PHOSPHATIDYLINOSITOL-4-PHOSPHATE-5-KINASEREGULATES FISSION YEAST CELL INTEGRITY THROUGHA PHOSPHOLIPASE C-MEDIATED PROTEIN KINASEC-INDEPENDENT PATHWAY

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【 学位論文題目 】

PHOSPHATIDYLINOSITOL-4-PHOSPHATE-5-KINASE
REGULATES FISSION YEAST CELL INTEGRITY THROUGH
A PHOSPHOLIPASE C-MEDIATED PROTEIN KINASE
C-INDEPENDENT PATHWAY
(PHOSPHATIDYLINOSITOL-4-PHOSPHATE-5-KINASEは、
分裂酵母の細胞統合性をPHOSPHOLIPASE Cを介するが、
PROTEIN KINASE Cとは独立した経路により制御する。)

審　査　委　員

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論文の内容要旨

Introduction

Phosphorylated phosphoinositides play a crucial role in a variety of distinct cellular processes including cell signaling, cell growth, membrane trafficking, transcription, and actin cytoskeletal arrangement. The major phosphoinositide PIP_2 is a substrate for phospholipase C, yielding two essential second messengers, IP_3 and diacylglycerol. In mammalian cells, IP_3 binds to specific receptors and induces the release of calcium from intracellular stores, whereas diacylglycerol activates PKC. We have been studying the phosphoinositide signal transduction pathway in fission yeast *S. pombe*. We have developed a genetic screen that utilizes the immunosuppressant drug FK506 and have searched for *its* mutations that display immunosuppressant- and temperature-sensitive phenotypes. By this genetic screen in our previous study, we have isolated *its3*$^+$ gene which encodes a PIP5K. The *its3-1* mutant had only low levels of PIP_2, consistent with the defective PIP5K activity. To unravel the signaling pathway regulated by the Its3 PIP5K, we searched for a novel phenotype of the *its3-1* mutant and found that the mutant was sensitive to micafungin, a (1,3)-beta-D-glucan synthase inhibitor, suggesting a cell wall integrity defect. In this study, we found that the overexpression of the *plc1*$^+$ gene encoding a phospholipase C, but not that of other genes encoding putative components downstream of PIP5K, suppressed the phenotypes of the *its3-1* mutant. Unexpectedly, PKCs are not involved in the suppression. These findings suggest that Its3 regulates cell integrity through a Plc1-mediated PKC-independent pathway, in addition to the Rgf1/Rho pathway.

Materials and Methods

Gene Expression - For ectopic expression of proteins, we used the thiamine-repressible *nmt1* promoter. Expression was repressed by the addition of 4 μg/ml thiamine to EMM, and was induced by washing and incubating the cells in EMM lacking thiamine.

Assays and Miscellaneous Methods - Techniques in light and fluorescent microscopy such as DIC microscopy and the localization of GFP-tagged proteins were performed as described previously. Actin staining using rhodamine-labeled phalloidin was performed as described previously. Tetrad analysis to examine the genetic interaction of *its3-1* mutant with other mutants was performed as described previously. IP_3 receptor-binding assay was performed as described previously.

Results

The its3-1 Mutant and PKC Mutants Showed Hypersensitivity to Cell Wall-Damaging Agents - The *its3-1* mutant showed hypersensitivity to micafungin, an inhibitor of (1,3)-beta-D-glucan synthase. We then examined the effect of micafungin on the mutants of putative downstream components of PIP5K signaling, phospholipase C (Δ*plc1*), and PKCs (Δ*pck1* and Δ*pck2*). At low concentration of micafungin (0.3μg/ml), the growth of Δ*pck2* and *its3-1* mutant cells were markedly inhibited as compared with those of the Δ*pck1* cells which were significantly inhibited but to a lesser extent. On the other hand, the growth of Δ*plc1* and wild-type cells was not affected by the micafungin at low concentration.

The bgs1-i2, a Mutant Allele of the (1,3)-beta-D-Glucan Synthase Gene was Synthetically Lethal with its3-1 Mutation - The *bgs1-i2*, a mutant allele of the *bgs1*$^+$ gene isolated as an *its* mutant showing immunosuppressant- and temperature-sensitive phenotypes in our genetic screening, was synthetically lethal with *its3-1* mutation. In wild type cells, GFP-Bgs1 localized to the septum during cytokinesis and to one or both tips during cell growth. However in *its3-1* mutant cells, GFP-Bgs1 was observed only at the ring area and was no longer observed at the cell tips.

Lack of Rgf1-GFP Localization at the Cell Tips in its3-1 Mutant - In the wild-type cells, Rgf1-GFP localized to the septum ring and to the cell tips. In *its3-1* mutant cells in contrast, although Rgf1-GFP could be detected in the ring area, it was hardly observed at the cell tips.

Overexpression of Plc1, but not That of PKCs, Suppressed the its3-1 Mutant Phenotypes - The *its3-1* mutants grew equally well as compared with the wild type cells at 27°C, but showed high temperature sensitivity at 33°C and could not grow on the plate containing FK506 (0.5μg/ml) or micafungin (0.6μg/ml) at 27°C. The overexpression of Plc1, but not that of Pck1 or Pck2, suppressed these phenotypes. In addition, the overexpression of Rho1, Rho2, Rgf1 or Pck1/Pck2 failed to suppress these mutant phenotypes.

PKCs are not Downstream of Plc1 in Fission Yeast - We examined whether the overexpression of Plc1 could suppress the micafungin-sensitive phenotype of Δ*pck2*, and whether the overexpression of the PKCs could suppress the zinc- sensitive phenotype of Δ*plc1*. Results showed that there was no suppression of the phenotype in each case. We then examined whether or not Pck2-overexpression would cause toxicity in Δ*plc1* and *its3-1* mutant cells. Results showed that the overexpression of Pck2 caused growth defects in Δ*plc1* as well as in *its3-1* mutant cells to the same extent as that observed in wild-type cells. The micafungin sensitivity of the Δ*pck2*Δ*plc1* double mutant was strongly suppressed by the overexpression of Pck2, and was also significantly suppressed by the overexpression of Pck1. However, with the overexpression of Plc1, no change in the micafungin sensitivity of the double mutant was observed. These findings support our hypothesis that PKCs are not downstream of Plc1 in fission yeast.

Deletion of Pck1 or Pck2 Could not Abolish the Effect of Plc1 Overexpression on its3-1 Mutant Phenotypes - We then examined the effects of the overexpression of Plc1 on the temperature sensitivities of the *its3-1*Δ*pck1* and *its3-1*Δ*pck2* double mutants. The temperature sensitivity of both double mutants was suppressed by Plc1 overexpression. Taken together with the above data showing that PKCs are not downstream of Plc1, the findings suggest that the suppression of the *its3-1* mutant phenotype by Plc1 overexpression is not mediated by the activation of PKCs.

Two Distinct Cell Integrity Signaling Pathways Downstream of Its3 PIP5K - The above results indicate that proper PIP_2 level is important for cell integrity. The overexpression of Its3 from the attenuated *nmt1* promoter completely halted the growth of *bgs1-i2* cells, suggesting that this cell integrity mutant is sensitive for the increased cellular PIP_2 level. Also, the *bgs1-i2* cells overexpressing Its3 PIP5K showed extremely aberrant morphology with elongated, swollen and branched cells. On the other hand, the overexpression of Plc1 had no effect on the growth of both cell types, suggesting that the growth inhibitory effect of Its3 PIP5K overexpression is directly mediated by PIP_2 and is not due to Plc1 activation.

Discussion

This study demonstrates for the first time the evidence in identifying a novel function of PIP5K that regulates fission yeast cell integrity through a phospholipase C-mediated PKC-independent pathway. The presence of PKC-like proteins in fission yeast such as Pck1 and Pck2 suggests that the PKC-mediated phosphorylation pathway, activated by diacylglycerol or Ca^{2+} in the mammalian cells, is conserved from yeast to human. However, our present *in vivo* study shows that the fission yeast PKCs are not downstream of Plc1, known to generate diacylglycerol, which activates PKC in mammalian cells. It is suggested that lower eukaryotic PKCs are more functionally similar to the mammalian PKN/PRK protein kinase subfamily, rather than to the conventional PKCs which are activated by diacylglycerol and Ca^{2+}.

神戸大学大学院医学系研究科　（博士課程）

論文審査の結果の要旨

受付番号	甲第1685号	氏名	鄧　璐
論文題目 Title of Dissertation	PHOSPHATIDYLINOSITOL-4-PHOSPHATE-5-KINASE REGULATES FISSION YEAST CELL INTEGRITY THROUGH A PHOSPHOLIPASE C-MEDIATED PROTEIN KINASE C-INDEPENDENT PATHWAY PHOSPHATIDYLINOSITOL-4-PHOSPHATE-5-KINASEは、分裂酵母の細胞統合性をPHOSPHOLIPASE Cを介するが、PROTEIN KINASE Cとは独立した経路により制御する		
審査委員 Examiner	主　査 Chief Examiner　横崎　宏 副　査 Vice-examiner　山村博子 副　査 Vice-examiner　岡村均		
審査終了日	平成　17　年　8　月　17　日		

（要旨は　１０００字～２０００字程度）

ホスホイノシチドは細胞内シグナル伝達、細胞増殖、小胞輸送、転写、アクチン細胞骨格制御などの様々な細胞機能において重要な役割を果たしている。主要なホスホイノシチドであるPIP_2はphospholipase Cの基質であり、分解されて二つの主要なセカンドメッセンジャーであるIP_3とジアシルグリセロールを生じる。哺乳動物細胞では、IP_3は特異的受容体に結合し細胞内ストアからのカルシウム遊離を引き起こす。一方、ジアシルグリセロールはプロテインキナーゼC（PKC）の活性化を引き起こす。申請者らは免疫抑制薬FK506を用いた分裂酵母S. pompeの遺伝学的スクリーニング法を開発し、免疫抑制薬および温度感受性表現型（immunosuppressant- and temperature- sensitive, *its*）を示す変異体解析を行い、Phosphatidylinositol-4-phosphage-5-kinase (PIP5K) をコードする*its3*$^+$遺伝子を単離している。本研究では、Its3 PIP5Kにより制御されるシグナル伝達系を解析し、以下の結果を得た。

*its3-1*変異体は(1,3)-beta-D-glucan synthase阻害剤であるmicafunginに超感受性を示した。そこで、PIP5Kシグナルの下流に位置すると想定されるphospholipase C (*Δplc1*)および PKC (*Δpck1* および*Δpck2*)破壊株に対するmicafunginの効果を検討したところ、低濃度のmicafungin (0.3 μg/ml)は*Δplc1* および野性株の増殖に影響を与えなかったが、PKCおよび*its3-1*変異体の増殖は抑制され、中でも*Δpck2* と *its3-1* の抑制が顕著であった。同様に*its*変異体として単離された(1,3)-beta-D-glucan synthase遺伝子 *bgs1*$^+$変異アレル *bgs1-i2* は *its3-1* 変異と合成致死を示した。野生株ではGFP-Bgs1は中隔と細胞の両端に局在したが、*its3-1*変異体ではその局在は中隔にのみ見られた。*its3-1*変異体における同様の局在異常はBgsの上流に位置するRgfに関しても確認された。

*its3-1*変異体のits表現型はPlc1の過剰発現により抑制されたが、Pck1やPck2には その効果が無く、さらに、Rho1、Rho2、Rgf1あるいはPck1/Pck2同時発現でもits表現型の抑制は見られなかった。そこで、Plc1あるいはPKCの過剰発現が、それぞれ*Δpck2*変異体のmicafungin感受性あるいは*Δplc1*変異体の亜鉛感受性に対する効果を検索したところ、いずれにも抑制作用は見られず、Pck2過剰発現は*Δplc1*、*its3-1*変異体に野生

株と同様の増殖抑制をもたらした。一方、*Δpck2Δpck1* 二重変異体の micafungin 感受性は Pck2 あるいは Pck1 過剰発現により抑制されが、Plc1 過剰発現ではその様な抑制効果は見られなかった。さらに、*its3-1Δpck1* および *its3-1Δpck2* 二重変異体に Plc1 を過剰発現させたところ、両者の温度感受性の抑制が認められた。従って、分裂酵母においては PKCs は Plc1 の下流に位置せず、Plc1 過剰発現による *its3-1* 変異体表現型の抑制は PKC の活性化によりもたらされないことが示された。

細胞内での適切な PIP_2 レベルが分裂酵母細胞の統合性に重要な役割を演ずることが示唆されたため、最後に *its3-1* 変異と合成致死を示し細胞内 PIP_2 レベルの低い *bgs1-i2* 変異体に対する Its3 PIP5K の過剰発現の効果を検討したところ、その増殖は完全に抑制され、延長、腫大、分岐等の異常な細胞形態を示したことより、細胞内 PIP_2 レベルの上昇に強い感受性を示すことが示唆された。一方、*bgs1-i2* 細胞および野生株に Plc1 を過剰発現してもこれらの増殖に影響を及ぼさなかったことから、Its3 PIP5K の過剰発現は Plc1 の活性化を経ることなく直接に PIP_2 によりもたらされると考えられた。

以上のごとく本研究は、分裂酵母における phosphatidylinositol-4-phosphate-5-kinase の細胞機能を解析したものであるが、従来明らかではなかったその細胞統合性における役割を見出し、かつ phsopholipase C は介するが PKC とは独立した新しい経路の存在を明らかにした重要な発見として、価値ある集積であると認める。よって、本研究者は博士（医学）の学位を得る資格があると認める。