Genetic characterization of genes encoding enzymes catalyzing addition of phospho-ethanolamine to the glycosylphosphatidylinositol anchor in \textit{Saccharomyces cerevisiae}

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(Received 1 July 2002, accepted 3 September 2002)


\textit{MPC1}/\textit{GPI13}/\textit{YLL031C}, one of the genes involved in the addition of phospho-ethanolamine to the glycosylphosphatidylinositol (GPI) anchor core, is an essential gene. Three available temperature-sensitive mutant alleles, \textit{mpc1-3}, \textit{mpc1-4}, and \textit{mpc1-5}, displayed different phenotypes to each other and, correspondingly, these mutants were found to have different mutations in the \textit{MPC1} ORF. Temperature-sensitivity of \textit{mpc1-5} mutants was suppressed by 5 mM \textit{ZnSO}_4 and by 5 mM \textit{MnCl}_2. Multicopy suppressors were isolated from \textit{mpc1-5} mutant. Suppressors commonly effective to \textit{mpc1-4} and \textit{mpc1-5} mutations are \textit{PSD1}, encoding phosphatidylserine decarboxylase, and \textit{ECM33}, which were found to suppress the temperature-sensitive phenotype shown by the \textit{fsr2-1} and \textit{las21A} mutants, those of which have defects in the GPI anchor synthesis. \textit{PSD2}, encoding another phosphatidylserine decarboxylase that is localized in Golgi/vacuole, was found to be able to serve as a multicopy suppressor of \textit{mpc1} and \textit{fsr2-1} mutants but not of the \textit{las21A} mutant. In contrast to \textit{psd1A}, \textit{psd2A} showed a synthetic growth defect with \textit{mpc1} mutants but not with \textit{fsr2-1} or \textit{las21A}. Furthermore, \textit{psd1A psd2A mpc1} triple mutants did not form colonies on nutrient medium unless ethanolamine was supplied to the medium, whereas \textit{psd1A psd2A fsr2-1} or \textit{psd1A psd2A las21A} triple mutants grew on nutrient medium without supplementation of ethanolamine. These observations suggest that Mpc1 preferentially utilizes phosphatidylethanolamine produced by Psd2 that is localized in Golgi/vacuole. \textit{fsr2-1 dpl1A psd1A} strains showed slower growth than \textit{fsr2-1 dpl1A psd2A}, suggesting that Fsr2 enzyme depends more on Dpl1 and Psd1 for production of phosphatidylethanolamine. Las21 did not show preference for the metabolic pathway to produce phosphatidylethanolamine.

\section*{INTRODUCTION}

Glycosylphosphatidylinositol (GPI) anchor proteins are major extracellular proteins and widely found in eukaryotic organisms. Some GPI anchor proteins are localized to a microdomain in the plasma-membrane and exert various functions such as in signal transduction (Stulnig et al., 1997; Mouillet-Richard et al., 2000), in prion formation (Taraboulos et al., 1995; Mouillet-Richard et al., 1997), and in cell-cell recognition (Lipke et al., 1989; Caro et al., 1997). Proteins to be destined to become GPI anchor proteins have a common feature of the amino acid sequence: the signal sequence at the N-terminus and the hydrophobic C-terminal sequence containing the site (the \(\omega\) site) where peptide bond is cleaved and bound to ethanolamine of the GPI anchor by transamidation (Sharma et al., 1999). Thus ethanolamine in the GPI anchor is essential for GPI anchor protein synthesis.

The GPI anchor core is produced in the ER by sequential addition of monosaccharide and phospho-ethanolamine to phosphatidylinositol (PI). The synthesis proceeds in the order of the transfer of N-acetyl-glucosamine (GlcNac) to PI, de-N-acetylation of GlcNac-PI, the successive addition of three \(\alpha\)-mannoses, and the addition of phospho-ethanolamine to mannoses (Takeda and Kinoshita, 1995). The global picture of the GPI anchor synthesis can be drawn but many of enzyme reactions are yet to be characterized by in vitro experiments using purified enzymes. Since the GPI anchor synthesis pathway is

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well conserved from yeast to mammals to the extent that some enzymes are exchangeable between different organisms (Leidich et al., 1995; Hiroi et al., 1998; Inoue et al., 1996; Watanabe et al., 1999; Naganuma et al., 2000), yeast affords a useful system for studying GPI metabolism and the biological significance of GPI proteins.

Three yeast genes, LAS21/GPI7 (Toh-e and Oguchi, 1998; Benachour et al., 1999), MCD4/FSR2 (Matsumoto et al., 1986; Mondesert et al., 1997), and MPC1/GPI13 (Toh-e and Oguchi, 2000; Flury et al., 2000; Taron et al., 2000), encode proteins that are reported to be involved in the reactions transferring phospho-ethanolamine of phosphatidylethanolamine (PE) to the mannose moiety of the GPI anchor. Each of three mannososes receives one phospho-ethanolamine. The structure of Las21, Fsr2, and Mpc1 is similar in that the amino acid sequence of the N-terminal one third is cytoplasmic and similar to each other and that the remaining portion is multiply spanning membrane but does not show a sequence similarity (Nelissen et al., 1997). In spite of their similarity, they play a distinctive function in the cell. Mcd4/Fsr2 and Mpc1/Gpi13 transfer phospho-ethanolamine to the first and third mannose, respectively (Gaynor et al., 1999; Flury et al., 2000; Taron et al., 2000). Gpi7/Las21 may transfer phospho-ethanolamine to the second mannose (Benachour et al., 1998), but this reaction may not be necessary or proceed spontaneously. The ethanolamine moiety of the GPI anchor acts as a receptor for protein to be fused to the GPI anchor, therefore, the steps catalyzed by these proteins are essential. In accordance with this, the two genes, MPC1 and FSR2, are essential for growth. Intracellular localization of Fsr2 was deduced from its amino acid sequence to be in the ER (Gaynor et al., 1999) and Mpc1/Gpi13 was reported to be localized to the ER by biochemical fractionation experiments (Flury et al., 2000). Biochemical fractionation and protease treatment indicated that Las21 is localized to the plasma membrane (Benachour et al., 1998). Las21 is different from Fsr2 and Mpc1 in that the former is nonessential. Furthermore, Las21 has a heavy metal associated motif at its N-terminus that has some role in the copper metabolism although it overlaps with a putative signal sequence. Las21 may have a different role other than GPI anchor synthesis (Toh-e and Oguchi, 2001).

The donor of phospho-ethanolamine in GPI anchor synthesis is known to be PE (Menon and Stevens, 1992). PE is produced by two pathways; (1) from phosphatidylserine (PS) by phosphatidylserine decarboxylase at mitochondria (Psd1) and at Golgi/vacuole (Psd2) and (2) by the Kennedy pathway. Phospho-ethanolamine produced by Dpl1 from dihydrosphingosine-1-phosphate is incorporated into PE. Psd1, that is located at mitochondria, explains a major phosphatidylserine decarboxylase activity in the cells. The fact that enzymes transferring PE to the GPI anchor are localized to different organelles indicates that PE produced must access to the enzymes localized in the different compartments.

Since GPI anchor proteins play various functions, it is of interest to elucidate how production of these proteins are regulated and how they are delivered to the site of action. To this end, it is necessary to uncover the metabolic pathway of GPI anchor synthesis and the regulation of each enzymatic step. Since GPI anchor proteins are bound to the nitrogen of ethanolamine by transamidation, incorporation of ethanolamine into the GPI anchor is essential for production of GPI anchor proteins that contain proteins with an essential function for life. This is consistent with the fact that FSR2/MCD4 and MPC1/GPI13 are essential genes.

In the previous report (Toh-e and Oguchi, 2000), we described temperature-sensitive mutants of MPC1. The sites of mutations of mpc1-3 and mpc1-4 are found in the C-terminal membrane spanning domain and those of mpc1-5 in the N-terminal hydrophilic domain. These mutants displayed different phenotypes in response to Zn\(^{2+}\) and Mn\(^{2+}\) ions. Multicopy suppressors of the mpc1-4 mutant or the mpc1-5 mutant or both are described and we discuss spatial specificity of reactions transferring phospho-ethanolamine to the GPI anchor core.

MATERIALS AND METHODS

**Microbial techniques.** Yeast strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. Methods for yeast genetics and for yeast cultures were described previously (Sherman, 1991). ZnSO\(_4\), MnCl\(_2\), and ethanolamine were added to YPD (yeast extract-peptone-dextrose) or omission medium at the concentration of 5 mM after autoclaving when needed. A permissive temperature for a temperature-sensitive mutant was 25°C and a restrictive temperature was 34 – 37°C depending on a mutant. Yeast transformation experiments were carried out as described previously (Ito et al., 1983; Schiestl and Gietz, 1991). *Escherichia coli* strain DH5α (endA1 gyrA96 hsdR17(rk--mk--) recA1 relA1 supE44 thi-1 deoR ΔlacZYA-argF(U169)lacZAM15 F'λθ) was used for construction and propagation of plasmids. *E. coli* was grown in LB medium (0.8 g Bacto-tryptone, 5 g Bacto-yeast extract, and 5 g NaCl) at 37°C. Sodium ampicillin was added as appropriate. Agar was added to 2% to prepare solid medium.

**DNA manipulations.** The methods for DNA engineering were described by Sambrook et al. (1989). Yeast genomic DNA was isolated by the method described by Hoffman and Winston (1987). DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977), and the adenine residue of the putative translation initiation codon ATG was defined as the
+1 nucleotide. DNA fragments were amplified by polymerase chain reaction (PCR) using appropriate primers and template. Oligo DNA primers used in this study were listed in Table 3. W303D cells were disrupted with glass beads by vortexing and insoluble material was removed by centrifugation at $15,000 \times g$ for 5 minutes. The resulting supernatant was used as template unless otherwise noted.
**Construction of plasmids.** TOP2041 (psd1::HIS3): A DNA fragment containing about 1 kb upstream of the PSD1 ORF was amplified using a pair of primer, PSD1-1 and PSD1-2, and digested with NotI and BamHI. A DNA fragment containing about 1 kb of downstream of the PSD1 ORF was amplified using a pair of primer, PSD1-3 and PSD1-4, and digested with SalI and BamHI. The resulting two kinds of DNA fragments were inserted between the NotI and SalI sites of pSR303 (Sikorski and Hieter, 1989) to generate TOP2041. To disrupt genomic PSD1, TOP2041 was linearized by BamHI and used as donor DNA to transform a wild type strain from His– to His+. A net consequence of this transformation experiment is replacement of the PSD1 ORF with the pSR303 sequence. Correct disruption was verified by PCR.

TOP2076 (psd2::TRP1) and TOP2153 (dpl1::URA3): These plasmids were constructed by a similar strategy described above, and used for disrupting the genomic counterpart. The functional PSD2 gene was cloned by amplifying the 3.2kb DNA fragment using a pair of primer PSD-1 and PSD2-4. The resultant DNA fragment was cut with BamHI and then inserted at the BamHI site of YEplac112 (Gietz and Sugino 1988) to generate TOP2109.

TOP2107 (MPC1-8GFP): The N-terminally truncated MPC1 gene without stop codon was amplified by PCR using a pair of primer MPC1-x and MPC1-y. The resulting DNA fragment was digested with HindIII and BamHI and inserted into the HindIII and BamHI sites of pGFP vector (Araki and Toh-e, Unpublished) in frame with respect to the Mpc1 and GFP. TOP2107 was linearized at the BstEII site within the MPC1 ORF and introduced into the wild type strain. The net result is replacement of the MPC1 gene with the MPC1-8GFP and the N-terminally truncated mpc1 gene.

**Cytological methods.** Microscopic observations were made according to the method described by Yoshida and Toh-e (2001) and Iwase and Toh-e (2001). Cells in logarithmic phase were harvested and fixed with 3.7% formalin at a room temperature for 30 minutes. Indirect immunofluorescence staining was carried out using anti-HA monoclonal antibody (3F10) (Roche) as primary antibody and anti-mouse IgG rat antibody conjugated with Alexa 546 (Molecular Probe) as secondary antibody. DNA was stained with Hoechst 33342 (Hiraoka et al., 2000). Photographs were taken by a cooled CCD camera using an IP lab software equipped to an epifluorescence microscope Olympus BX60 with an objective UplanFI 100x.

**RESULTS AND DISCUSSION**

**Localization of Mpc1-8GFP.** GPI anchor biosynthesis proceeds in the ER. In accordance with this, many of enzymes involved in this pathway are reported to be localized to the ER. Three enzymes are participating in the addition of phospho-ethanolamine to mannos residues of the core...
Fig. 1. Intracellular localization of Mpc1. (A) YAT3041 (MPC1-8GFP) cells in logarithmic phase at 25°C in YPD were harvested and resuspended in PBS (phosphate buffered salin). Hundred µl of suspension was added Hoechst 33342 at 1 µg/ml. After 10 minutes at room temperature, GFP signals and Hoechst signals in cells were observed by epifluorescent microscope BX60. (B) YAT3041 cells carrying pRS313 growing logarithmically at 25°C were harvested and fixed with 3.7% formaldehyde for 30 minutes at room temperature. Cells were processed according to the indirect immunofluorescence method using anti-HA monoclonal antibody as primary antibody and anti-mouse IgG rat antibody conjugated with Alexa 546 as secondary antibody. (C) YAT3041 cells carrying pSR313-SEC71-HA were similarly processed and observed as in (B).
of the GPI anchor: they are Las21/Gpi7 (Toh-e and Oguchi, 1998; Benachour et al., 1999), Fsr2/Med4 (Matsumoto et al., 1986; Mondesert et al., 1997), and Mpc1/Gpi13 (Toh-e and Oguchi, 2000; Flury et al., 2000; Taron et al., 2000), and are structurally similar to each other (Nelissen et al., 1997). Flury et al. (1998) reported that Las21/Gpi7 is localized to plasma membrane and Gaynor et al. (1999) deduced the localization of Med4 at the ER from its amino acid sequence. By exploiting Med4-GFP fusion protein, Packeiser et al. (1999) reported that Med4 was localized to the vacuolar membrane. Since the Med4-GFP construct lost some C-terminal amino acid residues including the ER retention signal, localization of Med4 should be reexamined experimentally. Flury et al. (2000) reported that Yll031c/Gpi13 is localized to the ER by biochemical fractionation experiments. We examined the localization of Mpc1 by constructing a strain (YAT3041) containing MPC1-8GFP in place of MPC1. When this 8GFP was fused with a nuclear protein, the fusion protein was localized to the nucleus, indicating that MPC1-8GFP is appropriate for analyzing localization of Mpc1. YAT3041 cells in logarithmic phase were stained with Hoechst33342, and GFP and DNA fluorescence were observed. As shown in Fig.1A, the GFP sig-

![Fig. 2. Variable severity of temperature-sensitivity of growth displayed by mpc1 mutants. Wild type, YAT2572 (mpc1-3), YAT2573 (mpc1-4), and YAT2574 (mpc1-5) strains were streaked across YPD plates, one of which was incubated at the indicated temperature.](image-url)
nal was observed at membranous structure surrounding the nucleus. This localization is similar to that of a typical ER protein. To confirm that Mpc1 is localized to the ER, we introduced pRS313-SEC71-HA and pRS313 separately into YAT3041, and the transformants were subjected to a cytological observation. Although the Mpc1-8GFP gene contains two copies of HA, the HA staining in YAT3041[pRS313] was negligible (Fig. 1B). In YAT3041[pRS313-SEC71-HA] cells, the GFP signal and HA staining were superimposable (Fig. 1C), indicating that Mpc1 is localized to the ER as reported by Flury et al. (2000).

**Phenotypes of mpc1-3, mpc1-4, and mpc1-5 mutants**

We first tested the difference in temperature-sensitivity among mpc1 mutants. Each mutant and wild type strains were streaked across YPD plates, one of which was incubated at the indicated temperature. As shown in Fig. 2, the severity of temperature-sensitivity is in a decreasing order of mpc1-3, mpc1-4, and mpc1-5. The motif, HE/DxxH or HxxE/DH, is seen in the Zn\(^{2+}\) binding protein (Tomoyasu et al., 1995; Kochi et al., 1994; Luciano et al., 1998). Mpc1 contains one such motif HxxEH (+395 – +399) within the N-terminal hydrophilic region. We examined if Zn\(^{2+}\) could recover the growth of any of a mpc1, fsr2-1, and las21Δ temperature-sensitive mutants. The wild type strain, fsr2-1, las21Δ, and mpc1 mutants were streaked across YPD plates or that containing 5 mM ZnSO\(_4\) and one YPD plate was incubated at 25°C and one YPD plate and YPD with Zn\(^{2+}\) were incubated at a restrictive temperature. As shown in Fig. 3, the temperature-sensitivity of the mpc1-5 mutant, but not others, was cured by the presence of Zn\(^{2+}\). This result indicates that Zn\(^{2+}\) ion affect Mpc1 in an allele specific manner. The above observations are interesting in relation to the reports that Zn\(^{2+}\) stimulates GPI anchor

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**Fig. 3.** Zn\(^{2+}\) recovered the ability of the mpc1-5 mutant to grow at a higher temperature. W303-1B (wild type), YAT2572 (mpc1-3), YAT2573 (mpc1-4), YAT2574 (mpc1-5), YAT2547 (fsr2-1) were streaked across two YPD plates and one plate containing YPD with 5 mM ZnSO\(_4\) (Zn plates). The YPD plate (A) was incubated at 25°C and one of the YPD plates (B) and the Zn plate were incubated at 36°C (C).

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**Fig. 4.** Mutation sites of mpc1 mutants. The ORF of each mpc1 mutant was sequenced. Amino acid substitutions were identified by DNA sequencing. Putative membrane spanning domains are indicated as black boxes. Motifs conserved among phosphodiesterases are shown as open boxes.
synthesis and that a Zn\textsuperscript{2+} chelator 1,10-phenanthroline inhibits GPI anchor synthesis (Sevlever et al., 2001; Mann and Sevlever, 2001). Mann and Sevlever (2001) showed that phospho-ethanolamine transfer to the first mannose of the GPI anchor was not inhibited by 1,10-phenanthroline, suggesting that the inhibition point by this drug is later than that point, most probably the step catalyzed by Mpc1.

**Mutation sites of mpc1 mutants** The DNA sequence (–267 ~ +3410) of the *MPC1* locus was cloned by PCR using two pairs of primer (MPC1-7 and MPC1-10, and MPC1-MUNK1 and MPC1-9), and genomic DNA prepared from each of *mpc1* mutants. The nucleotide sequence of the ORF of each of three mutant genes was determined and the amino acid sequences deduced from the nucleotide sequences were compared (Fig. 4). The amino acid substitutions found in Mpc1-3 are I367T, V463A, S556P, V722A, D887G and T901A. Those in Mpc1-4 are I367T, S556P, D887G, and T901A. Four sites are common in

![Fig. 5. Multicopy suppressors. Multicopy plasmid carrying one of *PSD1*, *PSD2*, and *ECM33* was introduced into YAT2573 (*mpc1-4*), YAT2574 (*mpc1-5*), YAT2626 (*las21::LEU2*), or YAT2547 (*fsr2-1*). Empty vector or the cognate gene was also introduced into an appropriate host for comparison.](image-url)
Mpc1-3 and Mpc1-4 and two more sites are changed in the former, and the phenotype of the mpc1-3 mutant was severer than that of the mpc1-4 mutant. All the amino acid substitutions except one at position 367 of Mpc1-3 and Mpc1-4 are in the C-terminal hydrophobic region. The mutation sites of Mpc1-5 are quite different from those in the other two mutants; three amino acid substitutions occurred in the N-terminal hydrophilic region, H198R, L231S, and N411T. In the previous section, the temperature-sensitive growth of the mpc1-5 mutant was cured by Zn\(^{2+}\), but contrary to our expectation, the mutation sites of the mpc1-5 mutant was not in the HxxEH motif.

**Multicopy suppressors of mpc1** To explore factors that interact with Mpc1, we screened for multicopy suppressors of the mpc1-5 mutation. Two types of suppressors were isolated (Fig. 5); one is commonly effective to mpc1-4 and mpc1-5, and the other is effective only on the mpc1-5 mutation.

![Multicopy suppressors](image)

**Fig. 6. mpc1-5 specific multicopy suppressors.** Plasmid carrying the indicated gene was introduced into YAT2574 (mpc1-5). Plates at the left were incubated at 25°C for 3 days. A plate at the right in the top row was incubated at 35.6°C for 3 days, the middle row at 35.4°C for 3 days, and the bottom row at 35.3°C for 4 days.
ECM33: As shown in Fig.5, ECM33 on a multicopy vector suppressed both the mpc1-4 and mpc1-5 mutations. Previously, this gene was isolated as a multicopy suppressor of las21Δ (Toh-e and Oguchi, 1999). Ecm33 is one of GPI anchor proteins but its function remains unknown. ECM33 overexpression overrode the temperature-sensitivity of the fsr2-1 mutant.

PSD1 and PSD2: The PSD1 gene encodes mitochondrial phosphatidylserine decarboxylase (Trotter et al., 1993). Multicopy of PSD1 suppressed las21Δ and fsr2-1 (Fig. 5) as well as mpc1-4 and mpc1-5, whereas ethanolamine (5 mM) did not recover the ability of any of las21Δ, fsr2-1, and mpc1 mutants to grow at a restrictive temperature. There is another phosphatidylserine decarboxylase encoded by PSD2. In contrast to Psd1, this enzyme is localized at the Golgi/vacuole and comprises about 5% of total phosphatidylserine decarboxylase activity (Birner et al., 2001). We cloned the PSD2 gene by the PCR-aided gene amplification method and constructed a multicopy plasmid containing the PSD2 gene. The multicopy PSD2 gene efficiently suppressed the mpc1-4, mpc1-5, and fsr2-1 mutations but not the las21Δ mutation (Fig. 5; data not shown). The product of phosphatidylserine decarboxylase reaction is PE, which in turn serves as substrate for the reactions catalyzed by Mpc1, Fsr2, and possibly Las21. This is consistent with the suppression pattern shown by multicopy of PSD1 or PSD2, which probably pushes the reactions forward by raising substrate concentration of the enzyme reactions. In accordance with this, overexpression of either PSD1 or PSD2 could not suppress other enzyme defect in such as Gpi1/Las27 (data not shown) that catalyzes the addition of GlcNac to PI (Leidich et al., 1994). In contrast to the suppression of fsr2-1 and mpc1 mutants by overproduction of PE, the suppression of las21Δ by the overexpression of PSD1 cannot be explained by the same mechanism described above.

Fig. 7. Mn²⁺ remedied temperature-sensitive growth of the mpc1-5 mutant. W303-1B (wild type), YAT2626 (las21::LEU2), YAT2572 (mpc1-3), YAT2573 (mpc1-4), YAT2574 (mpc1-5), YAT2547 (fsr2-1) were streaked across two YPD plates and YPD with MnCl₂ plates. A, YPD with 5 mM MnCl₂ plate incubated at 36°C for 3 days; B, YPD with MnCl₂ plate incubated at 25°C for 3 days; C, YPD plate incubated at 36°C for 3 days; D, YPD plate incubated at 25°C for 3 days.
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because of the absence of Las21 in las21Δ cells. This result suggests that the reaction catalyzed by Las21 can spontaneously occur. Dpl1 is enzyme that catalyzes hydrolysis of dihydrosphingosine-1-phosphate to produce phospho-ethanolamine that is used for producing PE via the Kennedy pathway. Overexpression of DPL1 did not suppress mpc1 mutations. This result is consistent with the report that the ranking of the contribution of PE biosynthesis is in the order of Psd1, Psd2, and Dpl1 (Birner et al., 2001).

SMF2: Overexpression of SMF2, NOP1, SSD1, and YBR233w suppressed only mpc1-5 (Fig. 6). Here we focused only on SMF2. Smf2 is a putative manganese transporter. To examine whether manganese ion is effective as suppressor of mpc1-5, mpc1, fsr2-1, and las21Δ mutations, growth of these strains was tested on YPD plates with 5 mM Mn2+ at permissive and restrictive temperatures. As shown in Fig. 7, every strain tested grew on YPD with 5 mM Mn2+ at 25°C. mpc1-3, fsr2-1 and las21Δ strains did not grow at 36°C on YPD with 5 mM Mn2+ whereas the mpc1-5 strain, and mpc1-4 although weakly, grew at 36°C in the presence of Mn2+. The mpc1-3 strain did not grow on YPD containing 5 mM MnCl2 even at 34°C.

There are three conserved motifs in the N-terminal half of Mpc1, Fsr2, and Las21; motifs of phosphodiesterases and nucleotide phosphotransferases (Galperin et al., 1997; Gaynor et al., 1999). Amino acid residues comprising a metal binding domain is contained in this motif. It should be noted that 1,10 phenanthroline, a specific Zn2+ chelator, inhibits GPI anchor synthesis at the step of phospho-ethanolamine addition (Mann and Sevlever, 2001), suggesting that Zn2+ interacts with wild type Mpc1 enzyme. It is of interest that mutation sites of the mpc1-5 gene reside in between the first and second phosphodiesterase motif conserved among Mpc1, Fsr2, and Las21. These mutations may affect interaction between the metal and Mpc1 so that the mutant enzyme activity becomes dependent on Zn2+ at a restrictive temperature. Mn2+ may become accessible to the Mpc1-5 enzyme due to its amino acid changes and support enzyme function at a higher temperature.

**Mpc1 may preferentially utilize PE produced by Psd2**
The fact that overproduction of phosphatidylethanolamine decarboxylase recovers the growth of mpc1

![Fig. 8](attachment:image.png) Synthetic effect between genes belonging to the GPI anchor synthesis pathway and the phosphatidylethanolamine synthesis pathway. Diploids of the indicated genotype were dissected on a YPD plate. Asterisks were put on the right hand side of double mutants in (A) – (D). Triple mutants were similarly indicated by the asterisks in (E) and (F). Triple mutants in (G) were deduced from segregation of markers and were indicated by the asterisks.
organella is essential for GPI anchor synthesis. PE thus produced is conveyed into the Golgi/vacuole, where PE is produced by phosphatidylserine decarboxylase. PE thus produced is transferred back to the ER to serve as substrate for GPI anchor synthesis. Thus lipid transport between organella is essential for GPI anchor synthesis.

When overexpressed, PSD1 and PSD2 suppressed mpc1 mutations at a similar efficiency. These two phosphatidylserine decarboxylases are distributed differently in the cell, and it is of interest to elucidate whether Mpc1 has a preference for one of these two enzymes in the phospho-ethanolamine transfer reaction. To this end, we constructed multiple mutant strains with respect to mpc1, psd1, and psd2. As described previously (Trotter et al., 1995), the psd1Δ psd2Δ strain displays ethanolamine requirement in synthetic medium but grows in YPD without supplementation of ethanolamine. Multiple mutants including the las2Δ and fsr2-1 strains were constructed and used as comparison. When the psd2Δ strain was crossed to mpc1-3 or mpc1-4 strain, double mutant psd2Δ mpc1 strains always produced smaller colonies than parental strains or wild type strain (Fig. 8A and C). This is in quite a contrast to the results that psd1Δ mpc1 double mutants showed a comparable growth as that of wild type strain (Fig. 8B and D). When diploid between psd1Δ psd2Δ and psd2Δ mpc1 was dissected, psd1Δ psd2Δ mpc1 triple mutant segregants never appeared on a dissection plate (Fig. 8G). Twenty-four more tetrads of diploids equivalent to that shown in Fig.8G were inspected with the same result. It is in a clear contrast that both psd1Δ psd2Δ fsr2-1 and psd1Δ psd2Δ las2Δ grew on YPD without supplementation of ethanolamine (Fig. 8E and F). Altogether, Mpc1 uses PE produced by Psd1 and Psd2, and that produced by Psd2 more efficiently than that produced by either Psd1 or by the Kennedy pathway. When diploid psd1Δ psd2Δ x psd2Δ mpc1-3 was dissected on YPD containing 5 mM ethanolamine, some psd1Δ psd2Δ mpc1-3 clones were recovered as tiny colonies. This observation indicates that an amount of PE produced by the Kennedy pathway under the regular conditions is not enough for supporting Mpc1-3 activity in the triple mutant.

Psd1 and Psd2 produce PE in the different compartment, and PE produced is conveyed into the ER. Since Psd1 has a major contribution in PE synthesis, it should be elucidated why Mpc1 depends more on Psd2 in GPI anchor synthesis. As pointed out by Vudugiriene et al. (1999), GPI anchor synthesis is carried out in a subcompartment of the ER, which contacts with mitochondria. This is consistent with our observation that psd2Δ mpc1 double mutants show a slow growth, but not lethal, indicating that mitochondrial phosphatidylserine decarboxylase functions for GPI anchor synthesis in these double mutants. The fact that PE produced by Psd2 in Golgi/vacuole serves as better substrate for Mpc1 may imply that the ER contacting with Golgi/vacuole is the site of Mpc1 function.

Temperature sensitive growth of fsr2-1 is suppressed by overexpression of PSD1 or PSD2 suggests that Fsr2-1 protein has a reduced affinity to PE. If this is the case, a reduction of a PE level in the fsr2-1 strain is expected to cause a further growth defect in fsr2-1 strain. However, a psd1Δ psd2Δ fsr2-1 mutant strain showed a comparable growth to that displayed by a fsr2-1 mutant, suggesting that Fsr2-1 efficiently uses PE produced by the Kennedy pathway at 25°C. To explore contribution of Dpl1 to supply PE to Fsr2, a cross was made between a fsr2-1 dpl1::URA3 strain and a psd1Δ psd2Δ strain to construct fsr2-1 dpl1Δ, fsr2-1 dpl1Δ psd1Δ, and fsr2-1 dpl1Δ psd2Δ strains. Comparing colony sizes on a dissection plate (YPD with 5 mM ethanolamine), fsr2-1 dpl1Δ psd1Δ colonies were always smaller than those of fsr2-1 dpl1Δ psd2Δ. This finding was confirmed by a spot test shown in Fig. 9. From these results, we suggest that Fsr2 preferentially use PE produced by the Kennedy pathway and by Psd1.

Conclusion: Mpc1/Gpi13/Yll031c is localized at the ER. Mpc1 interacts with Zn²⁺ and Mn²⁺. It preferentially uses PE produced by Psd2 that is localized to the Golgivacuole, whereas Fsr2/Mcd4 preferentially uses PE produced via Psd1 that is localized in mitochondria and Dpl1. These results suggest that GPI anchor synthesis proceeds in a local domain of the ER as proposed by Vudugiriene et al. (1999) and that the efficiency of GPI anchor synthesis in these double mutants. The fact that PE produced by Psd2 in Golgi/vacuole serves as better substrate for Mpc1 may imply that the ER contacting with Golgi/vacuole is the site of Mpc1 function.
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synthesis depends on interactions between organella.

The authors would like to express their thanks to Akihiko Nakano (Riken) for plasmid possessing SEC71-HA.

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