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Isolation of a Fission Yeast Mutant Cell Affected in MAP Kinase Signaling and Sterol Biosynthesis

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We have previously demonstrated that calcineurin and the Pmk1 MAP kinase pathway play an antagonistic role in Cl^- homeostasis. Using this relationship, we screened for mutations that show *vic* (viable in the presence of immunosuppressant and chloride ion) phenotype and isolated a *vic6* mutant cell.

The *vic6* mutant cells also showed sensitivity to high temperature. Using this phenotype, we isolated *hmg1*$^+$ gene, encoding a HMG-CoA reductase. Consistently, the *vic6* mutant cells exhibited hypersensitivity to miconazole, an inhibitor of ergosterol biosynthesis and showed aberrant intracellular localization of filipin, suggesting that the mutant cells are affected in the sterol biosynthesis. In addition, overexpression of the *hmg1*$^+$ gene complemented the phenotype of *vic1-1/cpp1-v1* mutant cells, an allele of the gene encoding a farnesyltransferase, whereas overexpression of the *cpp1*$^+$ gene exacerbated the temperature-sensitive phenotype of the *vic6* mutant cells.

The mitogen-activated protein kinase (MAPK) signaling is one of the most important intracellular signaling that plays a crucial role in cell proliferation, cell differentiation, and cell cycle regulation (4,6,9,12). The Pmk1 MAPK, a homologue of the mammalian extracellular signal-regulated kinase (ERK)/MAPK, regulates cell morphology and cell integrity in fission yeast *Schizosaccharomyces pombe* (*S. pombe*) (15,18). Calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase, is conserved from yeast to human (2,8,17) and is a molecular target for immunosuppressive drugs, such as cyclosporin A (CsA) and FK506 (7). These drugs induce their biological effects by forming an initial complex with cytosolic proteins termed immunophilins (cyclophilin and FKBP12). These drug-immunophilin complexes then bind to and inhibit calcineurin.

We have been studying basic cellular functions using fission yeast because this system is amenable to genetics and has many advantages in terms of relevance to higher systems. We have previously demonstrated that calcineurin plays an essential role in maintaining chloride ion homeostasis and acts antagonistically with the Pmk1 MAPK pathway (13,14 Figure.1). Based on this genetic interaction between calcineurin and Pmk1 MAPK, we screened for mutations that show *vic* (viable in the presence of immunosuppressant and chloride ion) phenotype and isolated the *vic* mutants (10). In the present study, we isolated and characterized *vic6* mutant cells and cloned *hmg1*$^+$ gene as a multicopy suppressor of the

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temperature-sensitive phenotype. Further analysis revealed that the mutated gene might be implicated in the sterol biosynthesis and that overexpression of *hmg1*$^+$ gene suppressed the phenotype of *cpp1-v1*, an allele of the *cpp1*$^+$ gene, encoding a farnesyltransferase. On the other hand, overexpression of the *cpp1*$^+$ gene exacerbated the temperature sensitivity of the *vic6* mutant cells suggesting a possible link between sterol biosynthesis and the MAPK pathway.

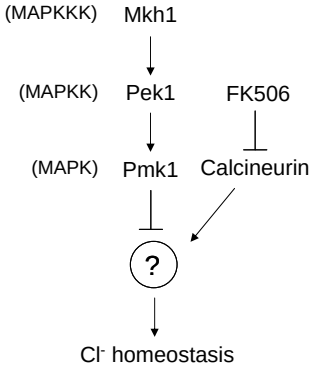

Figure 1 Calcineurin and the Pmk1 MAPK pathway play an antagonistic role in Cl^- homeostasis.

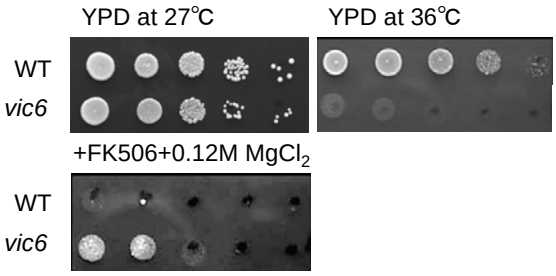

Figure 2 The phenotypes of *vic6* cells. Wild-type and *vic6* mutant cells were dropped onto the plates as indicated and then incubated for 4 days at 27°C or 3 days at 36°C, respectively.

MATERIALS AND METHODS

Genetic Methods and Bioinformatics

Standard fission yeast molecular genetic methods were used except where noted (11, 15). Database searches were performed using the National Center for Biotechnology Information BLAST network service (www.ncbi.nlm.nih.gov) and the Sanger Center *S. pombe* data base search service(www.sanger.ac.uk).

Microscopy and Miscellaneous Methods

Methods in light microscopy, such as fluorescence microscopy and differential interference contrast (DIC) microscopy, were performed as described (5). For staining of sterol, we used the fluorescent probe filipin, a polyene antibiotic that forms specific complexes with free 3-β-hydroxysterols (3). Cells were grown to exponential phase in YPD medium at 27°C, and the filipin was added to the medium and cells were observed immediately.

RESULTS AND DISCUSSION

Isolation of *vic* Mutant

The *vic* mutants were isolated in a screen of cells that had been mutagenized with nitrosoguanidine as described previously (19). Mutants were spread on YPD plates to give ~1000 cells/plate, and the plates were incubated at 27°C for 4 days. The plates were then replica-plated at 27°C onto plates containing 0.5 μg/ml FK506 and 0.2 M $MgCl_2$. Mutants that grew in the plates were selected and designated as *vic* mutants. As shown in Table I, genes for several *vic* mutants have been identified. The *vic1*$^+$ gene encodes a β subunit of farnesyltransferase which acts upstream of Pmk1 MAPK signaling (10). Notably, many *vic*

mutants were resulted from mutation in the genes encoding members of Pmk1 MAPK signaling (Table I). These results are consistent with our hypothesis that calcineurin and the Pmk1 MAPK pathway play an antagonistic role in Cl^- homeostasis.

Table I. Genes and gene products for *vic* mutants

Mutant	Gene	Gene product
vic1	*cpp1*$^+$	Farnesyltransferase β subunit
vic8, vic13, vic16, vic18, vic19	*pck2*$^+$	Protein Kinase C
vic9, vic14, vic17, vic21	*pmk1*$^+$	MAPK (*S. pombe* MAPK)
vic12, vic22	*pek1*$^+$	MAPKK (*S. pombe* MEK)
vic7, vic10, vic11, vic15, vic20, vic23	*mkh1*$^+$	MAPKKK (*S. pombe* MEKK)

Isolation and Characterization of the *vic6* Mutant Cell

The *vic*6 mutant cell was also isolated from the screening for *vic* mutants as described above. As shown in Figure.2, the *vic6* mutant cells grew in the presence of FK506 and 0.12 M $MgCl_2$ at 27°C wherein wild-type cells failed to grow. The *vic6* mutant cells failed to grow at 36°C whereas wild-type cells grew normally.

Isolation of the *hmg1*$^+$ Gene, Encoding a HMG-CoA Reductase, that Suppressed the Temperature-sensitive Phenotype of the *vic6* Mutant Cells

To clone the responsible gene by complementation, the temperature sensitivity of the *vic6* mutant cells was used. The *vic6* mutant cells were grown at 27°C and transformed with an *S. pombe* genomic DNA library constructed in the multicopy vector pDB248 (1). Leu^+ transformants were replica-plated onto YPD plates at 36°C, and the plasmid DNA was recovered from transformants that showed plasmid-dependent rescue. These plasmids complemented the temperature sensitivity. By DNA sequencing, one of the suppressing plasmid was identified to contain the *hmg1*$^+$ gene, which encodes a 1053-amino acids homolog of mammalian HMG-CoA reductase. The multicopy plasmid containing the *hmg1*$^+$ gene suppressed the temperature sensitive phenotype as shown in Figure.3A. However, overexpression of the *hmg1*$^+$ gene failed to suppress the *vic* phenotype as shown in Figure.3B. Consistently, further analysis suggested that the *vic6* mutant cell has at least two mutations (data not shown).

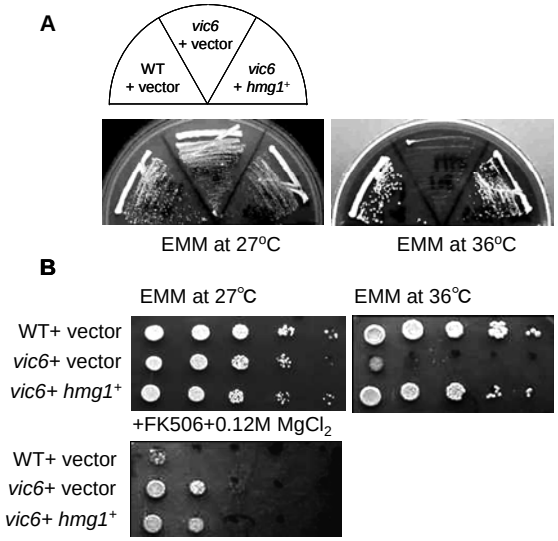

Figure3 Overexpression of the *hmg1*$^+$ gene suppressed the temperature-sensitive phenotype of the *vic6* mutant cells, but not its *vic* phenotype. (A) Expression of *hmg1*$^+$ gene suppressed the temperature- sensitive phenotype of the *vic6* mutant cells. Cells were streaked onto EMM plates and then incubated for 4 days at 27°C or 3 days at 36°C, respectively. (B) The *hmg1*$^+$ gene failed to suppress the *vic* phenotype. Wild-type and *vic6* mutant cells were dropped onto the plates as indicated and then incubated for 4 days at 27°C or 3 days at 36°C, respectively.

The *vic6* Mutant Cells Were Hypersensitivity to Miconazole and Showed Aberrant Intracellular Localization of Filipin

The above results suggest that the sterol biosynthesis pathway may be defective in the *vic6* mutant cells. We then examined the effect of miconazole**,** an inhibitor of ergosterol biosynthesis. As shown in Figure.4A, the growth of the *vic6* mutant cells was significantly inhibited by miconazole as compared with that of wild-type cells. Next, we examined the cell morphology and the localization of the sterol, using the fluorescent probe filipin, a polyene antibiotic that forms specific complexes with free 3-β-hydroxysterols. The sterol is the final product for sterol biosynthesis pathway. As shown in Figure.4B, in wild-type cells filipin is enriched in the plasma membrane at the growing cell tips and as reported by Wachtler *et al*. (16). On the other hand, filipin fluorescence was weakly observed at the plasma membrane outside the growing cell tips of the *vic6* mutant cells (arrowheads).

Taken together with the result that the *hmg1*$^+$ gene is isolated as a multicopy suppressor, these results suggest that sterol biosynthesis and/or sterol transport was defective in the *vic6* mutant cells.

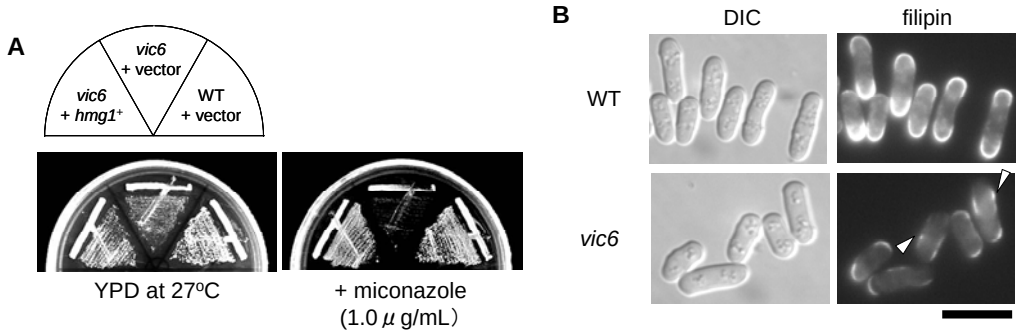

Figure4 The *vic6* mutant cells showed hypersensitivity to miconazole and aberrant localization of filipin. (A) The *vic6* mutant cells were hypersensitive to miconazole, an inhibitor of ergosterol biosynthesis. (B) Cell morphology and intracellular localization of filipin. DIC; differential interference contrast. Arrowheads indicate filipin fluorescence that was weakly observed at the plasma membrane outside the growing cell tips. Bar, 10 μm.

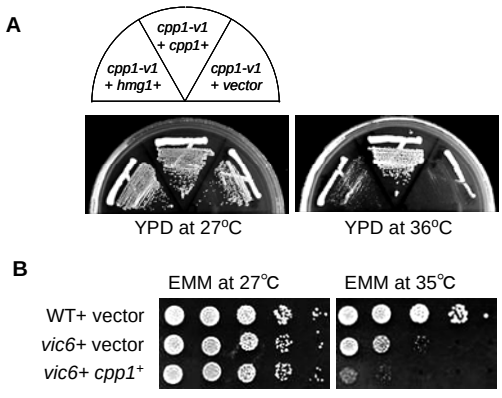

Figure5 Genetic interaction between the *hmg1*$^+$ gene and the *cpp1*$^+$ gene. (A) Overexpression of the *hmg1*$^+$ gene suppressed the temperature sensitive phenotype of the *vic1-1/cpp1-v1* mutant. Cells were streaked onto YPD plates and then incubated for 4 days at 27°C or 3 days at 36°C, respectively. (B) Overexpression of the *cpp1*$^+$ gene exacerbated the temperature sensitivity of the *vic6* mutant cells. Wild-type and the *vic6* mutant cells were dropped onto the plates and then incubated for 4 days at 27°C or 3 days at 35°C, respectively.

The *hmg1*$^+$ Gene Complemented the Temperature Sensitivity of *vic1-1/cpp1-v1* Mutant

Next, we examined the genetic interaction between the *hmg1*$^+$ gene and the responsible gene for *vic1* mutant, *cpp1*$^+$, encoding a β subunit of farnesyltransferase (Table I). Overexpression of *hmg1*$^+$ gene complemented the temperature-sensitive phenotype of *vic1-1/cpp1-v1* mutant (Figure.5A), but not that of *cpp1* deletion (data not shown). The farnesyltransferase is located downstream from HMG-CoA reductase in the sterol biosynthesis pathway and catalyzes the posttranslational modification of Ras family proteins. Cpp1 functions upstream Pmk1 MAPK signaling through Rho2 regulation (10). These results suggest that overexpression of the *hmg1*$^+$ gene increased the intermediates of the sterol biosynthesis, including farnesyl diphosphate, which is used as the source for farnesylation. This result prompted us to investigate whether overexpression of the *cpp1*$^+$ gene affects the phenotype of *vic6* mutant cells. Interestingly, overexpression of the *cpp1*$^+$ gene resulted in exacerbation of temperature-sensitive phenotype of the *vic6* mutant cells (Figure.5B). These results suggest a possible relationship between MAPK signaling and sterol biosynthesis pathway in fission yeast. Presumably, overexpression of the *cpp1*$^+$ gene in the *vic6* mutant cells may promote overconsumption of farnesyl diphosphate and shortage of the downstream products in the pathway, thereby exacerbating the temperature sensitivity of the mutant. Consistently, *vic6* mutant cells showed hypersensitivity to miconazole and aberrant localization of filipin, suggesting that sterol biosynthesis is decreased in the *vic6* mutant cells.

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