Geranylgeranyl Diphosphate Synthase in Fission Yeast Is a Heteromer of Farnesyl Diphosphate Synthase (FPS), Fps1, and an FPS-like Protein, Spo9, Essential for Sporulation

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Both farnesyl diphosphate synthase (FPS) and geranylgeranyl diphosphate synthase (GGPS) are key enzymes in the synthesis of various isoprenoid-containing compounds and proteins. Here, we describe two novel *Schizosaccharomyces pombe* genes, *fps1*⁺ and *spo9*⁺, whose products are similar to FPS in primary structure, but whose functions differ from one another. Fps1 is essential for vegetative growth, whereas, a *spo9* null mutant exhibits temperature-sensitive growth. Expression of *fps1*⁺, but not *spo9*⁺, suppresses the lethality of a *Saccharomyces cerevisiae* FPS-deficient mutant and also restores ubiquinone synthesis in an *Escherichia coli ispA* mutant, which lacks FPS activity, indicating that *S. pombe* Fps1 in fact functions as an FPS. In contrast to a typical FPS gene, no apparent GGPS homologues have been found in the *S. pombe* genome. Interestingly, although neither *fps1*⁺ nor *spo9*⁺ expression alone in *E. coli* confers clear GGPS activity, coexpression of both genes induces such activity. Moreover, the GGPS activity is significantly reduced in the *spo9* mutant. In addition, the *spo9* mutation perturbs the membrane association of a geranylgeranylated protein, but not that of a farnesylated protein. Yeast two-hybrid and coimmunoprecipitation analyses indicate that Fps1 and Spo9 physically interact. Thus, neither Fps1 nor Spo9 alone functions as a GGPS, but the two proteins together form a complex with GGPS activity. Because *spo9* was originally identified as a sporulation-deficient mutant, we show here that expansion of the forespore membrane is severely inhibited in *spo9Δ* cells. Electron microscopy revealed significant accumulation membrane vesicles in *spo9Δ* cells. We suggest that lack of GGPS activity in a *spo9* mutant results in impaired protein prenylation in certain proteins responsible for secretory function, thereby inhibiting forespore membrane formation.

INTRODUCTION

Prenyltransferases are key enzymes in isoprenoid biosynthesis, and a number of prenyltransferases have been described in a variety of organisms. Among them, farnesyl diphosphate synthase (FPS) and geranylgeranyl diphosphate synthase (GGPS) have been well characterized (for review, see Koyama, 1999; Szkopinska and Plochocka, 2005). FPS catalyzes the sequential condensation of dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) with isopentenyl diphosphate (IPP). The product, farnesyl diphosphate (FPP), is the precursor for several important metabolites, including sterols, dolichols, ubiquinones, and

heme a (for review, see Kawamukai, 2002). GGPS catalyzes a similar condensation reaction as FPS. It uses DMAPP, GPP, and FPP as allylic substrates to generate an amphipathic molecule containing four isoprene units, GGPP, which is a precursor of carotenoids and chlorophylls. FPP and GGPP are also substrates for protein prenylation mediated by farnesyl transferase and geranylgeranyl transferase. Protein prenylation is a posttranslational modification by which isoprenoid compounds are covalently attached to cysteine residues at or near the C terminus of proteins (Casey 1992, 1994; Schafer and Rine, 1992; Omer and Gibbs, 1994). Prenylated proteins, which include small GTP-binding proteins such as Ras and Rab, account for ~2% of total cellular proteins (Epstein *et al.*, 1991). Prenylation has generally been found to be essential for proper localization of membrane proteins.

Sporulation in the fission yeast *Schizosaccharomyces pombe* is equivalent to gametogenesis in higher eukaryotes. This unique process includes two overlapping processes, meiosis and spore formation. Four haploid nuclei produced by meiosis are packaged into individual spores. During meiosis II, membrane vesicles accumulate in the vicinity of the spindle pole body, a structure equivalent to the centrosome in animal cells, and they fuse to generate a double unit membrane, termed the forespore membrane. The forespore membrane expands by fusing with membrane vesicles, and eventually it encapsulates each of the four nuclei produced by meiosis.

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Abbreviations used: DMAPP, dimethylallyl diphosphate; FARM, first aspartate-rich motif; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; SARM, second aspartate-rich motif; TLC, thin-layer chromatography.

Spore wall material is deposited in the luminal space of the forespore membrane, and its inner membrane becomes the plasma membrane of the spores. Assembly of forespore membranes provides a model system for studying the de novo biogenesis of membrane compartments within the cytoplasm (Yoo *et al.*, 1973; Tanaka and Hirata, 1982; Hirata and Shimoda, 1992, 1994). Many sporulation-deficient *S. pombe* mutants have been isolated (Bresch *et al.*, 1968; Kishida and Shimoda, 1986). Thus far, we have analyzed *spo3*⁺, *spo4*⁺, *spo6*⁺, *spo14*⁺, *spo15*⁺, *spo19*⁺, and *spo20*⁺ (Ikemoto *et al.*, 2000; Nakamura *et al.*, 2000, 2001, 2002; Nakase *et al.*, 2001; Nakamura-Kubo *et al.*, 2003). *spo3*⁺, *spo14*⁺, and *spo20*⁺ have been shown to be involved in expansion of the forespore membrane. *spo3*⁺ encodes a membrane protein. Spo3 is expressed exclusively during meiosis and localizes to the forespore membrane (Nakamura *et al.*, 2001). Both *spo14*⁺ and *spo20*⁺ encode components of a vesicle-trafficking pathway (Nakase *et al.*, 2001; Nakamura-Kubo *et al.*, 2003). In addition to these *spo*⁺ genes, several other genes are necessary for expansion of the forespore membrane. *psy1*⁺ and *sec9*⁺, both of which encode components of the plasma membrane target membrane-associated soluble N-ethylmaleimide-sensitive factor-attachment protein receptor complex, are required for both vegetative growth and sporulation (Nakamura *et al.*, 2001, 2005). *vps* genes, which are involved in vacuolar protein sorting, are also responsible for expansion of the forespore membrane

(Iwaki *et al.*, 2003; Onishi *et al.*, 2003; Koga *et al.*, 2004; Kashiwazaki *et al.*, 2005).

Among many sporulation-deficient mutants, *spo9* has not been well characterized. In the present study, we isolated and analyzed the *spo9*⁺ gene, which encodes a protein with significant homology to a typical FPS. We also identified another gene, *fps1*⁺, which encodes FPS. Although *fps1*⁺ was found to be essential for growth, *spo9*⁺ was required for proper assembly of the forespore membrane. Despite its high sequence homology with FPS, Spo9 was largely responsible for GGPS activity. We report the novel finding that *S. pombe* GGPS consists of a heteromer made up of a typical FPS protein, Fps1, and an FPS-like protein, Spo9.

MATERIALS AND METHODS

Strains, Media, and Plasmids

Yeast strains used in this study are listed in Table 1. For *S. pombe*, complete medium (YE) was used for vegetative growth. Malt extract medium MEA and synthetic sporulation media SSA, SSL-N, and MM-N were used for mating and sporulation. *Saccharomyces cerevisiae* strains were grown in either YPD, YP-galactose, or selective minimal medium (SC) supplemented with appropriate amino acids, unless otherwise indicated. Plasmids used in this study are listed in Table 2.

Cloning of *spo9*⁺ and *fps1*⁺

The *spo9*-B261 mutant YF2 was transformed with the *S. pombe* genomic library, pTN-L1 (Nakamura *et al.*, 2001). Leu⁺ transformants on SSA (~7500

Table 1. Strains used in this study

Strain	Genotype	Source
<i>S. pombe</i>		
MKW5 (FY7456) ^a	<i>h</i> ⁹⁰	Nakamura-Kubo <i>et al.</i> (2003)
TN8 (FY7132) ^a	<i>h</i> ⁹⁰ <i>leu1</i> -32	Nakamura <i>et al.</i> (2001)
TN75	<i>h</i> ⁹⁰ / <i>h</i> ⁹⁰ <i>ade6</i> -M210/ <i>ade6</i> -M216 <i>leu1</i> -32/ <i>leu1</i> -32 <i>ura4</i> -D18/ <i>ura4</i> -D18	Nakamura <i>et al.</i> (2001)
B261 (FY12194) ^a	<i>h</i> ⁹⁰ <i>spo9</i> -B261 <i>ade6</i> -M210	Bresch <i>et al.</i> (1968)
YF2 (FY13113) ^a	<i>h</i> ⁹⁰ <i>spo9</i> -B261 <i>leu1</i> -32	This study
YF5	<i>h</i> ⁹⁰ <i>spo9</i> -B261 <i>leu1</i> << GFP- <i>psy1</i> <i>leu1</i> -32	This study
YF9	<i>h</i> ⁹⁰ / <i>h</i> ⁹⁰ <i>fps1</i> Δ:: <i>ura4</i> ⁺ / <i>fps1</i> ⁺ <i>ade6</i> -M210/ <i>ade6</i> -M216 <i>leu1</i> -32/ <i>leu1</i> -32 <i>ura4</i> -D18/ <i>ura4</i> -D18	This study
YF16	<i>h</i> ⁹⁰ <i>spo9</i> Δ:: <i>ura4</i> ⁺ <i>leu1</i> -32 <i>ura4</i> -D18	This study
YF51	<i>h</i> ⁹⁰ <i>leu1</i> << GFP- <i>ypt7</i> <i>leu1</i> -32	This study
YF54	<i>h</i> ⁹⁰ <i>spo9</i> Δ:: <i>ura4</i> ⁺ <i>leu1</i> <<GFP- <i>ypt7</i> <i>leu1</i> -32 <i>ura4</i> -D18	This study
YF69	<i>h</i> ⁹⁰ <i>leu1</i> -32 <i>ura4</i> -D18 pAL(<i>fps1</i> -GFP) pDS473(<i>spo9</i>)	This study
YF75	<i>h</i> ⁹⁰ <i>leu1</i> -32 <i>ura4</i> -D18 pTN54 pDS473(<i>spo9</i>)	This study
YF76	<i>h</i> ⁹⁰ <i>leu1</i> -32 <i>ura4</i> -D18	This study
YF77	<i>h</i> ⁹⁰ <i>fps1</i> Δ:: <i>ura4</i> ⁺ <i>ade6</i> <i>leu1</i> -32 <i>ura4</i> -D18 pREP81(<i>fps1</i>)	This study
YF78	<i>h</i> ⁹⁰ <i>fps1</i> Δ:: <i>ura4</i> ⁺ <i>ade6</i> <i>leu1</i> -32 <i>ura4</i> -D18 pREP81(ERG20)	This study
YF81	<i>h</i> ⁹⁰ <i>spo9</i> Δ:: <i>ura4</i> ⁺ <i>leu1</i> -32 <i>ura4</i> -D18 pAL(<i>spo9</i>)	This study
YN68	<i>h</i> ⁹⁰ <i>leu1</i> << GFP- <i>psy1</i> <i>leu1</i> -32	Nakase <i>et al.</i> (2004)
<i>S. cerevisiae</i>		
W303 (BY4502) ^a	MAT a /α <i>ade2</i> -1/ <i>ade2</i> -1 <i>his3</i> -11,15/ <i>his3</i> -11,15 <i>leu2</i> -3,112/ <i>leu2</i> -3,112 <i>trp1</i> -1/ <i>trp1</i> -1 <i>ura3</i> -1/ <i>ura3</i> -1	R. Rothstein (Columbia Univ.)
SFNY368	MATα <i>bts1</i> Δ:: <i>URA3</i> <i>ura3</i> -52 <i>leu2</i> -3,112	Jiang <i>et al.</i> (1995)
YF73	MAT a /α ERG20/ <i>erg20</i> Δ:: <i>URA3</i> <i>ade2</i> -1/ <i>ade2</i> -1 <i>his3</i> -11,15/ <i>his3</i> -11,15 <i>leu2</i> -3,112/ <i>leu2</i> -3,112 <i>trp1</i> -1/ <i>trp1</i> -1 <i>ura3</i> -1/ <i>ura3</i> -1	This study
YF79	MATα <i>erg20</i> Δ:: <i>URA3</i> <i>ade2</i> -1 <i>his3</i> -11,15 <i>leu2</i> -3,112 <i>trp1</i> -1 <i>ura3</i> -1 pYEL2(ERG20)	This study
YF80	MATα <i>erg20</i> Δ:: <i>URA3</i> <i>ade2</i> -1 <i>his3</i> -11,15 <i>leu2</i> -3,112 <i>trp1</i> -1 <i>ura3</i> -1 pYEL2(<i>fps1</i>)	This study
AH109	MATα <i>ura3</i> -52 <i>his3</i> -200 <i>ade2</i> -101 <i>trp1</i> -901 <i>leu2</i> -3,112 <i>gal4</i> Δ <i>met</i> ⁻ <i>gal80</i> Δ LYS2:: <i>GAL1</i> _{UAS} - <i>GAL1</i> _{TATA} - <i>HIS3</i> <i>GAL2</i> _{UAS} - <i>GAL2</i> _{TATA} - <i>ADE2</i> <i>ura3</i> :: <i>MEL1</i> UAS- <i>MEL1</i> _{TATA} - <i>lacZ</i>	Takara-Bio
<i>E. coli</i>		
SF7	Δ <i>ispA</i> :: <i>neo</i> Δ(<i>srl-recA</i>)306::Tn10	Fujisaki <i>et al.</i> (2005)
DH5α	<i>supE44</i> Δ <i>lacU169</i> (φ80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook <i>et al.</i> (1989)
DH10B	Δ(<i>mrr-hsd</i> RMS- <i>mcrBC</i>) <i>mcrA</i> <i>recA1</i>	Sambrook <i>et al.</i> (1989)

^a The strain was obtained from the Yeast Genetic Resource Center of Japan supported by the National BioResource Project (YGRC/NBRP) (<http://yeast.lab.nig.ac.jp/nig/>). The *S. pombe* strains constructed in this study are deposited at the YGRC/NBRP.

Table 2. Plasmids used in this study

Plasmid	Characteristics	Source
<i>S. pombe</i>		
pAL-KS	<i>ars1</i> , <i>LEU2</i> -based vector	Tanaka <i>et al.</i> (2000)
pREP1	<i>ars1</i> , <i>LEU2</i> -based vector carrying a thiamine-repressible <i>nmt1</i> promoter	Maundrell (1993)
pREP81	<i>ars1</i> , <i>LEU2</i> -based vector carrying a thiamine-repressible <i>nmt81</i> promoter	Maundrell (1993)
pREP1(spo9)	<i>spo9</i> ⁺ in pREP1	This study
pREP1(fps1)	<i>fps1</i> ⁺ in pREP1	This study
pREP1(ERG20)	<i>ERG20</i> in pREP1	This study
pREP1(BTS1)	<i>BTS1</i> in pREP1	This study
pREP81(fps1)	<i>fps1</i> ⁺ in pREP81	This study
pREP81(ERG20)	<i>ERG20</i> in pREP81	This study
pBS(fps1)	<i>fps1</i> ⁺ in pBluescript II-KS ⁽⁺⁾	This study
pTN143	GFP and <i>nmt1</i> terminator in pAL-KS	Ikemoto <i>et al.</i> (2000)
pTN54	<i>ars1</i> , <i>LEU2</i> -based vector carrying an N-terminal GFP tag and <i>nmt41</i> promoter	Nakamura <i>et al.</i> (2001)
pAL(fps1-GFP)	<i>fps1</i> ⁺ in pTN143	This study
pAL(spo9)	<i>spo9</i> ⁺ in pAL-KS	This study
pDS473a	<i>ars1</i> , <i>ura4</i> ⁺ -based vector carrying an N-terminal GST tag and <i>nmt42</i> promoter	Forsburg and Sherman (1997)
pDS473(spo9)	<i>spo9</i> ⁺ in pDS473a	This study
pTN381(GFP-ypt7)	<i>leu1</i> ⁺ -based vector carrying an N-terminal GFP tag and <i>ypt7</i> ⁺ native promoter	Kashiwazaki <i>et al.</i> (2005)
pAL(GFP-psy1)	<i>LEU2</i> -based vector carrying an N-terminal GFP tag and <i>psy1</i> ⁺ native promoter	Nakamura (unpublished)
<i>S. cerevisiae</i>		
pYES2	2 μ origin, <i>URA3</i> -based vector carrying a galactose-inducible <i>GAL1</i> promoter	Invitrogen
pYEL2	<i>LEU2</i> in pYES2	This study
pYEL2(spo9)	<i>spo9</i> ⁺ in pYEL2	This study
pYEL2(fps1)	<i>fps1</i> ⁺ in pYEL2	This study
pYEL2(ERG20)	<i>ERG20</i> in pYEL2	This study
pYEL2(BTS1)	<i>BTS1</i> in pYEL2	This study
pGBKT7	2 μ origin, <i>TRP1</i> -based vector carrying a DNA-binding domain of Gal4 and <i>ADH1</i> promoter	Takara-Bio
pGBK(spo9)	<i>spo9</i> ⁺ in pGBKT7	This study
pGBK(fps1)	<i>fps1</i> ⁺ in pGBKT7	This study
pGADT7	2 μ origin, <i>LEU2</i> -based vector carrying a activation domain of Gal4 and <i>ADH1</i> promoter	Takara-Bio
pGAD(spo9)	<i>spo9</i> ⁺ in pGADT7	This study
pGAD(fps1)	<i>fps1</i> ⁺ in pGADT7	This study
<i>E. coli</i>		
pGEX-KG	<i>amp</i> based vector carrying an N-terminal GST tag	Guan and Dixon (1991)
pGEX(spo9)	<i>spo9</i> ⁺ in pGEX-KG	This study
pGEX(fps1)	<i>fps1</i> ⁺ in pGEX-KG	This study
pMAL-c	<i>amp</i> based vector carrying an N-terminal maltose-binding protein (MBP) tag	New England Biolabs (Ipswich, MA)
pMAL(spo9)	<i>spo9</i> ⁺ in pMAL-c	This study
pMAL(fps1)	<i>fps1</i> ⁺ in pMAL-c	This study
pMAL(BTS1)	<i>BTS1</i> in pMAL-c	This study
pMAL(spo9+fps1)	<i>spo9</i> ⁺ and <i>fps1</i> ⁺ in pMAL-c	This study
pMAL(spo9+fps1-R104Q)	<i>spo9</i> ⁺ and <i>fps1</i> -R104Q in pMAL-c	This study
pMAL(spo9-R109Q+fps1)	<i>spo9</i> -R109Q and <i>fps1</i> ⁺ in pMAL-c	This study
pMAL(spo9-R109Q+fps1-R104Q)	<i>spo9</i> -R109Q and <i>fps1</i> -R104Q in pMAL-c	This study
pBluescript II-KS ⁽⁻⁾	<i>amp</i> -based vector	Stratagene
pBH	Human <i>GGPS1</i> in pBluescript II-KS ⁽⁻⁾	Kainou <i>et al.</i> (1999)

colonies) were then exposed to iodine vapor (Gutz *et al.*, 1974). The few colonies that turned brown were taken as candidates for sporulation-proficient transformants. Two plasmids were isolated from these candidates and their nucleotide sequences were determined. One of these plasmids, pYF1, was further analyzed (Table 2).

The *fps1*⁺ gene was cloned as follows. Polymerase chain reaction (PCR) primers 5'-ATATTTTCTCTCAACCTCTAGTCTTT-3' and 5'-GCGCG-CTCGAC(Sall)ATTATTAATGGATTGCTG-3' were used to amplify a 3.5-kb fragment encompassing the entire *fps1*⁺ gene, and the resulting BamHI-Sall fragment was subcloned into pBluescript II-KS⁽⁺⁾ (Stratagene, La Jolla, CA) to generate pBS(fps1).

Disruption of *spo9*⁺ and *fps1*⁺

Disruptions of the *spo9*⁺ and *fps1*⁺ genes by using *ura4*⁺ were constructed as follows. PCR primers 5'-AGAGAGCGGCCGC(NotI)AAAACGGATCCA-GATGGTTA-3' and 5'-AGAGACTCGAG(XhoI)TTCGGAGCGCTTTCGC-GTAGAATT-3' were used to amplify a 2.7-kb fragment encompassing the entire *spo9*⁺ gene. The resulting NotI-XhoI fragment was then subcloned into pGEM-T easy vector (Promega, Madison, WI) to generate pGEM-(spo9). After digestion with HindIII and XbaI, pGEM(spo9) was filled in and ligated to a HindIII linker. The resulting plasmid was digested with HindIII, and a 1.7-kb *ura4*⁺ fragment was inserted at the HindIII site. The

resulting plasmid was digested with BglIII and BamHI and used to transform strain TN75.

To disrupt *fps1*⁺, pBS(*fps1*) was digested with PstI and EcoRI to remove a 1.1-kb fragment of the open reading frame (ORF). A HindIII linker was then inserted into at these restriction sites. A 1.7-kb *ura4*⁺ fragment was inserted at the HindIII site. The resulting plasmid was digested with ClaI and BamHI and used to transform strain TN75. Disruptions were confirmed by Southern hybridization of genomic DNA (data not shown).

Southern and Northern Blot Analyses

Genomic DNA was digested, separated in a 1% agarose gel, and then transferred onto nylon membranes for Southern blotting. Total RNA was isolated from cells harvested at specific times. The isolated RNA was separated on a 1% agarose gel, transferred to a nylon membrane, and subjected to Northern blot analysis (Thomas, 1980).

Nucleotide Sequence Analysis of the *spo9*-B261 Mutant Allele

The entire *spo9*⁺ ORF and promoter region were amplified by PCR with primers 5'-CTCCACTCGAG(XhoI)CATATAGCCATGGGTTCTC-3' and 5'-AGAGACTCGAG(XhoI)TTCGGAGCGCTTTCGCGTAGAATT-3', by using genomic DNA from the *spo9*-B261 mutant as template. The amplified DNA fragment was cloned into pGEM-T easy vector and sequenced.

Ubiquinone Extraction and Measurement

Ubiquinone was extracted as described previously (Kainou *et al.*, 1999). The extracted crude ubiquinone was analyzed by normal phase thin layer chromatography (TLC) with an authentic standard UQ6. Normal phase TLC was carried out on a Kiesel gel 60 F254 plate (Merck, Frankfurt, Germany) by using benzene:acetone (97:3, vol/vol). The UV-visualized band containing ubiquinone was collected from the TLC plate and extracted with chloroform:methanol (1:1, vol/vol). Samples were dried, and the pellets were redissolved in ethanol. The purified ubiquinone was further analyzed by high-performance liquid chromatography using ethanol as the solvent.

Prenyl Diphosphate Synthase Assay

Prenyl diphosphate synthase activity was measured by detection of [¹⁴C]IPP incorporated into reaction products as described previously (Kainou *et al.*, 2001; Saiki *et al.*, 2003). *E. coli* DH5α harboring a plasmid containing the human *GGPS1* gene was incubated to late log phase in LB medium containing ampicillin at 37°C. Cells were harvested by centrifugation, suspended in buffer A (100 mM potassium phosphate, pH 7.4, 5 mM EDTA, and 1 mM 2-mercaptoethanol), and ruptured by sonication (six 30-s pulses with 30-s intervals in an ice bath between sonications). After centrifugation of the homogenate, the supernatant was used as a crude enzyme extract. Similarly, *S. pombe* cells were grown to mid- to late log phase in minimum medium. Cells were harvested by centrifugation and suspended in buffer A. The washed cells were ruptured by vigorous mixing with glass beads 14 times for 30 s with 60-s intervals in an ice bath between mixings. After centrifugation of the homogenate, the supernatant was used as a crude enzyme extract. The assay reaction mixture contained 1.0 mM MgCl₂, 0.1% (wt/vol) Triton X-100, 50 mM potassium phosphate buffer, pH 7.5, 10 μM [¹⁴C]IPP (specific activity 0.92 TBq/mol), 5 μM FPP, and 200 μg of crude extract containing the enzyme in a final volume of 0.4 ml. Sample mixtures were incubated for 60 min at 30°C. Reaction products such as prenyl diphosphates were extracted with 1-butanol-saturated water and hydrolyzed with acid phosphatase. Hydrolysis products were extracted with hexane and analyzed by reversed phase TLC by using acetone:water (19:1, vol/vol). Radioactivity on the TLC plate was detected with an imaging analyzer BAS1500-Mac (Fuji Film, Tokyo, Japan). The plate was exposed to iodine vapor to detect marker prenols.

Yeast Two-Hybrid Analysis

For yeast two-hybrid analysis, PCR primers 5'-GCGCGCCATGG(NcoI)AAATGGTCAACGATTTTAA-3' and 5'-GCGCGCTCGAG(XhoI)CTACCGTACCTTTTGTA-3' were used to amplify the *spo9*⁺ gene. The amplified fragment was digested with NcoI and XhoI and inserted into the corresponding sites of pGBKT7 (*GAL4* DNA binding domain; Takara-Bio, Otsu, Japan) and pGADT7 (*GAL4* activation domain; Takara-Bio), yielding pGBK(*spo9*) and pGAD(*spo9*), respectively. Similarly, PCR primers 5'-GCGCGCCATGG(NcoI)AGATGCTCTGCAGTTGATAA-3' and 5'-GCGCGCTCGAG(XhoI)TTACTTATTTCTTTTGTA-3' were used to amplify the *fps1*⁺ gene. The amplified fragment was digested with NcoI and XhoI, and inserted into the corresponding sites of pGBKT7 and pGADT7 yielding pGBK(*fps1*) and pGAD(*fps1*), respectively. These plasmids were introduced into *S. cerevisiae* AH109 (Takara-Bio), and transformants were replicated onto selective minimal medium supplemented with appropriate amino acids, except for His to assay for protein interactions *in vivo*.

Coimmunoprecipitation Assays

The wild-type strain (YF76) was transformed with pDS473(*spo9*) plus pAL(*fps1*-GFP), or with pDS473(*spo9*) plus pTN54 to yield YF69 and YF75 strains, respectively. YF69 and YF75 were grown overnight at 28°C in MM. The cells were harvested in log phase, resuspended in TES (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 150 mM NaCl) and then ruptured with glass beads. The lysate was centrifuged at 800 × *g* for 5 min to remove cell debris. The supernatant was then centrifuged at 13,000 × *g* for 20 min to prepare a soluble fraction. The soluble fraction was incubated with mouse anti-green fluorescent protein (GFP) antibody (Roche Diagnostics, Mannheim, Germany) at 4°C for 1 h, and then mixed with protein G-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). After further incubation at 4°C for 1 h, the solution was centrifuged at 800 × *g* for 5 min. The pellet was washed with TES three times and resuspended in sample buffer. Samples were electrophoresed in SDS-polyacrylamide gels, and the target polypeptides were detected by Western blot analysis using rat anti-GFP (a generous gift from S. Fujita) or mouse anti-glutathione *S*-transferase (GST) (Sigma-Aldrich, St. Louis, MO) antibody.

Subcellular Fractionation

pTN381(GFP-ypt7) was digested with SnaBI and the linear plasmid was integrated into the *leu1* locus of wild type (TN8) and the *spo9Δ* mutant (YF16) to yield YF51 and YF54, respectively. The YF51 and YF54 strains were grown overnight at 28°C in MM. The cells were harvested in log phase, ruptured with glass beads as described above, and centrifuged at 800 × *g* for 5 min. The supernatant was further centrifuged at 100,000 × *g* for 1 h to separate the soluble fraction, the pellet being resuspended in an equal volume of resuspension buffer. The samples from each fraction were subjected to Western blot analysis by using either mouse anti-GFP (Roche Diagnostics), rabbit anti-Rhb1 (Nakase *et al.*, 2006), or rabbit anti-Spo14 (Nakamura-Kubo *et al.*, 2003) antibodies.

Fluorescence Microscopy

Cells were fixed as described previously (Hagan and Hyams, 1988) by use of glutaraldehyde and paraformaldehyde. The Psy1 protein was visualized by fusing it to GFP. The nuclear chromatin region was stained with 4,6-diamidino-2-phenylindole (DAPI). Stained cells were observed under a fluorescence microscope (model BX51; Olympus, Tokyo, Japan), and images were obtained using a CoolSNAP charge-coupled device camera (Roper Scientific, San Diego, CA).

Electron Microscopy

Cells were mounted on copper grids to form a thin layer, and then they were submerged in liquid propane cooled with liquid N₂. Frozen cells were transferred to 2% OsO₄ in anhydrous acetone, held at -80°C for 48 h in a solid CO₂-acetone bath, and then transferred to -35°C for 2 h, 4°C for 2 h, and room temperature for 2 h. After washing with anhydrous acetone three times, samples were infiltrated with increasing concentrations of Spurr's resin in anhydrous acetone, and finally with 100% Spurr's resin. Samples were then polymerized at 50°C for 5 h and 60°C for 50 h. Thin sections were cut on a Reichert Ultracut S ultramicrotome, and then stained with uranyl acetate and lead citrate. Sections were viewed on an electron microscope H-7600 (Hitachi, Tokyo, Japan) at 100 kV.

RESULTS

spo9⁺ Encodes a Farnesyl Diphosphate Synthase-like Protein

To further investigate phenotypes of *spo9*-B261 mutant, we monitored vegetative growth and sporulation. As reported previously, the *spo9*-B261 mutant exhibits defects in ascospore formation (Bresch *et al.*, 1968) (Figure 1A). In addition, the *spo9*-B261 mutant also exhibited temperature-sensitive vegetative growth (Figure 1B). These results indicate that the *spo9*⁺ gene is required not only for sporulation but also for vegetative growth. To identify the *spo9*⁺ gene product and its biological function, we isolated the *spo9*⁺ gene by functional complementation (see *Materials and Methods*). Two plasmids were isolated that were able to rescue both the temperature sensitivity and sporulation deficiency of *spo9*-B261. Subcloning and partial sequencing revealed that the *spo9*-complementing ability was due to one ORF composed of 1.2 kb (SPBC36.06c) (Figure 2A). The *spo9*⁺ gene encodes a 40.0-kDa protein consisting of 351 amino acid residues (Figure 2B). The predicted Spo9 protein shares 42 and 38%

Figure 1. *spo9* mutant exhibits a sporulation deficiency and temperature-sensitive growth. (A) Wild type (TN8), *spo9-B261* (YF2), and *spo9Δ* (YF16) were incubated at 28°C on sporulation medium (MEA) for 2 d. Bar, 10 μm. (B) The same strain as shown in A was streaked on complete medium (YEA) and incubated at 25 or 36°C for 3 d.

identity and 64 and 61% similarity with the *S. cerevisiae* FPS, Erg20, and human FPS, respectively (Figure 2B). FPS catalyzes chain elongation of the C₅ substrate DMAPP to the C₁₅ product FPP by addition of two molecules of IPP. Spo9 contains two aspartate-rich domains, first aspartate-rich motif: consensus DDXX(XX)D (FARM), and second aspartate-rich motif: consensus DDXXD (SARM), conserved among many prenyltransferases, including FPSs and GGPs (Koyama *et al.*, 1993; Chen *et al.*, 1994; Song and Poulter, 1994; Szkopinska and Plochocka, 2005) (Figure 3A).

To investigate the biological function of *spo9*⁺, a null mutant was created by conventional gene disruption by using *ura4*⁺ as a marker (Figure 2A). The *spo9* deletion mutant (*spo9Δ*) was viable but displayed temperature sensitivity and sporulation deficiency like the original *spo9-B261* mutant (Figure 1, A and B). Because most of the meiosis-defective mutants isolated to date are unable to sporulate (Bresch *et al.*, 1968; Kishida and Shimoda, 1986), one cannot rule out the possibility that the *spo9Δ* mutant has a defect in meiosis. Therefore, we analyzed the meiotic nuclear divisions in *spo9Δ*. The first and second meiotic divisions were found to proceed with kinetics similar to that observed in an isogenic wild-type strain, with the final yield of tetranucleate cells reaching ~90% (data not shown). These results suggest that the *spo9Δ* mutant is able to complete meiosis but that it is defective in ascospore formation.

Sequencing of the *spo9-B261* allele revealed a direct duplication of 20-base pairs (from the 926th to 945th nucleotide of wild-type *spo9*⁺) (Figure 2C). This duplication caused a frameshift, resulting in a truncated protein of 272 amino acid residues. This truncated protein lacked the conserved hydrophilic C terminus, which is important for FPS activity (Song and Poulter, 1994). The *spo9-B261* mutant protein seems to have lost all activity, like the *spo9* disruptant.

Although the *S. pombe* genes responsible for mating, meiosis, and sporulation are generally transcribed under conditions of

nutritional starvation (Yamamoto *et al.*, 1997; Watanabe *et al.*, 2001; Shimoda, 2004), Northern analysis revealed that *spo9*⁺ transcription occurred during vegetative growth and that it was not enhanced, but rather diminished, after the shift to a nitrogen-free medium (Figure 4).

S. pombe Has a Second FPS-like Gene

A homology search performed against the fission yeast genome database (The Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom; <http://www.sanger.ac.uk/>) detected another gene encoding an FPS-like protein (SPAC6F12.13C). We designated this gene *fps1*⁺. Fps1 shares 44, 58, and 48% identity and 64, 73, and 66% similarity with fission yeast Spo9, *S. cerevisiae* Erg20, and human FPS, respectively. Like Spo9, Fps1 also contains two aspartate-rich domains, which are conserved among FPSs and GGPs as described above (Figure 3A). The *fps1*⁺ mRNA was also detectable during vegetative growth, but transcription did not increase after nitrogen starvation (Figure 4). It was also noted that the level of transcription of *fps1*⁺ was apparently higher than that of *spo9*⁺. Next, we disrupted the *fps1*⁺ gene. Tetrad analysis of the heterozygous diploid (YF9: *fps1*⁺/*fps1Δ*) indicated that only two spores per ascus were viable and produced Ura⁻ colonies. Microscopic observation of the inviable spores showed that most germinated, but they seemed to have undergone only one or two divisions before growth arrest. Therefore, we conclude that *fps1*⁺ is essential for cell viability, but not for germination.

Genetic Relationship among *S. pombe* and *S. cerevisiae* FPS- and GGPs-related Genes

To test the possibility of functional redundancy between Fps1 and Spo9, we examined whether overexpression of *fps1*⁺ could rescue the phenotypes of *spo9Δ*. *fps1*⁺ was placed under control of the thiamine-repressible *nmt1* promoter. The resultant expression plasmid, pREP1(*fps1*), rescued both the temperature-sensitive growth and sporulation deficiency, when introduced into *spo9Δ* cells (Figure 5, C and D). In contrast, pREP1(*spo9*) could not rescue the lethality of the *fps1* null mutant (data not shown).

In *S. cerevisiae*, FPS is encoded by the *ERG20* gene. *ERG20* was originally identified by genetic analysis of an inviable ergosterol-deficient mutant (Chambon *et al.*, 1990; Blanchard and Karst, 1993). To assess the level of functional relatedness between *S. pombe* and *S. cerevisiae* FPSs, *spo9*⁺ and *fps1*⁺ were expressed under control of the *GAL1* promoter in an *erg20Δ* mutant of *S. cerevisiae*. Ectopic expression of *fps1*⁺ but not *spo9*⁺ rescued the lethality of the *erg20* null mutation in *S. cerevisiae* (Figure 5A). In a reciprocal manner, *S. cerevisiae* *ERG20* was placed under the control of the thiamine-repressible *nmt1* promoter and introduced into *S. pombe* *fps1Δ* and *spo9Δ* mutants. *ERG20* rescued both the lethality of the *fps1Δ* mutant (Figure 5B) and the temperature sensitivity and sporulation deficiency of a *spo9Δ* mutant (Figure 5, C and D). These data establish that fission yeast Fps1 is able to fulfill all essential Erg20 functions.

GGPs and FPS belong to a family of prenyltransferases that catalyze consecutive condensations of IPP with allylic primer substrates. A number of genes of GGPs (Misawa *et al.*, 1990; Ohnuma *et al.*, 1994; Jiang *et al.*, 1995; Zhu *et al.*, 1997; Kainou *et al.*, 1999; Kuzuguchi *et al.*, 1999; Okada *et al.*, 2000; Engprasert *et al.*, 2004) and FPS (Chambon *et al.*, 1990; Fujisaki *et al.*, 1990; Koyama *et al.*, 1993; Delourme *et al.*, 1994; Cunillera *et al.*, 1996; Koyama, 1999; Szkopinska and Plochocka, 2005) have been isolated from various organisms. Sequence alignment of the encoded enzymes reveals limited partial homology (Figure 3A). Apparently, the enzymes have been

Figure 2. Structure of the *spo9⁺* gene. (A) Restriction map, subcloning, and construction of null mutants. The arrow indicates the region and direction of the *spo9⁺* ORF, which encodes a protein composed of 351 amino acid residues. All the subclones were derived from pYF1.

Figure 3. (A) Comparison of the amino acid sequences of conserved regions of Fps1 and Spo9 and other members of FPSs and GGPSs. ScBts1, *S. cerevisiae* GGPS, Bts1 (Q12051); AtGGPS, *Arabidopsis thaliana* GGPS6 (BAA23157); and HsGGPS, *Homo sapiens* GGPS1 (AAH05252). FARM and SARM are boxed. The GQ motif is shown in bold. The arrow indicates the fifth amino acid before the FARM motif, which is important for determination of the final product. (B) A phylogenetic tree of GGPSs and FPSs from various organisms calculated by the neighbor joining method. Fps1 and Spo9 are marked by asterisks. Abbreviations: Ag, *Abies grandis*; At, *A. thaliana*; Bm, *Bombyx mori*; Bt, *Bos taurus*; Cp, *Claviceps purpurea*; Dj, *Dendroctonus jeffreyi*; Dm, *Drosophila melanogaster*; Ec, *E. coli*; Gf, *Gibberella fujikuroi*; Gg, *Gallus gallus*; Hb, *Hevea brasiliensis*; Hs, *Homo sapiens*; Kg, *Kitasatospora griseola*; Kl, *Kluyveromyces lactis*; La, *Lupinus albus*; Lc, *Lactarius chrysorrheus*; Ld, *Leishmania donovani*; Mm, *Mus musculus*; Mp, *Myzus persicae*; Mt, *Methanothermobacter thermautotrophicus*; Nc, *Neurospora crassa*; Pa, *Parthenium argentatum*; Rn, *Rattus norvegicus*; Sa, *Sulfolobus acidocaldarius*; Sar, *Streptomyces argenteolus*; Sc, *S. cerevisiae*; Sm, *Sphaceloma manihoticola*; Sp, *S. pombe*; Tb, *Trypanosoma brucei*; Tg, *Toxoplasma gondii*; Tt, *Thermus thermophilus*; and Zm, *Zea mays*.

grouped based on their primary structures (Chen *et al.*, 1994) (Figure 3B). However both enzymes share two aspartate-rich motifs as described above. A BLAST search of the nonredundant proteins in the database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) was carried out using human GGPS (accession no. AAH05252) as a template to identify its *S. pombe* orthologue. We selected proteins whose e-value is <1.0. Two *S. pombe* prenyl diphosphate synthases, Dps1 (e-value 4e-06) and Fps1 (e-value 2e-02) were hit. Dps1 has been known to function as a component of decaprenyl diphosphate syn-

thase (Saiki *et al.*, 2003), but Fps1 remained to be elucidated. These facts suggest that *S. pombe* has no apparent orthologue of GGPS as deduced from the primary structure, and they also imply the possibility that Fps1 and/or Spo9 function(s) as a GGPS. To test this possibility, we examined the relationship between *S. pombe* FPS-like proteins and *S. cerevisiae*

Figure 4. Transcription of *spo9*⁺ and *fps1*⁺ genes. Wild type (MKW5) precultured in growth medium (MM+N) was incubated in liquid sporulation medium (MM-N) at 28°C. After the shift, samples were harvested at the indicated times and subjected to Northern blotting. Top, *spo9*⁺ mRNA; middle, *fps1*⁺ mRNA; bottom, rRNA stained with ethidium bromide.

Figure 2 (cont). Complementation by each subclone: +, complemented; -, did not complement. Restriction enzyme sites: A, AvrII; B, BamHI; Bg, BglII; H, HindIII; S, StuI; and X, XbaI. (B) Comparison of the amino acid sequences of two *S. pombe* FPS-like proteins, Spo9 (CAA19054), and Fps1 (CAB11097) and other members of the FPS family. SpSpo9, *S. pombe* Spo9; SpFps1, *S. pombe* Fps1; ScErg20, *S. cerevisiae* FPS (CAA89462); AtFPS, *Arabidopsis thaliana* FPS2 (AAB07247); and HsFPS, *Homo sapiens* FPS (P14324). Identical amino acid residues are shown in white against black; similar residues are shaded. (C) Determination of *spo9*-B261 mutation. Duplicated nucleotide region is shown in white against black.

Figure 5. (A) *S. cerevisiae* strains YF79 [*erg20Δ* harboring pYEL2(ERG20)] or YF80 [*erg20Δ* harboring pYEL2(*fps1*)] was incubated on YP-galactose (induced) or YPD medium (repressed) at 30°C for 2 d. (B) *S. pombe* strains YF77 [*fps1Δ* harboring pREP81(*fps1*)] and YF78 [*fps1Δ* harboring pREP81(ERG20)] were incubated on MM medium with (repressed) or without thiamine (induced) at 25°C for 3 d. (C) Overexpression of various genes in *spo9Δ*. *S. pombe* strain YF16 (*spo9Δ*) was transformed with either empty pREP1, pREP1(*fps1*), pREP1(*spo9*), pREP1(ERG20), or pREP1(BTS1). The Leu⁺ transformants were incubated on sporulation medium (MEA) at 28°C for 2 d. Bar, 10 μm. (D) The same transformants as shown in C were streaked on YEA medium and incubated at 25 or 36°C for 3 d. (E) Overexpression of various genes in *S. cerevisiae bts1Δ*. *S. cerevisiae* strain SFNY368 (*bts1Δ*) was transformed with empty pYEL2, pYEL2(BTS1), pYEL2(*spo9*), pYEL2(*fps1*), or pYEL2(ERG20). The Leu⁺ transformants were incubated on YP-galactose medium at 30°C for 3 d and at 15°C for 9 d.

GGPS. Both *fps1*⁺ and *spo9*⁺ complemented the cold sensitivity of an *S. cerevisiae bts1Δ* mutant (Figure 5E) and reciprocally, *BTS1* complemented the temperature sensitivity and sporulation deficiency of an *S. pombe spo9Δ* mutant (Figure 5, C and D), but not the lethality of the *fps1Δ* mutant (data not shown). *Erg20* also complemented the cold sensitivity of the *S. cerevisiae bts1Δ* mutant (Figure 5E). However, *BTS1* could not rescue the lethality of the *S. cerevisiae erg20Δ* mutation (data not shown).

S. pombe fps1⁺ Complements an *E. coli ispA* Mutant

We next examined whether *Fps1* and *Spo9* possessed FPS activity by expressing the genes in an *E. coli ispA* mutant that lacks FPS (Fujisaki *et al.*, 1990). The ubiquinone level in the *ispA* mutant is significantly lower than that in wild type,

Figure 6. *fps1*⁺, but not *spo9*⁺, complements an *E. coli ispA* deletion mutant. Ubiquinone was extracted from *E. coli* wild-type strain DH5α and SF7 (*ispA*) harboring pGEX-KG, pGEX(*fps1*) or pGEX(*spo9*). UQ6 was included as an internal standard. The amount of UQ-8 plus UQ-7 was estimated to be 64, 9.2, 24, and 8.6 ng per A₆₀₀ for growth in DH5α (wild type), SF7 harboring pGEX-KG, pGEX(*fps1*), and pGEX(*spo9*), respectively.

because ubiquinone is one of the end products of FPP (Fujisaki *et al.*, 2005, Figure 6, A and B). If *fps1*⁺ and/or *spo9*⁺ encoded an FPS, we presumed that the ubiquinone level of the *ispA* strain expressing these genes would be restored. Ubiquinone was extracted from *ispA* strains either harboring the empty vector, *fps1*⁺, or *spo9*⁺-carrying plasmids, and it was assayed by HPLC. As shown in Figure 6, the ubiquinone level in *fps1*⁺-expressing cells (Figure 6C) was significantly higher than in cells harboring the control vector (Figure 6B). In contrast, the level in the *spo9*⁺-expressing strain was almost identical to that in the control strain (Figure 6D). These data suggest that *Fps1* but not *Spo9* has FPS activity.

Fps1 and *Spo9* Are Sufficient for GGPS Activity

Phenotypes of mutants of *S. pombe* and *S. cerevisiae* harboring disruptions in FPS- and GGPS-related genes are summarized in Table 3 and indicate that the behavior of *spo9*⁺ is similar to that of *BTS1*. Although *S. pombe* has no apparent GGPS-like genes, these data suggest the possibility that *Spo9* functions as a GGPS in *S. pombe*. GGPP is also known as a precursor of carotenoids. Several bacterial groups, including *Erwinia*, possess a gene cluster, *crt*, required for carotenoid biosynthesis that contains *crtE* (geranylgeranyl diphosphate synthase), *crtX* (zeaxanthin β-glucosidase), *crtY* (lycopene cyclase), *crtI* (phytoene desaturase), *crtB* (phytoene synthase), and *crtZ* (β-carotene hydroxylase) genes (Misawa *et al.*, 1990). Because *E. coli* does not possess GGPS, *E. coli* cells transformed with plasmid pACCAR25Δ*crtE* containing all the *crt* genes except for *crtE* from *E. uredovora*, are only able to accumulate zeaxanthin, which has a yellow color, if GGPP is produced (Zhu *et al.*, 1997; Kainou *et al.*, 1999; Engprasert *et al.*, 2004). Therefore, we tested whether *spo9*⁺ has GGPS activity by use of this carotenoid formation test. *fps1*⁺, *spo9*⁺, and *S. cerevisiae BTS1* were cloned into a bacterial expression plasmid, and introduced into

Table 3. Genetic relationship among *S. cerevisiae* and *S. pombe* FPS- and GGPS- like proteins

Phenotype		Complementation by			
		<i>ERG20</i>	<i>BTS1</i>	<i>fps1</i> ⁺	<i>spo9</i> ⁺
<i>S. cerevisiae</i>					
<i>erg20Δ</i>	Lethal	+	–	+	–
<i>bts1Δ</i>	Cold sensitive	+	+	+	+
<i>S. pombe</i>					
<i>fps1Δ</i>	Lethal	+	–	+	–
<i>spo9Δ</i>	Temperature sensitive	+	+	+	+
	Sporulation deficient	+	+	+	+

+, complemented; –, did not complement.

DH10B harboring pACCAR25ΔcrtE. *BTS1* transformants (positive control) produced the expected yellow pigment (Figure 7). However, neither *fps1*⁺ nor *spo9*⁺ transformants formed yellow colonies. Because it is known that FPS and GGPS from many organisms form a dimer and that dimer formation is essential for enzyme activity (Tarshis *et al.*, 1994; Szkopinska and Plochocka, 2005), we speculated that if coexpression of Fps1 and Spo9 is needed for GGPS activity, cotransformation of these genes in *E. coli* might result in formation of a functional GGPP-producing enzyme. As shown in Figure 7, transformants expressing *fps1*⁺ and *spo9*⁺ simultaneously produced a notable yellow pigment. These data suggest that Fps1 and Spo9 together form a heteromeric GGPS.

We next examined whether Fps1 and Spo9 physically interact each other, by use of the yeast two-hybrid assay. The assay indicated a positive interaction between Fps1 and Spo9 (Figure 8A). An interaction was also observed between Fps1 and Fps1 and between Spo9 and Spo9, but the level was considerably lower than between Fps1 and Spo9 (Figure 8A). To assess the association of Fps1 and Spo9 in *S. pombe* cells, a chimeric protein consisting of Fps1 fused to GFP was coexpressed with GST-tagged Spo9 in *S. pombe*. Fps1-GFP was immunoprecipitated with anti-GFP antibody, and copurification of GST-Spo9 was verified by Western blotting

Figure 7. Coexpression of Spo9 and Fps1 is required for GGPS activity. *E. coli* DH10B carrying pACCAR25ΔcrtE was transformed with pMAL-c, pMAL(BTS1), pMAL(spo9), pMAL(fps1), pMAL(spo9+fps1), pMAL(spo9-R109Q+fps1), pMAL(spo9+fps1-R104Q), or pMAL(spo9-R109Q+fps1-R104Q); plated on LB medium supplemented with ampicillin and chloramphenicol; and incubated at 28°C for 3 d.

Figure 8. Spo9 interacts with Fps1. (A) Yeast two-hybrid analysis. Plasmids expressing the respective Gal4 activation domain (AD) and Gal4 DNA-binding domain (BD) fusions were tested for two-hybrid interaction. (B) Coimmunoprecipitation of Spo9 and Fps1. Cell extracts were prepared from vegetative cells expressing tagged proteins, GFP and GST-Spo9 or Fps1-GFP and GST-Spo9, and subjected to immunoprecipitation with anti-GFP antibody. Precipitates were analyzed by Western blotting by using anti-GFP, or anti-GST antibody.

by using anti-GST antibody. As shown in Figure 8B, GST-Spo9 copurified with Fps1-GFP but not with unfused GFP. The interaction between Spo9 and Fps1 was also observed in an *E. coli* lysate (data not shown). Together, these data indicate that Fps1 and Spo9 interact directly with each other.

To understand the role of Fps1 and Spo9 in GGPS activity, we constructed two mutants, Fps1^{R104Q} and Spo9^{R109Q}, in which the conserved arginine just after the FARM motif was replaced with glutamine. These arginines seem to be critical for function of Fps1 and Spo9 because *fps1*-R104Q and *spo9*-R109Q were unable to complement the phenotype of *fps1Δ* and *spo9Δ*, respectively. An *E. coli* strain that harbored plasmid pACCAR25ΔcrtE coexpressing either Fps1 plus Spo9^{R109Q} or Fps1^{R104Q} plus Spo9 failed to produce the yellow pigment. Surprisingly, the same *E. coli* strain expressing Fps1^{R104Q} plus Spo9 formed yellow colonies (Figure 7). These data suggest that GGPS is dependent on the enzymatic activity of Spo9 but not on that of Fps1.

If *spo9*⁺ encodes a component of GGPS, GGPS activity should be detected in *S. pombe* wild type, but not in the *spo9Δ* mutant. In vitro GGPS activity was then measured in cell-free extracts from these strains. Cells were homogenized, and [¹⁴C]IPP and FPP were used as substrates to detect prenyl diphosphate synthase activity. The extracts were subsequently hydrolyzed with acid phosphatase, and the products were separated by reversed phase TLC. Although wild type was able to produce geranylgeraniol (GGOH), the *spo9Δ* strain barely showed any GGPS activity (Figure 9). The GGOH spot in the *spo9Δ* strain was intensified by the overexpression of the *spo9*⁺ gene. These data indicate that Spo9 indeed has GGPS activity.

Figure 9. GGPS activity is significantly reduced in *spo9* mutant. GGPS was assayed using [1-¹⁴C]IPP and FPP as substrates with crude extracts from wild-type (TN8), *spo9Δ* (YF16), and the *spo9Δ* harboring *spo9+* (YF81) cells and *E. coli* DH5α harboring pBH expressing human GGPS1. The reaction products were hydrolyzed by acid phosphatase, and the resulting alcohols were analyzed by reversed phase TLC. An arrow indicates the product of GGOH. Other than GGOH, there are three intense spots; the uppermost and the lowest spots are farnesol and decaprenol, respectively, and a middle one is presumably ergosterol. These spots are indicated by arrowheads.

Protein prenylation is a posttranslational modification by which isoprenoid compounds are covalently attached to cysteine residues at or near the C terminus of proteins (Casey 1992, 1994; Schafer and Rine, 1992; Omer *et al.*, 1994). A number of proteins are known to be prenylated, and in the most cases, the prenylation is essential for their membrane localization. Indeed, in the *S. cerevisiae* *bts1Δ* mutant, the membrane association of the small GTPases, Ypt1 and Sec4, is defective due to lack of geranylgeranylation (Jiang *et al.*, 1995). Ypt7, a fission yeast orthologue of Rab7 GTPase, mediates fusion of endosomes to vacuoles and homotypic vacuole fusion (Iwaki *et al.*, 2004). Ypt7 has a Cys-X-Cys at its C terminus, which is recognized by geranylgeranyl transferase. If *spo9+* encodes a sole GGPS, disruption of the gene should result in depletion of GGPP and loss of geranylgeranylation of Ypt7. To test this hypothesis, we examined the membrane association of Ypt7 in the *spo9Δ* mutant. Cell extracts were fractionated into a membrane fraction and a cytosolic fraction, and the distribution of Ypt7 was examined in each by Western blot analysis. Spo14, a membrane-bound protein was used as a probe for the membrane fraction (Nakamura-Kubo *et al.*, 2003). Most Ypt7 was detected in the membrane fraction prepared from wild-type cells. However, in *spo9Δ* cells, Ypt7 was detected in both cytosolic

Figure 10. *spo9* mutation causes mislocalization of Ypt7 but not Rhb1. Wild-type cells (YF51) carrying GFP-Ypt7 and *spo9Δ* cells (YF54) were ruptured, and then they were subjected to differential centrifugation to fractionate into a P100 membrane fraction and an S100 supernatant. Each fraction was resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting using either anti-GFP, anti-Rhb1, or anti-Spo14 (control) antibody.

and membrane fractions (Figure 10). Thus, the membrane association of Ypt7 is partially defective in *spo9Δ* cells. In the unfractionated extract from the *spo9Δ* cells, Ypt7 was detected as a doublet. In contrast, in both cytosolic and membrane fractions from *spo9Δ* cells, Ypt7 was detected as a single band, although migration of Ypt7 detected in the membrane fraction was slightly faster than that in the cytosolic fraction. Generally, the prenylated protein migrates faster than its unmodified form (Figure 10). These data support the notion that the extent of geranylgeranylation was lower in *spo9Δ* cells. Rhb1, a fission yeast orthologue of the human small GTPase Rheb, is known to be farnesylated and the farnesylation is important for membrane association (Yang *et al.*, 2001; Nakase *et al.*, 2006). If Spo9 mainly functions as a GGPS and not an FPS, the distribution of Rhb1 should not be affected by the *spo9* mutation. As shown in Figure 10, the distribution of Rhb1 in the *spo9Δ* cells in cytosolic and membrane fractions was almost identical to that in wild-type cells. Together, these data are consistent with the possibility that Spo9 is an essential component of GGPS in fission yeast.

The *spo9* Mutant Is Defective in Forespore Membrane Formation

Phenotypic analyses of many sporulation-deficient mutants have revealed that most are defective in forespore membrane formation (Nakamura *et al.*, 2001, 2005; Nakase *et al.*, 2001; Nakamura-Kubo *et al.*, 2003; Onishi *et al.*, 2003; Koga *et al.*, 2004; Kashiwazaki *et al.*, 2005). To examine in detail how the *spo9* mutation impairs sporulation, the assembly of forespore membrane in the *spo9-B261* mutant was observed using GFP-tagged Psy1, a syntaxin-like protein (Nakamura *et al.*, 2001). Progression of meiosis was monitored by observing the formation and elongation of spindle microtubules. In wild-type cells, most haploid nuclei produced by meiotic second divisions were encapsulated by the forespore membrane. In the *spo9-B261* mutant, forespore membrane formation initiated normally during meiosis II (Figure 11A), but subsequent development was blocked (Figure 11B). Only 40% of the zygotes contained four complete sets of nucleated prespores (membrane-bounded spore precursors), although they were remarkably small (type I in Figure 11B). Anucleated prespores were observed in 50% of the zygotes (type II in Figure 11B). In type III, assembly of the

Figure 11. Aberrant assembly of the forespore membrane in *spo9* mutant. (A) Assembly of the forespore membrane during metaphase II. Wild type (YN68) and *spo9-B261* (YF5) were cultured in SSL-N to induce meiosis at 28°C for 8 h. Fixed cells were doubly stained with anti- α -tubulin and DAPI. (B) Classification of terminal phenotypes of the forespore membrane in *spo9* zygotes. Strain, culture conditions and staining procedures are the same as described in Figure 11A. Type I, forespore membranes engulfed each nucleus but prespores were remarkably small. Type II, four aggregates of GFP-Psy1 were formed close to nuclei. Type III, forespore membrane formation arrested. Bars, 10 μ m.

forespore membrane seemed to be arrested halfway (Figure 11B). These results indicate that forespore membrane formation initiates normally, but that subsequent development and integrity of the forespore membrane are impaired in *spo9*. Essentially identical data were obtained when *spo9* Δ cells were used.

To investigate the structure of the forespore membrane in more detail, we observed the fine structures of *spo9* Δ cells by thin-section electron microscopy. As shown in Figure 12, membrane vesicle accumulation was notable in the cytoplasm of immature *spo9* Δ asci, but such vesicle accumulation was not observed in wild-type asci. The vesicles in the *spo9* Δ mutant had an average diameter of 79 ± 42 nm, consistent with previous measurements of post-Golgi secretory vesicles in *S. cerevisiae*. Furthermore, Golgi-like structures were often observed in the *spo9* Δ mutant (Figure 12B). In addition, apparently abnormal forespore membranes were also observed. Anucleated spore-like bodies were often observed as well (Figure 12, B–D). These data are essentially identical to those obtained by fluorescence microscopy described above. Thus, *spo9*⁺ is important for the assembly of the forespore membrane, especially the expansion of the membrane.

DISCUSSION

Isolation and Characterization of Two Novel Genes Encoding a Farnesyl Diphosphate Synthase-like Protein in *S. pombe*

Both FPS and GGPS are highly conserved enzymes from bacteria to humans. Here, we isolated two novel genes, *fps1*⁺

Figure 12. Fine structures of *spo9* Δ asci. Mature spores in wild type (A) and anucleated spore-like bodies in *spo9* Δ mutant (B–D). N, SPB, and FSM denote the nucleus, spindle pole body, and forespore membrane, respectively. Note that many membrane vesicles (indicated by arrows) and Golgi-like structures (indicated by arrowheads) are present in the cytoplasm of *spo9* Δ . Bar, 0.5 μ m.

and *spo9*⁺. Both Fps1 and Spo9 share significant identity (40–50%) with typical FPSs, but only modest identity (~20%) with typical GGPSs. Thus, both Fps1 and Spo9 more closely resemble FPS rather than GGPS in amino acid sequence. Based on database search and phylogenetic analysis, *S. pombe* has no other GGPS-encoding gene. In *S. cerevisiae*, Erg20 and Bts1 are specifically recognized as an FPS and a GGPS, respectively. Overexpression of *fps1*⁺ in an *E. coli* *ispA* mutant that lacks FPS restored the production of ubiquinone, which is one of the end products of FPP, suggesting that Fps1 functions as a typical FPS in *S. pombe*. Because GGPP can be a substrate for octaprenyl diphosphate synthase, a key enzyme of ubiquinone synthesis, it is also possible that the same complementation of the *ispA* mutation is due to the GGPS activity of Fps1. However, this possibility seems unlikely by the following reasons. First, the overexpression of Fps1 but not *S. cerevisiae* GGPS Bts1 could complement the lethality of an *S. cerevisiae* *erg20* mutant that lacks FPS. Second, *ERG20* but not *BTS1* could complement the lethality of an *S. pombe* *fps1* Δ mutant. Third, overexpression of *fps1*⁺ alone in *E. coli* cells cannot produce the substantial amount of GGPP by judging from the result of carotenoid producing assay (Figure 7). In similar experiments, no evidence was obtained to support the possibility that Spo9 functions as an FPS, although it resembled other FPSs in primary structure. The failure of *spo9*⁺ to complement the phenotype of either *fps1* Δ or *erg20* Δ makes it unlikely that Spo9 functions as an FPS.

S. pombe Geranylgeranyl Diphosphate Synthase Is Composed of a Typical FPS, Fps1, and an FPS-like Protein, Spo9

Although *S. pombe* has two FPS-like proteins, a protein that shares significant similarity with GGPS in the *S. pombe* genome database was not found. Several lines of evidence obtained in this study suggest that both Spo9 and Fps1 form a heteromer and that this complex functions as GGPS. First, *spo9*⁺ was able to complement the phenotype of an *S. cer*

visiae *bts1* Δ mutant, which lacks GGPS, and *BTS1* was able to rescue *spo9* Δ . Second, GGPS activity was significantly lower in *S. pombe* *spo9* Δ cells than in wild type. Third, the *spo9* mutation perturbed the membrane association of a geranylgeranylated protein but not that of a farnesylated protein. Fourth, Spo9 directly interacts with Fps1. Fifth, *E. coli* cells that express a set of genes required for carotenoid biosynthesis but without a GGPS gene, only produced carotenoid when *spo9*⁺ and *fps1*⁺ were expressed simultaneously.

Most short-chain prenyltransferases are known to function as homodimers (Koyama, 1999). However, in other cases, as in spearmint (Burke *et al.*, 1999), geranyl diphosphate synthase forms a heterodimer and decaprenyl diphosphate synthase in *S. pombe* and humans forms a heterotetramer (Saiki *et al.*, 2003, 2005). Each of these heteromers includes a subunit that has significant homology with typical prenyltransferases that contain two aspartate-rich motifs. For example, Dps1, a component of decaprenyl diphosphate synthase from *S. pombe* has a high similarity (~40%) with typical long-chain prenyl diphosphate synthases, whereas the other component, Dlp1, has only weak similarity (~20%) with prenyl diphosphate synthases (Suzuki *et al.*, 1997; Saiki *et al.*, 2003, 2005). In marked contrast to these proteins, both subunits of *S. pombe* GGPS share high similarity with FPSs, but only weak similarity with typical GGPSs. We attempted to determine whether the *S. pombe* GGPS is composed of a heterodimer or a heterotetramer by a gel filtration analysis. However, neither protein could be detected in any fractions corresponding to heterodimers or heterooligomers (data not shown). This fact suggests that the interaction between Fps1 and Spo9 is so weak that the complex readily dissociates during gel filtration. In the experiment showing the coimmunoprecipitation of Spo9 and Fps1 in *S. pombe* cells, a density of Spo9 relative to Fps1 in lane 2 (IP) is lower than that in lane 2 (lysates) (Figure 8B). This result might be attributed to their dissociation during the immunoprecipitation. Alternatively, the result could be interpreted by the formation of homodimers of Fps1, as revealed in Figure 8A.

We obtained clear evidence that *S. pombe* GGPS is composed of Fps1 and Spo9, but at the same time, we cannot rule out the possibility that Fps1 might have weak GGPS activity for the following reasons. First, although GGPS activity was significantly reduced in *spo9* Δ cells, it was not absent (Figure 9). In addition, geranylgeranylation-dependent membrane association of Ypt7 was observed to some extent in *spo9* Δ cells (Figure 10). These results suggest that GGPP is still synthesized in *spo9* Δ cells. Second, overexpression of *fps1*⁺ was able to suppress the temperature sensitivity and sporulation deficiency of *spo9* Δ , and GGPS activity was detected in those cells (data not shown). Several FPSs have been known to retain GGPS activity as well (Chen and Poulter, 1993; Fujiwara *et al.*, 2004). It is reasonable that GGPS activity was detected under nonphysiological conditions, i.e., overexpression of the Fps1 protein. At present, we cannot examine the GGPS activity and membrane association of Ypt7 and Rhlb1 in the *fps1* disruptant, because *fps1*⁺ is essential for growth. Analysis of conditional *fps1* mutants will allow us to elucidate the role of Fps1 as a GGPS.

Previous studies have revealed that the amino acid residue located on the fifth position before the FARM of FPS is important for the determination of chain length of a final product. In all of known FPSs, including *S. pombe* Fps1, the corresponding amino acid is either phenylalanine or tyrosine, but the corresponding amino acid is cysteine in Spo9 (Figure 3A). Ohnuma *et al.* (1996) constructed 20 FPS mutant proteins from *Bacillus stearothermophilus*, each of which has a

different amino acid at position 81 located on the fifth position before the FARM, and revealed that the average chain length of products is inversely proportional to the accessible surface area of substituted amino acids. Interestingly, *B. stearothermophilus* FPS Y81C mutant, in which tyrosine 81 was replaced with cysteine, effectively produced GGPP (Ohnuma *et al.*, 1996). These data suggest the possibility that cysteine 95 is important for the Spo9 function as GGPS. To test this possibility, we constructed the mutant protein Spo9^{C95F}, in which cysteine 95 was replaced with phenylalanine, and introduced it into *fps1* Δ . However, the mutant protein could not complement the lethality of *fps1* Δ , and it did complement the phenotypes of *spo9* Δ . Conversely, the *fps1*-F90C mutant gene could complement the lethality of *fps1* Δ . Thus, it is unlikely that cysteine 95 is the only important determinant for the function of *S. pombe* Spo9 as a GGPS.

Role of Spo9 in Sporulation

Our study also demonstrates that GGPS plays a crucial role in sporulation. Fluorescence microscopy by using the forespore membrane marker protein GFP-Psy1 revealed that the forespore membrane formation initiates normally, but that the subsequent process, expansion of the membrane was severely impaired in *spo9* Δ . GGPP is the precursor for several important metabolites, including dolichols, ubiquinones, and heme a in *S. pombe*. One possibility is that the decrease in these metabolites leads to the defect in sporulation. If so, various steps in sporulation should be affected. However, the *spo9* mutation perturbed only expansion of forespore membrane. The other events of sporulation, the meiotic nuclear division and the initiation of forespore membrane formation, seemed to proceed normally in *spo9* Δ . Therefore, it seems unlikely that the decrease of end products such as heme a and/or ubiquinone directly affects the sporulation-defective phenotype of *spo9* Δ . At least there is no defect in sporulation in ubiquinone-deficient strains (unpublished observations). Alternatively, it is possible that the *spo9* mutation perturbs the membrane association of a geranylgeranylated protein necessary for sporulation. Indeed, the membrane association of Ypt7, a putative geranylgeranylated protein, is significantly diminished in *spo9* Δ . Our previous study revealed that Ypt7 is essential for proper spore formation (Kashiwazaki *et al.*, 2005). Thus, the defective membrane association of Ypt7 could plausibly result in the *spo9* Δ mutant phenotype. Nonetheless, the phenotype of *spo9* Δ is much severe than that of *ypt7* Δ , suggesting that other geranylgeranylated proteins are involved in sporulation as well (Giannakouros *et al.*, 1992). In an *S. cerevisiae* *bts1* mutant, the failure to geranylgeranylate Ypt1 and Sec4 leads to a defect in the membrane association of these proteins. The *bts1* mutation may impair intracellular membrane trafficking, because these proteins play essential roles in this process (Rossi *et al.*, 1991; Jiang *et al.*, 1995). A number of genes that play roles in the general secretion machinery have been reported to be necessary for sporulation in both *S. cerevisiae* and *S. pombe* (Neiman, 1998; Nakase *et al.*, 2001; Nakamura *et al.*, 2001, 2005; Jantti *et al.*, 2002; Nakamura-Kubo *et al.*, 2003; Nakanishi *et al.*, 2006). Among them, *sec4* mutant also exhibits a sporulation deficiency in *S. cerevisiae* (Neiman, 1998). *S. pombe* possesses a Sec4 homologue, Ypt2, which also has a consensus sequence for geranylgeranylation (Haubruck *et al.*, 1990). Defective geranylgeranylation of Ypt2 in the *spo9* mutant might cause a sporulation deficiency. This possibility is further supported by our observation that many membrane vesicles accumulate in the cytoplasm during sporulation in *spo9* Δ cells.

In summary, we report here that *S. pombe* FPS is composed of Fps1 and that GGPS is composed of a typical FPS, Fps1, and an FPS-like protein, Spo9. To our knowledge, a heteromer of two FPS-like proteins is a novel type of GGPS that has not been previously reported. We speculate that *S. pombe* developed this GGPS by duplicating an FPS gene followed by limited mutational changes in the duplicated copy resulting in the present heteromeric structure.

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