1Δ, psd2Δ, and psd3Δ strains each grew about as well as the
wild-type parental S. pombe strain on both YEAU and EMM.
These results demonstrate that no single psd gene is essential
for S. pombe cell growth and proliferation.

To determine whether the psd genes encode proteins that
share overlapping or redundant functions necessary for the
growth of S. pombe cells, we carried out a series of genetic
crosses to generate psd1Δ psd2Δ, psd1Δ psd3Δ, and
psd2Δ psd3Δ double mutants, as well as a psd1Δ psd2Δ psd3Δ triple
mutant (the psd1-3Δ mutant). Each of the psdΔ mutants was
tested for growth on YEAU and EMM. As shown in Fig. 2B,
the psd1Δ psd2Δ, psd1Δ psd3Δ, and psd2Δ psd3Δ double mu-
tants grew similarly to wild-type S. pombe cells on both YEAU
and EMM agar media. In contrast, the psd1Δ-3Δ triple mutant
grew slowly on YEAU medium and not at all on EMM.

Although on YEAU and EMM agar media, as well as in
YEAU liquid medium, the psdΔ single and double mutants
grew similarly to wild-type S. pombe cells, we noted that in
liquid EMM, the psd2Δ mutant as well as the psd1Δ and

FIG. 1. Sequence organization and phylogenetic relationships of S.
pombe, S. cerevisiae, mammalian, and representative bacterial PSD
proteins. (A) The structural organizations of the Psd1-Sp, Psd2-Sp,
Psd3-Sc, Psd1-Sc, Psd2-Sc, human Psd (Psd-Hs), and
Fusobacterium nucleatum PSD (PSD-Fn) proteins were determined by use of the
Conserved Domain Database (CDD) (16) integrated with the National
Center for Biotechnology Information BLASTP application (http://
blast.ncbi.nlm.nih.gov/Blast.cgi). PSD superfamily domains and C2
domains are indicated. (B) Alignment of the C2 domains of rat syn-
aptotagmin III (SNPG III) and Psd3-Sc. The alignment was generated
by the CDD program (16) integrated with the NCBI BLASTP appli-
cation (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Asterisks mark the four
conserved amino acids present in the conserved metal binding pocket of
Ca2+-binding C2 domains. (C) The phylogenetic relationships of the
PSD proteins diagrammed in panel A as well as the PSD from the ma-
rine bacterium Alcanivorax sp. were determined using the CLUSTAL
W application provided by the Protein Information Resource website,
hosted by Georgetown University Medical Center (http://pir.
georgetown.edu/pirwww). Branch lengths are drawn to scale.


EUKARYOT. CELL
psd2Δ psd3Δ mutants grew markedly more slowly than wild-type S. pombe cells (Table 2). Consistent with the results of growth assays on YEAU and EMM agar media, the psd1-3Δ triple mutant was found to grow very slowly relative to wild-type S. pombe cells in liquid EMM supplemented with ethanolamine, and its growth was arrested within about 1 generation after transfer from EMM supplemented with ethanolamine to EMM lacking ethanolamine (Table 2). Taken together, the results of these assays analyzing the growth of PSD mutants in rich and defined minimal media indicate that the psd1 to psd3 gene products share substantially overlapping but not completely redundant functions essential for S. pombe cell growth and proliferation.

The S. pombe psd1-3Δ mutant is partially rescued by supplementation of growth media with ethanolamine but not with choline. The S. pombe genome contains genes encoding proteins that share structural homology with enzymes comprising the CDP-ethanolamine and CDP-choline branches of the Kennedy pathway (Y. Matsuo and S. Marcus, unpublished data), which in budding yeast and mammalian cells are used to convert ethanolamine to PE and choline to phosphatidylcholine (PC), respectively (15, 34, 36). Previous studies of S. pombe PS synthase (pps1Δ) and phospholipid methyltransferase (cho1Δ) mutants suggested that both branches of the Kennedy pathway are functional in S. pombe (13, 17). We therefore performed experiments to determine whether supplementation of media with ethanolamine or choline can rescue the growth defect of the psd1-3Δ mutant. We found that supplementation of media with 1 mM ethanolamine strongly suppressed the growth-defective phenotype of the psd1-3Δ mutant on YE medium (Fig. 3A) but only partially rescued the growth defect of the mutant on EMM (Fig. 3B). In contrast, choline supplementation, even at a concentration as high as 100 mM, did not rescue the growth-defective phenotype of the psd1-3Δ mutant to any extent and, in fact, was inhibitory to the growth of this mutant on YE medium (Fig. 3C). Taken together, the results of these assays analyzing the growth of PSD mutants in rich and defined minimal media indicate that the psd1 to psd3 gene products share substantially overlapping but not completely redundant functions essential for S. pombe cell growth and proliferation.
together, these results indicate that PE is essential for *S. pombe* cell growth and, further, that the CDP-ethanolamine branch of the Kennedy pathway can only partially compensate for the essential functions conferred by *S. pombe* PSDs in synthetic defined minimal medium.

The *psd1-3* gene products are responsible for the majority of PE synthesis in *S. pombe*. We next carried out radiolabeling experiments to determine whether *S. pombe* cells harboring null mutations in all three *psd* genes have lower levels of PE than wild-type *S. pombe* cells. For this purpose, wild-type and *psd1-3A* *S. pombe* strains were labeled to uniformity with $^{32}$P$_i$ in nonsupplemented YEAU (wild-type cells) or YEAU supplemented with 1 mM ethanolamine (wild-type and *psd1-3A* cells). Lipids were extracted from the radiolabeled cells, and individual phospholipids were resolved by high-performance thin-layer chromatography and quantified by autoradiography and densitometry (see Materials and Methods). We found that *psd1-3A* cells cultured in ethanolamine-supplemented YEAU medium had substantially lower relative levels of PE than wild-type *S. pombe* cells, which displayed similar phospholipid profiles in nonsupplemented YEAU and YEAU containing ethanolamine (Fig. 4A). In addition, we noted that *psd1-3A* cells cultured under these conditions had markedly higher relative levels of PS than wild-type *S. pombe* cells (Fig. 4A).

We next carried out labeling experiments in EMM, which requires supplementation with ethanolamine to support the growth of *psd1-3A* cells. We first analyzed the phospholipid compositions of wild-type and *psd1-3A* cultures labeled with $^{32}$P$_i$ in EMM containing 1 mM ethanolamine. As with ethanolamine-supplemented YEAU medium, we found that *psd1-3A* cells cultured in EMM containing 1 mM ethanolamine had significantly lower PE levels than wild-type cells as well as higher levels of PS (Fig. 4B). Levels of phosphatidylinositol (PI) relative to PC were also higher in both wild-type and *psd1-3A* strains cultured under these conditions.

Next, we carried out labeling experiments to determine the effect of ethanolamine starvation on phospholipid levels in *psd1-3A* cells. For this purpose, wild-type and *psd1-3A* cells were labeled with $^{32}$P$_i$ in EMM containing 1 mM ethanolamine, washed with EMM, resuspended in EMM, and labeled for 8 additional h prior to the harvesting and extraction of lipids from the labeled cells. As shown in Fig. 4C, ethanolamine starvation led to a further reduction of PE levels in *psd1-3A* cells relative to those in wild-type cells. Taken together, the results of these in vivo labeling experiments indicate that PSD activity is responsible for the majority of PE synthesis in *S. pombe* and that, consistent with the results of the growth assays described above, the activity of the Kennedy pathway cannot fully compensate for the loss of PSD activity with respect to the maintenance of levels of PE relative to those of PS, PI, and PC.

The *psd1-3A* mutant exhibits severe morphology- and cytokinesis-defective phenotypes under ethanolamine-limited growth conditions. Microscopic analyses were carried out to determine whether *S. pombe* PSD mutants exhibit defects in cell morphology. When cultured in nonsupplemented YEAU medium, *psd1Δ*, *psd2Δ*, and *psd3Δ* single mutants, as well as *psd1Δ psd2Δ*, *psd1Δ psd3Δ*, and *psd2Δ psd3Δ* double mutants, appeared similar to wild-type *S. pombe* cells in morphology (Fig. 5). These results demonstrate that the loss of any two *psd*
genes does not result in obvious morphological abnormalities in *S. pombe* cells cultured in rich medium. To determine whether *S. pombe* cells lacking all three *psd* genes exhibit morphological abnormalities, the *psd1-3Δ* mutant was cultured in YE medium containing 1 mM ethanolamine to mid-log phase, washed, resuspended in nonsupplemented YE or YE containing 1 mM ethanolamine, and cultured for several generations prior to microscopic analysis. In YE medium supplemented with ethanolamine, *psd1-3Δ* cells exhibited a rod-shaped morphology (Fig. 6B) indistinguishable from that of wild-type *S. pombe* cells (Fig. 6A). In stark contrast, in *psd1-3Δ* cultures grown for 6 to 7 generations in nonsupplemented YE medium, we observed high frequencies of morphologically aberrant cells, including elongated, enlarged, bot-tle-shaped, and bulbous cells (Fig. 6C). Moreover, we noted that about 20% of septated *psd1-3Δ* cells contained more than one septum (Fig. 6C), including cells with double septa (two closely positioned septa), and that many septated cells exhibited mispositioned septa (not located near the middle of the cell) and/or misoriented septa (not perpendicular to the apparent growth axis of the cell). After culturing for 9 to 10 generations in YE medium lacking ethanolamine, we found that more than 70% of cells in *psd1-3Δ* cultures exhibited bottle-shaped, ovoid, or bulbous morphologies (Fig. 6D). In addition, more than 40% of cells in these cultures were septated, in contrast to about 10% of cells in the corresponding wild-type *S. pombe* cultures (Fig. 6E). Abnormally septated cells were also common in these cultures, and a low incidence of multisepated cells was also detected (about 2% of septated cells) (Fig. 6D, inset).

We next sought to determine whether multisepated cells in *psd1-3Δ* cultures grown in nonsupplemented YE medium would be capable of completing cytokinesis when transferred to medium supplemented with ethanolamine. For this purpose, *psd1-3Δ* cells were cultured for about 8 generations in YE medium, spotted onto a thin layer of YE agar containing 1 mM ethanolamine, and monitored by time lapse DIC microscopy. We found that in the majority of samples observed, multisepated cells were able to complete cytokinesis to produce daughter cells that were themselves able to grow and divide (Fig. 7). These results demonstrate that the multisepate phenotype of *psd1-3Δ* cells cultured in ethanolamine-limited medium is not a terminal phenotype. We noted that in some cases the time required for completion of separation of compartments within a multisepated cell varied greatly, as in the example documented in Fig. 7. In addition, we noted during

![Figure 5](image1.png)

**FIG. 5.** Microscopic analysis of *psdΔ* single and double mutants. Wild-type *S. pombe* cells, *psd1Δ*, *psd2Δ*, and *psd3Δ* single mutants, and *psd1Δ psd2Δ*, *psd1Δ psd3Δ*, and *psd2Δ psd3Δ* double mutants were cultured overnight in YEUA medium to mid-log phase and analyzed by photomicroscopy.

![Figure 6](image2.png)

**FIG. 6.** *psd1-3Δ* cells exhibit severe morphology- and cytokinesis-defective phenotypes. (A) Photomicrograph of wild-type *S. pombe* cells cultured to mid-log phase in YEUA medium. (B) Photomicrograph of *psd1-3Δ* cells cultured to mid-log phase in YEUA containing 1 mM ethanolamine (+ EA). (C and D) *psd1-3Δ* cells were cultured to mid-log phase in YEUA + EA, washed three times with YEUA, resuspended in nonsupplemented YEUA (− EA), cultured at 30°C, and subjected to photomicroscopy after approximately 7 (C) or 10 (D) generations of growth. (C) Arrows mark cells with highly aberrant septation. (Inset) Cell containing a double septum. (D) (Inset) Cell containing two septa. (E) Wild-type cells cultured in YEUA and *psd1-3Δ* cells cultured in YEUA − EA or YEUA + EA for approximately 10 generations were analyzed by photomicroscopy to determine the frequency of septated cells. The bar graph shows average results ± standard errors of the means for two independent experiments.
the course of this experiment that daughter cells produced from the cytokinesis of multiseptated cells were usually at least somewhat abnormal in shape and that progeny produced from these first-generation daughters of multiseptated cells were likewise often abnormal in shape (Fig. 7). These observations indicated that the reversion of psd1-3Δ cells to normal morphological phenotypes after growth under ethanolamine-limited conditions requires several generations of growth after subculturing to medium supplemented with ethanolamine.

Additional analyses revealed that psd1-3Δ cultures grown in EMM containing 1 mM ethanolamine (Fig. 8B) were generally similar in morphology to wild-type S. pombe cells (Fig. 8A), although approximately 10 to 15% of cells in psd1-3Δ cultures exhibited various degrees of morphological aberrancy, including ovoid, bottle-shaped, and, occasionally, bent cells. In contrast, the majority of cells in psd1-3Δ cultures incubated overnight in EMM lacking ethanolamine exhibited ovoid or bottle-shaped morphologies and were necrotic, lysed, and/or shrunken in appearance, a phenotype indicative of a defect in cell integrity (Fig. 8C). Taken together, the results of these microscopic analyses demonstrate that PE is required for proper regulation of cell morphology and cytokinesis in S. pombe and, moreover, that in rich medium, any one of the S. pombe psd gene products is sufficient for normal cytokinesis and maintenance of a normal cell shape.

Analysis of cell wall and actin cytoskeletal organization in psd1-3Δ cells. Given the severe defects in septation and morphology exhibited by psd1-3Δ cells under ethanolamine-limited growth conditions, we carried out experiments to investigate whether the mutant exhibits defects in cell wall and/or actin cytoskeletal organization. For analysis of cell wall organization, wild-type and psd1-3Δ S. pombe cells cultured to mid-log phase in YEAU medium were stained with the fluorescent dye calcofluor white and observed by fluorescence microscopy.

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For analysis of actin cytoskeletal organization, wild-type and psd1-3Δ cells cultured to mid-log phase in YEAU medium were stained with the fluorescent F-actin binding compound rhodamine-phalloidin and observed by fluorescence microscopy.

In wild-type S. pombe cells, F-actin is concentrated as dots or patches at the growing cell tips during interphase, while in dividing cells it is concentrated at the cell division site in the form of a contractile actin ring and, later in M phase, as actin patches on either side of the forming septum (Fig. 10A). In contrast, we noted that in multiseptated psd1-3Δ cells, contractile ring and septal actin structures frequently formed asynchronously in the
two daughter cells produced by an initial septation event (Fig. 10C and D). In addition, significantly misoriented actin rings were detected at a high frequency in multisepated psd1-3 Δ cells (Fig. 10D and E). Interestingly, despite the relatively high frequency of septation-defective phenotypes detected in psd1-3 Δ cells, nuclear staining experiments indicated only a very low frequency of cells (<0.2%) exhibiting discernible defects in nuclear segregation (data not shown).

**DISCUSSION**

Previous studies by other investigators have provided evidence of an essential role for PE in the execution of cytokinesis in mammalian cells (7) and of the potential involvement of this phospholipid in both cytokinesis and polarized cell growth in S. pombe and S. cerevisiae on the basis of its localization to the cell division plane and sites of cell growth, respectively, in both yeasts (11). In the present study, we investigated the cellular functions of PE in S. pombe by identifying, cloning, and functionally characterizing the three genes in this organism, psd1-3, encoding homologs of PSDs, enzymes that directly catalyze PE synthesis in both eukaryotic and prokaryotic organisms. Our results demonstrate that PSD activity is essential for the growth of S. pombe cells in synthetic minimal medium and for optimal cell growth in complex rich medium. Supplementation of growth media with ethanolamine, which can be converted to PE via the Kennedy pathway, strongly suppresses the growth defect of psd1-3 Δ cells in rich medium and partially suppresses the growth defect of the mutant in minimal medium, demonstrating that PE is essential for S. pombe cell growth. Importantly, we have shown that S. pombe mutants carrying null mutations in each of the organism’s three predicted PSD genes exhibit severe defects in cell shape and cytokinesis and that these phenotypes are strongly suppressed by the addition of ethanolamine to the growth medium. These findings demonstrate that PE plays critical roles in the control of both cytokinesis and cellular morphogenesis in S. pombe, and they establish the usefulness of this model organism for further investigations on the biological and molecular functions of PE in eukaryotic cells.

Analyses of phospholipid levels in psd1-3 Δ cells indicate that they maintain proportionately lower levels of PE relative to PS, PI, and PC, in comparison to wild-type S. pombe cells, even when cultured in medium containing ethanolamine. This finding demonstrates that PSDs are responsible for the majority of PE synthesis in S. pombe and, furthermore, that the CDP-ethanolamine branch of the Kennedy pathway cannot completely compensate for the loss of PSD function with respect to the maintenance of normal PE levels. Despite this, in rich medium supplemented with ethanolamine, psd1-3 Δ cells grow at rates comparable to those of wild-type S. pombe cells, maintain a normal cell shape, and carry out cytokinesis with apparent efficiency. Thus, in the absence of PSD activity, the Kennedy pathway is clearly sufficient to restore the minimal levels of PE required for cell growth as well as for normal cell shape and cytokinesis in S. pombe. An implication of these findings is that these processes in S. pombe are not dependent on a spatially defined source of PE synthesis provided by the PSD enzymes. In this regard, it will be of interest to determine whether components of the Kennedy pathway exhibit localization patterns that overlap with those of PSD proteins in S. pombe.

*S. pombe* mutants harboring null mutations in any one or two PSD genes exhibit no obvious growth-, morphology-, or cytokinesis-defective phenotypes when cultured in complex rich medium. However, when cultured in synthetic minimal medium, the psd2 Δ mutant and, to a greater extent, psd1 Δ psd2 Δ and psd2 Δ psd3 Δ double mutants were found to grow markedly more slowly than wild-type S. pombe cells and exhibited low frequencies of morphological defects. These findings suggest that the products of the psd1, psd2, and psd3 genes share substantially overlapping but not completely redundant functions necessary for normal *S. pombe* cell growth and morphogenesis.

In a previous study, we showed that *S. pombe* PS synthase (pps1 Δ) mutants are ethanolamine auxotrophs, which in the absence of ethanolamine supplementation exhibit severe defects in cytokinesis and morphology (17). In that study, we noted that pps1 Δ cultures grown in nonsupplemented rich medium contain relatively high frequencies of cells exhibiting abnormal cell wall-enriched aggregates, typically in close proximity to cell tips and/or septa. In addition, F-actin aggregates and thick, short F-actin filaments were also detected in pps1 Δ cells. Here we found that S. pombe psd1-3 Δ mutants, unlike the pps1 Δ mutant, do not exhibit obvious abnormalities in cell wall organization or abnormal F-actin aggregates when cultured in nonsupplemented rich medium. On the other hand, we found that in minimal medium lacking ethanolamine, psd1-3 Δ cultures, like cultures of pps1 Δ cells grown in non-ethanolamine-supplemented rich or minimal medium, contained high frequencies of cells that were necrotic or lysed in appearance, a phenotype indicative of a defect in cell integrity. Importantly, ethanolamine supplementation strongly suppresses the cell integrity-defective phenotype exhibited by psd1-3 Δ cells in liquid minimal medium, whereas this is not the case for the pps1 Δ mutant (17). Taken together, these phenotypic differences between *S. pombe* PS synthase and PSD mutants are consistent with the notion that PS has functions distinct from its role as a precursor for PE synthesis in *S. pombe*.

As already noted, PE has been shown to play critical roles in contractile ring disassembly and the completion of cytokinesis in mammalian cells (7). The results of the present study demonstrate that PE also plays a central role in the execution of cytokinesis at rates comparable to those of wild-type *S. pombe* cells.
cytokinesis in *S. pombe*. Several cytokinesis-defective phenotypes were detected in cultures of *S. pombe psd1-3Δ* cells grown under ethanolamine-limited conditions. First, the *psd1-3Δ* mutant exhibits a profound delay in cell separation, as evidenced by the high frequencies of septated and multiseptated cells detected in cultures of this mutant grown under ethanolamine-limited conditions. Second, *psd1-3Δ* cells frequently exhibit severe abnormalities in septum positioning, which in wild-type cells occurs in close proximity to the middle of the cell, as well as in septum orientation, which in wild-type cells is perpendicular to the central growth axis (5, 10). Microscopic analyses of actin-stained *psd1-3Δ* cultures grown under ethanolamine-limited conditions revealed that in multiseptated cells, contractile ring maturation and septum maturation frequently occurred asynchronously in the two daughter cells produced by an initial septation event. Not unexpectedly, given the observed defects in septum orientation, we noted that contractile rings were commonly misoriented in multiseptated *psd1-3Δ* cells. Additional studies will obviously be required to elucidate the molecular basis for the severe cytokinesis defects detected in *psd1-3Δ* cells and to determine whether it is analogous to the PE-dependent processes required for normal cytokinesis in mammalian cells, which, to our knowledge, likewise have yet to be well defined. In addition, although potential roles for PE in polarized growth have been suggested by investigations of the localization of this phospholipid in both budding and fission yeasts, we are not aware of studies implicating PE in the control of polarized growth in mammalian cells. Thus, it remains to be determined whether this represents a function of PE that is conserved in yeasts and higher organisms. Certainly, an intriguing speculation arising from this and related studies is that PE, which is spatially organized in plasma membranes (6, 11), might contribute to processes controlling the spatial organization of lipid-modified plasma membrane-associated small G proteins, such as Cdc42 and Rho, which play central roles in regulating actin cytoskeletal remodeling during polarized growth and cytokinesis in eukaryotic cells (8, 12). As PE deficiency has also been shown to cause cytokinesis defects in the bacterium *Escherichia coli*, it is possible that its contribution(s) to cell division processes include functions that are primordial in nature (5a, 9a, 20a).

Although apparently present in all eukaryotic cell membranes, PE has long been recognized to be enriched in mitochondrial membranes, and studies with a broad range of species, including mammalian cells, *S. cerevisiae*, and plants, have demonstrated a requirement for this phospholipid in mitochondrial integrity and function (4, 35, 37). For example, in mouse embryonic fibroblasts carrying a homoygous null mutation in the PSD gene, mitochondria were found to be fragmented and to exhibit abnormal morphologies (28). *S. cerevisiae* mutants deficient in mitochondrial PE exhibit a number of mitochondrion-defective phenotypes, including inability to grow in nonfermentable carbon sources, formation of high frequencies of respiration-deficient cells in culture, and defects in the assembly of mitochondrial protein complexes (3, 4, 30). While the primary focus of the present study was to address the contributions of PE to the control of cell morphology and cytokinesis in *S. pombe*, the results of preliminary experiments carried out in our laboratory indicate that, as in other eukaryotes, PE deficiency results in mitochondrial defects, including respiratory deficiency and abnormal mitochondrial localization (our unpublished data). Consistent with our preliminary findings, a global analysis of protein localization in *S. pombe*, examining the localization of C-terminally tagged yellow fluorescent protein fusion proteins corresponding to all known and predicted *S. pombe* open reading frames, found that Psd1 is localized to mitochondria and that Psd2 is localized both to mitochondria and to the nuclear envelope (18). In the same study, it was reported that Psd3 is localized to the cytosol and, in dividing cells, to the periphery of the site of septum formation. The localization reported for Psd3 is noteworthy, given our findings that *psd1-3Δ* mutants exhibit profound defects in the execution of cytokinesis.

In conclusion, our results demonstrate the value of *S. pombe* as a model organism for studies of the regulation and function of PE in eukaryotic organisms. Indeed, its highly polarized morphology and cell division characteristics make *S. pombe* ideally suited for investigations of the contributions of PE to processes governing the execution of cytokinesis and cellular morphogenesis, which may provide insight with regard to potentially analogous functions of PE in higher organisms.

ACKNOWLEDGMENT

This study was funded by National Institutes of Health grant R01GM068685 (to S.M.).

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