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

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Running Title: Characterization of *S. pombe* Phosphatidylserine Decarboxylase Genes

ABSTRACT

To investigate the contributions of phosphatidylethanolamine to growth and morphogenesis of the fission yeast, *Schizosaccharomyces pombe*, we have characterized three predicted genes in this organism, designated *psd1-3*, encoding phosphatidylserine decarboxylases, which catalyze the conversion of phosphatidylserine 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, *psd1-3Δ* mutants carrying deletions in all three *psd* genes grow slowly in rich medium and are inviable in minimal medium, indicating that the *psd1-3* 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 low levels of phosphatidylethanolamine relative to wild type cells, even in medium supplemented with ethanolamine, indicating that the Kennedy pathway can only partially compensate for the loss of phosphatidylserine 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 non-supplemented medium 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 illustrate the usefulness of this model eukaryote for investigating potentially conserved biological and molecular functions of phosphatidylethanolamine.

INTRODUCTION

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 phosphatidylserine (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 lyso-phosphatidylethanolamine (21, 29), a reaction that in the budding yeast, *Saccharomyces cerevisiae*, is 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 aforementioned routes of PE synthesis (22). In contrast, 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 gene *PSD1*, which encodes a mitochondrial localized PSD, exhibit phenotypes indicative of mitochondrial dysfunction, as do cells derived from mouse embryos carrying a disruption of the gene *Psid*, 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 from studies showing that a ubiquitin-like protein, Atg8p, required for yeast autophagy is conjugated to phosphatidylethanolamine 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 disassembly of the contractile ring and completion of cytokinesis in cultures of 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*, thus implicating PE in processes controlling polarized cell growth (11).

Although *S. pombe* mutants defective in enzymes that directly catalyze PE synthesis have not previously been described, we recently showed that mutants carrying a null mutation in the phosphatidylserine synthase gene, *pps1*, are ethanolamine auxotrophs that exhibit severe morphological and cytokinesis defective phenotypes in ethanolamine-limited growth conditions (17). These findings implicated PE in the regulation of cellular morphogenesis and cytokinesis in *S. pombe*. To investigate 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 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. *S. pombe* strains used in this study are listed in Table 1. The recombinant polymerase chain reaction (PCR) gene knockout method described by Krawchuk and Wahls (14) was used to generate *psd1::kanMX6, psd2::hphMX6,* and *psd3::ura4* deletion cassettes in which *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 plasmids pFA6a-kanMX6 (2), pFA6a-hphMX6, or pFA6a-ura4 as the template DNA together with the oligonucleotide primer sets psd1-F1 (5'-GGCAGTATGCTATTAGCAGG-3'), psd1-R1 (5'-GGGGATCCGTCGACCTGCAGCGTACGAGTTAGATTGTTTTTATGGAA-3'), psd1-F2 (5'-GTTTAAACGAGCTCGAATTCATCGATTAGCAAGCAATGCATATTGG-3'), psd1-R2 (5'-GTTAGAAGAAGTAAACATTC-3'), psd2-F1 (5'-CTCCATCTTATCAGCTAGAC-3'), psd2-R1 (5'-GGGGATCCGTCGACCTGCAGCGTACGAATCGGTATAAATTTCCTTAG-3'), psd2-F2 (5'-GTTTAAACGAGCTCGAATTCATCGATCCCATTTGCTTTTGATCTTC-3'), psd2-R2 (5'-GCCTCTTATAAGTCATCCAC-3'), psd3-F1 (5'-GTTCGTAACAAACTCCATCC-3'), psd3-R1 (5'-GGGGATCCGTCGACCTGCAGCGTACGATTCCCGTAGTGGAAAACAGC-3'), psd3-F2 (5'-GTTTAAACGAGCTCGAATTCATCGATCTTGCTTTTAACGACGACAG-3'), and psd3-R2 (5'-CATCATCCCAAATATCTCCC-3'). *S. pombe* haploid strain SP870 was transformed with the resulting *psd1::kanMX6, psd2::hphMX6*, or *psd3::ura4* cassettes and transformants were isolated on selective EMM minimal medium (*psd3::ura4* transformants) or YEAU containing G418 (*psd1::kanMX6* transformants) or hygromycin (*psd2::hphMX6*). 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 (0.5% yeast extract, 3% dextrose, 225 mg/L adenine, 225 mg/L uracil) 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/ml [^{32}P]orthophosphate/ml at 30°C with shaking to a density of 6 X 10^6 cells/ml and harvested by centrifugation for subsequent lipid extraction (see below). Where indicated, YEAU was supplemented with 1 mM ethanolamine. For analyses 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 X 10^5 cells/ml in EMM containing 1 mM ethanolamine and 20 μCi/ml [^{32}P]orthophosphate, and cultured to a density of 6 X 10^6 cells/ml. A portion of each culture was harvested by centrifugation for lipid extraction and the remainder washed with EMM containing 20 μCi/ml [^{32}P]orthophosphate, resuspended at 3 X 10^6 cells/ml in EMM containing 20 μCi/ml [^{32}P]orthophosphate, and incubated for 8 hours. For lipid extraction, one ml of each labeled cell culture was harvested by centrifugation. The cells were washed with 1 ml sterile water, resuspended in 1 ml 5% TCA, 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% of water, 39.5% ethanol, 13.9% of diethyl ether, 2.8% of pyridine, and 0.1% ammonium hydroxide) for one hour at 60°C with occasional swirling. Samples were centrifuged at 1800 x *g* for 3 min and 1 ml of the resulting supernatant volume was added to 0.25 ml extraction solvent II (water : chloroform : methanol; 9 : 60 : 30). After centrifugation at 200 x *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 K6 60 Å thin layer chromatography plates (Whatman) developed in chloroform : ethanol : water : triethylamine (30:35:7:35). Phospholipids were detected by autoradiography and quantified using the Quantity One program (BioRad).

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 culture supernatant was removed, leaving a volume of approximately 10 µl. Two µl of the concentrated cell suspension was mixed on a microscope slide with 2 µl of Calcofluor white stock solution (1 mg/ml H_20) 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 Sawin and Nurse (25). For time-lapsed microscopy of *psd1-3Δ* cells, Petri dishes containing a thin layer (8 ml) of YEAU agar containing 1 mM ethanolamine were prepared. About 10^4 cells were pipetted onto the agar, overlayed with a cover slip, 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/epifluorescence microscope system operated using Nikon NIS-Elements software. Images were captured using a CoolSNAP HQ2 monochrome CCD camera (Photometrics) and level-adjusted using Adobe PhotoShop CS3 software (Adobe Systems, Inc.).

RESULTS

Identification of fission yeast PS decarboxylase 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 *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 SPAC25B8.03), encoding proteins highly homologous in structure to *S. cerevisiae* Psd1 (*E* values of $6x10^{-85}$ and $5x10^{-51}$, respectively). A third predicted gene, which we have designated *psd3* (GenBank locus tag SPAC31G5.15), was identified that encodes a protein highly homologous in structure to *S. cerevisiae* Psd2 (*E* value of $9x10^{-96}$). Like *S. cerevisiae* Psd1, the *S. pombe psd1* and *psd2* gene products share high degrees of structural homology with mammalian PSD enzymes (*E* values of 10^{-71} and 10^{-41}, respectively) and moderate homologies with various bacterial PSDs (*E* values as high as $3x10^{-28}$). By 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 of $<10^{-16}$ and 10^{-9}, respectively) but exhibit substantial homology with PSDs found in a variety of bacterial organisms (*E* values as high as $4x10^{-52}$).

Sequence analysis of fission yeast PSDs. The structural organization of *S. pombe*, *S. cerevisiae*, and human PSD proteins is depicted in Fig. 1A. *S. pombe* Psd1 (Psd1-Sp) is most similar in structural organization to mammalian PSDs, represented in Fig. 1 by the human Psid protein (GenBank accession No. CR456540). In both *S. pombe* Psd2 (Psd2-Sp) and *S. cerevisiae* Psd1 (Psd1-Sc), the predicted PSD superfamily domain is disrupted by non-conserved sequence, which is quite extensive in the case of Psd2-Sp (Fig. 1A). Interestingly, both *S. pombe* Psd3 (Psd3-Sp) and *S. cerevisiae* Psd2 (Psd2-Sc) contain substantial non-

catalytic N-terminal sequence that comprises 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 non-catalytic N-terminal domains with the exception of a predicted protein kinase C conserved region 2 Ca2+-binding domain (C2), 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 synaptotagmins (23, 26). Particular C2 domains have been shown to bind to phospholipids, inositol polyphosphates, and other proteins. While some C2 domains have been shown to bind Ca2+, others appear to lack Ca2+-binding sites. However, it is noteworthy that the predicted C2 domains of both Psd3-Sp (Fig. 1B) and Psd2-Sc (data not shown) contain each of four amino acid residues that comprise the conserved metal binding pocket of the archetypal Ca2+-binding C2 domain.

Phylogenetic relationships of *S. pombe*, *S. cerevisiae*, human (Psid), and representative bacterial PSD proteins were determined using the CLUSTAL W program (31). A PSD from the marine bacterium Alcanivorax was identified from a BLASTP search as the bacterial PSD exhibiting the greatest degree of sequence homology with Psd1-Sp (*E* value of 10^{-26}), while a PSD from the bacterium *Fusobacterium nucleatum* showed the greatest degree of homology to Psd3-Sp (*E* value of $4x10^{-52}$). As shown in Fig. 1C, the yeast, human, and bacterial PSDs used for predicting phylogenetic relationships lie in three branches, two with multiple nodes, derived from a hypothetical common ancestor. Psd1-Sp and Psd2-Sp are predicted to share a common phylogenetic lineage with mammalian PSDs. Psd3-Sp and Psd2-Sc occupy nodes on a separate branch shared with the bacterial PSDs. Interestingly, Psd1-Sc lies in a phylogenetically separate branch from those occupied by the other PSDs included in this comparative analysis.

The *psd1-3* gene products share overlapping functions essential for normal growth of *S. pombe* cells. To determine the phenotypes resulting from deletion of the *psd1-3* genes, we used PCR to construct the gene knockout cassettes *psd1::kanMX6*, *psd2::hphMX6*, and

psd3::ura4 in which the *psd1-3* protein coding sequences are each replaced by genes encoding different selectable markers (Materials and Methods). The *psd1-3* knockout cassettes were transformed separately into wild type *S. pombe* strain SP870. Transformants were plated on media selective for growth of the respective knockout mutants, which were subsequently identified by colony PCR (Materials and Methods). The resulting *psd1Δ*, *psd2Δ*, and *psd3Δ* deletion mutants were tested for growth on complex rich medium (YEAU) and synthetic defined minimal medium (EMM). As shown in Fig. 2A, the *psd1Δ*, *psd2Δ*, and *psd3Δ* strains each grew about as well as the parental wild type *S. pombe* strain on both YEAU and EMM media. 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 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 latter of which will henceforth be referred to as the *psd1-3Δ* mutant. Each of the *psdΔ* mutants was tested for growth on YEAU and EMM media. As shown in Fig. 2B, the *psd1Δ psd2Δ*, *psd1Δ psd3Δ*, and *psd2Δ psd3Δ* double mutants grew similarly to wild type *S. pombe* cells on both YEAU and EMM agar media. By contrast, the *psd1-3Δ* triple mutant grew slowly on YEAU medium and not at all on EMM medium.

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 medium, the *psd2Δ* mutant as well as the *psd1Δ psd2Δ* and *psd2Δ psd3Δ* mutants grew markedly slower 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 medium supplemented with ethanolamine and arrested growth within about one 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 *psd1-3* 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 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 results), which in budding yeast and mammalian cells is used to convert ethanolamine to PE and choline to phosphatidylcholine (PC), respectively (15, 34, 36). Previous studies on *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 YEAU medium (Fig. 3A) but only partially rescued the growth defect of the mutant on EMM medium (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 YEAU medium (Fig. 3C). Taken together, these results indicate that PE is essential for *S. pombe* cell growth and, further, that essential functions conferred by *S. pombe* PSDs can only be partially compensated for in synthetic defined minimal medium by the CDP-ethanolamine branch of the Kennedy pathway.

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 reduced levels of PE in comparison to wild type *S. pombe* cells. To do this, wild type and *psd1-3Δ S. pombe* strains were labeled to uniformity with [^{32}P]orthophosphate in YEAU supplemented (wild type and *psd1-3Δ* cells) or not

(wild type cells) with 1 mM ethanolamine. Lipids were extracted from the radiolabeled cells and individual phospholipids were resolved by HPTLC and quantified by autoradiography and densitometry (Materials and Methods). We found that *psd1-3Δ* cells cultured in ethanolamine supplemented YEAU medium had substantially lower relative levels of PE in comparison to wild type *S. pombe* cells, which displayed similar phospholipid profiles in both YEAU and YEAU containing ethanolamine (Fig. 4A). In addition, we noted that *psd1-3Δ* cells cultured under these conditions had markedly higher relative levels of PS in comparison to wild type *S. pombe* cells (Fig. 4A).

We next carried out labeling experiments in EMM minimal medium, which requires supplementation with ethanolamine to support the growth of *psd1-3Δ* cells. We first analyzed phospholipid compositions of wild type and *psd1-3Δ* cultures labeled with [^{32}P]orthophosphate in EMM containing 1 mM ethanolamine. Similar to results obtained using ethanolamine-supplemented YEAU medium, we found that *psd1-3Δ* cells cultured in EMM containing 1 mM ethanolamine had significantly lower PE levels relative to 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 *psd1-3Δ* strains cultured under these conditions.

Next, we carried out labeling experiments to determine the effect of ethanolamine starvation on phospholipid levels in *psd1-3Δ* cells. To do this, wild type and *psd1-3Δ* cells were labeled with [^{32}P]orthophosphate in EMM containing 1 mM ethanolamine, washed with EMM, resuspended in EMM, and labeled for 8 additional hrs prior to harvesting and extraction of lipids from the labeled cells. As shown in Fig. 4C, ethanolamine starvation lead to a further reduction of PE levels in *psd1-3Δ* cells relative to 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 results of the above described growth assays, the loss of PSD activity cannot be fully compensated for by the activity of the Kennedy pathway with respect to maintenance of levels PE relative to PS, PI, and PC.

The *psd1-3Δ* mutant exhibits severe morphology and cytokinesis defective phenotypes in ethanolamine-limited growth conditions. Microscopic analyses were carried out to determine whether *S. pombe* PSD mutants exhibit defects in cell morphology. When cultured in non-supplemented 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 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 YEAU medium containing 1 mM ethanolamine to mid-log phase, washed, resuspended in YEAU or YEAU containing 1 mM ethanolamine (YEAU+EA), and cultured for several generations prior to microscopic analysis. In YEAU medium supplemented with ethanolamine, *psd1-3Δ* cells exhibited a rod-shaped morphology (Fig. 6B) that was indistinguishable from that of wild type *S. pombe* cells (Fig. 6A). In stark contrast, in *psd1-3Δ* cultures grown for 6-7 generations in non-supplemented YEAU medium, we observed high frequencies of morphologically aberrant cells, including elongated, enlarged, bottle-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 (not located near the cell middle) and/or misoriented septa (not perpendicular to the apparent growth axis of the cell). After culturing for 9-10 generations in YEAU 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 cultures of wild type *S. pombe* cells (Fig. 6E). Abnormally septated cells were also common in these cultures and a low incidence of multi-septated cells was also detected (about 2% of septated cells)(Fig. 6D, inset panel).

We next sought to determine whether multi-septated cells in *psd1-3Δ* cultures grown in non-supplemented YEAU medium would be capable of completing cytokinesis when transferred

to medium supplemented ethanolamine. To do this, *psd1-3Δ* cells were cultured for about eight generations in YEAU medium, spotted onto a thin layer of YEAU agar containing 1 mM ethanolamine, and monitored by time-lapse DIC microscopy. We found that in the majority of observed samples, multi-septated 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 multi-septate 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 multi-septated cell varied greatly, as in the example documented in Fig. 7. In addition, we noted during the course of this experiment that daughter cells produced from the cytokinesis of multi-septated cells were usually at least somewhat abnormal in shape and that progeny produced from these first generation daughters of multi-septated cells were likewise often abnormal in shape (Fig. 7). These observations indicate that the reversion of *psd1-3Δ* cells to normal morphological phenotypes after growth in 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 10-15% of cells in these cultures exhibited varying degrees of morphological aberrancy, including ovoid, bottle-shaped, and, occasionally, bent cells. By 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, or/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 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 in 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, *S. pombe* wild type and *psd1-3Δ* cells cultured to mid-log phase in YEAU medium were stained with the fluorescent cell wall binding dye Calcofluor and observed by fluorescence microscopy. Not unexpectedly, we found that *psd1-3Δ* cells exhibited a variety of abnormalities in septum organization and or structure, including double-walled (Fig. 9B) and deformed septa (Figs. 9C and D). Despite these defects, the vast majority of Calcofluor stained *psd1-3Δ* cells exhibited an intensity of both cell wall and septum wall fluorescence that was similar to wild type *S. pombe* cells (compare wild type cells in panel 9A with *psd1-3Δ* cells in panels 9B-D). We also noted that cultures of *psd1-3Δ* cells, unlike *S. pombe* phosphatidylserine synthase (*pps1Δ*) mutants (17), did not contain significant frequencies of cells with abnormal cell wall aggregates. Moreover, whereas *pps1Δ* cells are hypersensitive to the growth inhibitory effects of Calcofluor, we found that *psd1-3Δ* were no more sensitive to Calcofluor than wild type *S. pombe* cells (data not shown).

For analysis of actin cytoskeletal organization, wild type and *psd1-3Δ* cells cultured to mid-log phase in YEAU medium were stained with the florescent 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). Interphase *psd1-3Δ* cells exhibited actin dots at one or both cell tips that were generally similar in appearance to those detected in wild type *S. pombe* cells (Figs. 10B-C). By contrast, we noted that in multi-septated *psd1-3Δ* cells, contractile ring and septal actin structures frequently form asynchronously in the two daughter cells produced by an initial septation event (Figs. 10C and D). In addition, significantly misoriented actin rings were detected at high frequency in multi-

septated *psd1-3Δ* cells (Figs. 10D and E). Interestingly, despite the relatively high frequency of septation defective phenotypes detected in *psd1-3Δ* cells, nuclear staining experiments indicated only a very low frequency of cells (<0.2%) exhibiting discernable 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 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 *S. pombe* cell growth 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 *psd1-3Δ* 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 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 *psd1-3Δ* 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 maintenance of normal PE levels. Despite this, in rich medium

supplemented with ethanolamine, *psd1-3Δ* cells grow at rates comparable to wild type *S. pombe* cells, maintain 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 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, morphological, or cytokinesis defective phenotypes when cultured in complex rich medium. However, when cultured in synthetic minimal medium, the *psd2Δ* mutant and, to a greater extent, *psd1Δ psd2Δ* and *psd2Δ psd3Δ* double mutants were found to grow markedly slower 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 (*pps1Δ*) 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 *pps1Δ* cultures grown in non-supplemented 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 *pps1Δ* cells. Unlike the *pps1Δ* mutant, we found that *S. pombe psd1-3* mutants do not exhibit obvious abnormalities in cell wall organization or abnormal F-actin aggregates when cultured in non-supplemented rich medium. On the other hand, we found that in minimal medium lacking ethanolamine, *psd1-3Δ* cultures, similar to cultures of *pps1Δ* 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 *psd1-3Δ* cells in liquid minimal medium, whereas this is not the case for the *pps1Δ* mutant (17). Taken together, these phenotypic differences between *S. pombe* PS synthase and PSD mutants are consistent with the notion that PS has functions that are 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 in *S. pombe*. Several cytokinesis defective phenotypes were detected in cultures of *S. pombe psd1-3Δ* cells grown in ethanolamine-limited conditions. First, the *psd1-3Δ* mutant exhibits a profound delay in cell separation, as evidenced by the high frequencies of septated and multi-septated cells detected in cultures of this mutant grown in ethanolamine-limited conditions. Secondly, *psd1-3Δ* cells frequently exhibit severe abnormalities in septum positioning, which in wild type cells occurs in close proximity to the cell middle, as well as septum orientation, which in wild type cells is perpendicular to the central growth axis (5, 10). Microscopic analyses of actin stained psd1-3 cultures grown in ethanolamine-limited conditions revealed that in multi-septated cells, contractile ring and septum maturation frequently occur 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 mis-oriented in multi-septated *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 whether they are analogous to PE-dependent processes required for normal cytokinesis in mammalian cells, the latter of which, to our knowledge, have likewise yet to be well-defined. In addition, although potential roles for PE in polarized growth were suggested by investigations on 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).

Although apparently present in all eukaryotic cell membranes, it has been long-recognized that PE is enriched in mitochondrial membranes and studies in 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 homozygous 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 mitochondrial defective phenotypes, including inability to grow in nonfermentable carbon sources, formation of high frequencies of respiration-deficient cells in culture, and defects in 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*, 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 previous study on global analysis of protein localization in *S. pombe*, which examined the localization of C-terminal-tagged YFP fusion proteins corresponding to all known and predicted *S. pombe* open reading frames, reported that Psd1 is localized to mitochondria and that Psd2 is localized both mitochondria and 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 on the regulation and function of PE in eukaryotic organisms. Indeed, its highly polarized morphology and characteristics of cell division make *S. pombe* ideally suited for investigations on the contributions of PE to processes governing the execution of cytokinesis and cellular morphogenesis, which may be insightful with regard to potentially analogous functions of PE in higher organisms.

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LITERATURE CITED

1. **Alfa, C., P. Fantes, J. Hyams, M. McLeod, and E. Warbrick.** 1993. Experiments with fission yeast: A laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. **Bahler, J., J. Q. Wu, M. S. Longtine, N. G. Shah, A. McKenzie, 3rd, A. B. Steever, A. Wach, P. Philippsen, and J. R. Pringle.** 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast **14:**943-51.
3. **Birner, R., M. Burgermeister, R. Schneiter, and G. Daum.** 2001. Roles of phosphatidylethanolamine and of its several biosynthetic pathways in Saccharomyces cerevisiae. Mol Biol Cell **12:**997-1007.
4. **Birner, R., R. Nebauer, R. Schneiter, and G. Daum.** 2003. Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine biosynthetic machinery with the prohibitin complex of Saccharomyces cerevisiae. Mol Biol Cell **14:**370-83.
5. **Chang, F.** 2001. Studies in fission yeast on mechanisms of cell division site placement. Cell Struct Funct **26:**539-44.
6. **Emoto, K., T. Kobayashi, A. Yamaji, H. Aizawa, I. Yahara, K. Inoue, and M. Umeda.** 1996. Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis. Proc Natl Acad Sci U S A **93:**12867-72.
7. **Emoto, K., and M. Umeda.** 2000. An essential role for a membrane lipid in cytokinesis. Regulation of contractile ring disassembly by redistribution of phosphatidylethanolamine. J Cell Biol **149:**1215-24.
8. **Fukata, M., M. Nakagawa, and K. Kaibuchi.** 2003. Roles of Rho-family GTPases in cell polarisation and directional migration. Curr Opin Cell Biol **15:**590-7.
9. **Fullerton, M. D., F. Hakimuddin, and M. Bakovic.** 2007. Developmental and metabolic effects of disruption of the mouse CTP:phosphoethanolamine cytidylyltransferase gene (Pcyt2). Mol Cell Biol **27:**3327-36.

10. **Hayles, J., and P. Nurse.** 2001. A journey into space. Nat Rev Mol Cell Biol **2:**647-56.

11. **Iwamoto, K., S. Kobayashi, R. Fukuda, M. Umeda, T. Kobayashi, and A. Ohta.** 2004. Local exposure of phosphatidylethanolamine on the yeast plasma membrane is implicated in cell polarity. Genes Cells **9:**891-903.

12. **Kaibuchi, K., S. Kuroda, and M. Amano.** 1999. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem **68:**459-86.

13. **Kanipes, M. I., J. E. Hill, and S. A. Henry.** 1998. The Schizosaccharomyces pombe cho1+ gene encodes a phospholipid methyltransferase. Genetics **150:**553-62.

14. **Krawchuk, M. D., and W. P. Wahls.** 1999. High-efficiency gene targeting in Schizosaccharomyces pombe using a modular, PCR-based approach with long tracts of flanking homology. Yeast **15:**1419-27.

15. **Letts, V. A., L. S. Klig, M. Bae-Lee, G. M. Carman, and S. A. Henry.** 1983. Isolation of the yeast structural gene for the membrane-associated enzyme phosphatidylserine synthase. Proc Natl Acad Sci U S A **80:**7279-83.

16. **Marchler-Bauer, A., J. B. Anderson, M. K. Derbyshire, C. DeWeese-Scott, N. R. Gonzales, M. Gwadz, L. Hao, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, D. Krylov, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, S. Lu, G. H. Marchler, M. Mullokandov, J. S. Song, N. Thanki, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant.** 2007. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res **35:**D237-40.

17. **Matsuo, Y., E. Fisher, J. Patton-Vogt, and S. Marcus.** 2007. Functional characterization of the fission yeast phosphatidylserine synthase gene, pps1, reveals novel cellular functions for phosphatidylserine. Eukaryot Cell **6:**2092-101.

18. **Matsuyama, A., R. Arai, Y. Yashiroda, A. Shirai, A. Kamata, S. Sekido, Y. Kobayashi, A. Hashimoto, M. Hamamoto, Y. Hiraoka, S. Horinouchi, and M. Yoshida.** 2006. ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe. Nat Biotechnol **24:**841-7.

19. **Nakatogawa, H., Y. Ichimura, and Y. Ohsumi.** 2007. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. Cell **130:**165-78.

20. **Nebauer, R., S. Rosenberger, and G. Daum.** 2007. Phosphatidylethanolamine, a limiting factor of autophagy in yeast strains bearing a defect in the carboxypeptidase Y pathway of vacuolar targeting. J Biol Chem **282:**16736-43.

21. **Riekhof, W. R., and D. R. Voelker.** 2006. Uptake and utilization of lyso-phosphatidylethanolamine by Saccharomyces cerevisiae. J Biol Chem **281:**36588-96.

22. **Riekhof, W. R., J. Wu, J. L. Jones, and D. R. Voelker.** 2007. Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in Saccharomyces cerevisiae. J Biol Chem **282:**28344-52.

23. **Rizo, J., and T. C. Sudhof.** 1998. C2-domains, structure and function of a universal Ca2+-binding domain. J Biol Chem **273:**15879-82.

24. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

25. **Sawin, K. E., and P. Nurse.** 1998. Regulation of cell polarity by microtubules in fission yeast. J Cell Biol **142:**457-71.

26. **Sondermann, H., and J. Kuriyan.** 2005. C2 can do it, too. Cell **121:**158-60.

27. **Sou, Y. S., I. Tanida, M. Komatsu, T. Ueno, and E. Kominami.** 2006. Phosphatidylserine in addition to phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. J Biol Chem **281:**3017-24.

28. **Steenbergen, R., T. S. Nanowski, A. Beigneux, A. Kulinski, S. G. Young, and J. E. Vance.** 2005. Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects. J Biol Chem **280:**40032-40.

29. **Stein, Y., and O. Stein.** 1966. Metabolism of labeled lysolecithin, lysophosphatidyl ethanolamine and lecithin in the rat. Biochim Biophys Acta **116:**95-107.

30. **Storey, M. K., K. L. Clay, T. Kutateladze, R. C. Murphy, M. Overduin, and D. R. Voelker.** 2001. Phosphatidylethanolamine has an essential role in Saccharomyces cerevisiae that is independent of its ability to form hexagonal phase structures. J Biol Chem **276:**48539-48.

31. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:**4673-80.

32. **Trotter, P. J., J. Pedretti, and D. R. Voelker.** 1993. Phosphatidylserine decarboxylase from Saccharomyces cerevisiae. Isolation of mutants, cloning of the gene, and creation of a null allele. J Biol Chem **268:**21416-24.

33. **Trotter, P. J., and D. R. Voelker.** 1995. Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast Saccharomyces cerevisiae. J Biol Chem **270:**6062-70.

34. **Vance, J. E.** 2003. Molecular and cell biology of phosphatidylserine and phosphatidylethanolamine metabolism. Prog Nucleic Acid Res Mol Biol **75:**69-111.

35. **Vance, J. E.** 2008. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically-related aminophospholipids. J Lipid Res.

36. **Vance, J. E., and R. Steenbergen.** 2005. Metabolism and functions of phosphatidylserine. Prog Lipid Res **44:**207-34.

37. **Voelker, D. R.** 1997. Phosphatidylserine decarboxylase. Biochim Biophys Acta **1348:**236-44.

Table 1. *S. pombe* used in this study.

Strain	Relevant Genotype	Source
SP870	*h^{90} ade6-210 leu1-32 ura4-D18*	D. Beach
YMSM108	*h^{90} ade6-210 leu1-32 ura4-D18 psd1::kanMX6*	This study
YMSM109	*h^{90} ade6-210 leu1-32 ura4-D18 psd2::hphMX6*	This study
YMSM110	*h^{90} ade6-210 leu1-32 ura4-D18 psd3::ura4*	This study
YMSM111	*h^{90} ade6-210 leu1-32 ura4-D18 psd1::kanMX6 psd2::hphMX6*	This study
YMSM112	*h^{90} ade6-210 leu1-32 ura4-D18 psd1::kanMX6 psd3::ura4*	This study
YMSM113	*h^{90} ade6-210 leu1-32 ura4-D18 psd2::hphMX6 psd3::ura4*	This study
YMSM114	*h^{90} ade6-210 leu1-32 ura4-D18 psd1::kanMX6 psd2::hphMX6 psd3::ura4*	This study

Table 2. Growth of *psdΔ* mutants relative to wild type *S. pombe* cells in liquid media.

	Relative Growth[1]			
Strain	YEAU	YEAU+EA	EMM	EMM+EA
Wild Type	+++	+++	+++	+++
psd1Δ (YMSM108)	+++	+++	+++	+++
psd2Δ (YMSM109)	+++	+++	++	+++
psd3Δ (YMSM110)	+++	+++	+++	+++
psd1Δ psd2Δ (YMSM111)	+++	+++	+	+++
psd1Δ psd3Δ (YMSM112)	+++	+++	+++	+++
psd2Δ psd3Δ (YMSM113)	+++	+++	+	+++
psd1Δ psd2Δ psd3Δ (YMSM114)	+	+++	-	+

[1] All strains except YMSM114 were cultured at 30°C with shaking in the indicated liquid medium to mid-log phase, subcultured by dilution into prewarmed medium to a density of 1-4x10^5 cells/ml (slower growing strains subcultured to higher densities), and incubated for 24-28 hrs prior to determination of cell densities by hemacytometer counting (final densities ranged from 1.4-6x10^6 cells/ml). Generation times were estimated by calculating the number of cell doublings occurring during 24-28 hours of growth. YMSM114 cells were precultured to mid-log phase in medium supplemented with 1 mM ethanolamine, washed with non-ethanolamine supplemented medium, resuspended at about 2x10^5 cells/ml in pre-warmed non-ethanolamine supplemented medium, and incubated for 24 hours prior to determination of cell densities by hemacytometer counting. Wild type cells were cultured under the same conditions for determination of relative growth rates. +++, generation time similar to wild type *S. pombe* cells; ++, generation time approximately twice that of wild type cells; +, generation time three times or more that of wild type cells; -, no growth.

FIGURE LEGENDS

Fig. 1. Sequence organization and phylogenetic relationships of *S. pombe*, *S. cerevisiae*, mammalian, and representative bacterial PSD proteins. (A) Structural organization of *S. pombe* Psd1 (Psd1-Sp), Psd2 (Psd2-Sp), and Psd3 (Psd3-Sp); *S. cerevisiae* Psd1 (Psd1-Sc) and Psd2 (Psd2-Sc); human Psid (PSD-Hs); and *Fusobacterium nucleatum* PSD (PSD-Fn) proteins was determined via the Conserved Domain Database (CDD)(16) component integrated with the National Center for Biotechnology Information BLASTP application (http://blast.ncbi.nlm.nih.gov/Blast.cgi). PSD, phosphatidylserine decarboxylase superfamily domain. CD, protein kinase C conserved region 2 domain. (B) Alignment of the C2 domains of rat synaptotagmin III and *S. pombe* Psd3 (Psd3-Sp). Alignment was generated by the CDD program (16) integrated with the NCBI BLASTP application (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) Phylogenetic relationships of PSD proteins depicted in (A) as well as the PSD from the marine bacterium Alcanivorax were determined using the CLUSTAL W application provided by the Protein Information Resource (PIR) website hosted by Georgetown University Medical Center (http://pir.georgetown.edu/pirwww). Branch lengths are drawn to scale.

Fig. 2. Growth of *S. pombe* PSD mutants on rich and minimal media. (A) *S. pombe* wild type cells and *psd1Δ*, *psd2Δ*, and *psd3Δ* mutant strains were streaked onto complex rich medium (YEAU) or synthetic defined minimal medium (EMM) and incubated at 30°C for 3 and 5 days, respectively. (B) *S. pombe psd1Δ psd2Δ*, *psd1Δ psd3Δ*, *psd2Δ psd3Δ* double mutants and the *psd1-3Δ* triple mutant were streaked onto YEAU or EMM medium, as indicated, and incubated at 30°C for 3 and 5 days, respectively.

Fig. 3 The growth defect of *psd1-3Δ* cells is rescued by ethanolamine but not choline. (A) Wild type and *psd1-3*Δ cells were grown in YEAU containing 1 mM ethanolamine (YEAU+EA) to mid-log phase, harvested by centrifugation, and resuspended in YEAU or EMM at 10^7 cells/ml. The cell suspensions were serially diluted (1:10) and 4 µl of each dilution was spotted onto YEAU or YEAU+EA medium as indicated and the plates were incubated for 3 days at 30°C. (B) Serially diluted suspensions of wild type and *psd1-3*Δ cells prepared as described in (A) were spotted onto EMM or EMM containing 1 mM ethanolamine (EMM+EA) as indicated and the plates were incubated for 5 days at 30°C. (C) Serially diluted suspensions of wild type and *psd1-3*Δ cells prepared as described in (A) were spotted onto YEAU or YEAU containing 1 mM or 100 mM choline as indicated and incubated for 3 days at 30°C.

Fig. 4. Comparison of relative phospholipid compositions in *S. pombe* wild type and *psd1-3Δ* strains. (A) Wild type and *psd1-3Δ* cells were cultured at 30°C to mid-log phase in YEAU containing 20 µCi/ml [^{32}P]orthophosphate/ml (wild type) or the same medium supplemented with 1 mM ethanolamine (wild type and *psd1-3Δ*). Lipids were extracted from labeled cells, resolved by HPTLC, and quantitated by autoradiography and subsequent densitometric analysis of scanned films (Materials and Methods). Relative quantities of the four major phospholipids detected in each strain are shown in the bar graphs. (B) Wild type and *psd1-3Δ* cells were radiolabeled in EMM+EA containing 20 µCi/ml [^{32}P]orthophosphate/ml as described (Materials and Methods). Lipids were extracted from labeled cells and analyzed as described in (A). (C) Wild type and *psd1-3Δ* cells were radiolabeled in EMM containing 20 µCi/ml [^{32}P]orthophosphate/ml as described (Materials and Methods). Lipids were extracted from labeled cells and analyzed as described in (A). Bar graph shows average results ± SEM of two independent determinations.

Fig. 5. Microscopic analysis of *psdΔ* single and double mutants. *S. pombe* wild type cells; *psd1Δ*, *psd2Δ*, *psd3Δ* single mutants; and *psd1Δ psd2Δ*, *psd1Δ psd3Δ*, and *psd2Δ psd3Δ* double

mutants were cultured overnight in YEAU medium to mid-log phase and analyzed by photomicroscopy.

Fig. 6. *psd1-3*Δ cells exhibit severe morphology and cytokinesis defective phenotypes. (A) Photomicrograph of wild type *S. pombe* cells cultured to mid-log phase in YEAU medium. (B) Photomicrograph of *psd1-3Δ* cells cultured to mid-log phase in YEAU+EA. (C) and (D) *psd1-3Δ* cells were cultured to mid-log phase in YEAU+EA, washed 3x with YEAU, resuspended in YEAU, cultured at 30°C and subjected to photomicroscopy after approximately 7 generations of growth (C) and 10 generations of growth (D). Arrows in (C) mark cells with highly aberrant septation. Inset panel of (C) shows a cell containing a double septum. Inset panel of (D) shows a cell containing two septa. (E) Wild type cells cultured in YEAU and *psd1-3Δ* cells cultured in YEAU (-EA) or in YEAU+EA (+EA) for approximately 10 generations were analyzed by photomicroscopy to determine the frequency of septated cells. Bar graph shows average results ± SEM of two independent experiments.

Fig. 7. Completion of cytokinesis by a multi-septated *psd1-3Δ* cell upon transfer to ethanolamine containing medium. A culture of *S. pombe psd1-3Δ* cells was grown for about 8 generations in YEAU then spotted onto YEAU agar containing 1 mM ethanolamine and monitored by time-lapsed DIC microscopy. Panels show a series of time-lapse images of a multi-septated cell (0') that completes a series of cytokinesis events (30' – 340') to produce viable daughter cells that likewise grow and divide. The arrow points to a septum in the original parent cell that separates well after several other daughter progeny have grown and divided to produce new daughter cells.

Fig. 8. Microscopic analysis of *psd1-3Δ* cells cultured in synthetic minimal medium. Wild type and *psd1-3Δ* cells were cultured overnight in EMM (A) and EMM+EA (B), respectively, at 30°C to mid-log phase prior to DIC photomicroscopy. (C) *psd1-3Δ* cells were cultured in

EMM+EA to mid-log phase, washed with EMM, and resuspended in EMM and incubated overnight at 30°C prior to DIC photomicroscopy.

Fig. 9. Analysis of cell wall organization in *psd1-3Δ* cells.

Wild type (A) and *psd1-3Δ* cells (B-D) cultured in YEAU at 30°C for 8-9 generations to mid-log phase were stained with Calcofluor white (Materials and Methods) and visualized by fluorescence microscopy. Arrows in panels B-D mark *psd1-3Δ* cells displaying malformed and/or multiple septa.

Fig. 10. Analysis of actin cytoskeletal organization in *psd1-3Δ* cells. Wild type (A) and *psd1-3Δ* cells (B-E) cultured in YEAU at 30°C for 8-9 generations to mid-log phase were stained with rhodamine-phalloidin and visualized by fluorescence microscopy. Arrows in panel B mark bottle-shaped interphase cells. Arrow in panel C marks a cell with two non-synchronous septation events. Arrow in panel D marks a cell displaying improperly synchronized actin ring structures. Arrow in panel E marks cell with severely misoriented actin ring.