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BRIEF REPORT

Contribution of phosphatidylserine to Rho1- and Pkc1-related repolarization of the actin cytoskeleton under stressed conditions in *Saccharomyces cerevisiae*

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ABSTRACT

The budding yeast *Saccharomyces cerevisiae* undergoes polarized cell growth, which is established in association with actin polarization. Rho1, one of the Rho-type GTPases in *S. cerevisiae*, is crucial for maintaining polarized cell growth and actin polarization and controlling the downstream signaling pathway, the Pkc1-Mpk1 MAP kinase cascade, through a physical interaction with Pkc1, the sole protein kinase C in this yeast. The Pkc1-Mpk1 MAP kinase cascade is important for the repolarization of actin under heat shock-stressed conditions. We recently reported that phosphatidylserine (PS), a membrane phospholipid component, played a pivotal role in the physical interaction between Rho1 and Pkc1 as well as the activation of the Pkc1-Mpk1 MAP kinase cascade. However, it currently remains unclear whether PS is involved in actin polarization by regulating the physical interaction between Rho1 and Pkc1. We herein demonstrated that the C1 domain of Pkc1, which is responsible for the interaction with Rho1, was crucial for Rho1-regulated actin polarization. We also found that actin repolarization under heat shock-stressed conditions was impaired in a mutant defective in *CHO1* encoding PS synthase. These results suggest that PS contributes to actin polarization in which Rho1 and Pkc1 play a crucial role.

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Introduction

The budding yeast *S. cerevisiae* undergoes polarized growth, and the actin cytoskeleton is known to influence cellular polarity.[1] F-actin forms dynamic structures such as cortical actin patches and actin cables in yeast cells. Cortical actin patches are considered to represent sites of endocytosis. Rho-type GTPases form a subgroup of the Ras superfamily, and function as key regulators in many cellular processes, including actin polarization, endocytosis, and chemotaxis. There are 6 Rho-type GTPases in *S. cerevisiae*, i.e., Cdc42 and Rho1-5, and they participate in polarized cell growth.[1,2] Cdc42 acts on the selection of budding sites to establish polarized growth.[3] Polarized cell growth proceeds concomitantly with remodeling of the cell wall at the growth site. Rho1, which is a homolog of mammalian RhoA, is essential for polarized growth. Rho1 regulates the levels of 1,3-β-glucan, which is a major structural component of yeast cell wall, through the activation of the 1,3-β-glucan synthases, Fks1 and Fks2.[4,5] Rho1 also plays important roles in the organization of actin and cellular signaling. GTP-bound Rho1 binds directly to the formin Bni1, which is necessary for the formation of polarized actin.[6] Temperature-sensitive *rho1* mutants show growth defects and actin depolarization at non-permissive temperatures.[7] Rho1 is necessary for the cell wall integrity (CWI) signaling pathway, which plays roles in the remodeling of cell walls and expression of stress response genes.[5] Heat shock and cell wall stresses, such as treatments with cell wall-perturbing agents,[5] induce the activation of the CWI pathway. In the CWI pathway, GTP-bound Rho1 levels increase in response to heat shock stress, and activate the downstream effector Pkc1, the sole protein kinase C in this yeast, through a physical interaction.[5,8] Pkc1 is also involved in the organization of actin. Actin patches in logarithmically growing *S. cerevisiae* cells accumulate in the bud, which affects the polarity of the cell. Upon heat shock stress, actin patches rapidly depolarize, but repolarize in buds during the adaptation process to higher temperatures.[8-10] The Mpk1 mitogen-activated protein (MAP) kinase cascade downstream of Pkc1 is involved in the repolarization of actin under heat shock-stressed conditions.[8,10]

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Pkc1 physically interacts with GTP-bound Rho1 through the HR1 and C1 domains of Pkc1.[11,12] The yeast 2-hybrid assay revealed that the C1 domain binds more strongly to GTP-bound Rho1 than the HR1 domain.[12] We recently demonstrated that the C1 domain was necessary for the physical interaction between Rho1 and Pkc1 in co-immunoprecipitation experiments, and also in the stress-induced activation of the Pkc1-Mpk1 MAP kinase cascade.[13] Furthermore, we found that PS, a membrane phospholipid component, was important for the Rho1-Pkc1 interaction, as well as the activation of the Pkc1-Mpk1 MAP kinase cascade.[13] However, it currently remains unclear whether the PS-mediated physical interaction between Rho1 and Pkc1 is necessary for actin polarization.

In the present study, we focused on the relationship between the Rho1-Pkc1 interaction and actin polarization. We demonstrated that the Rho1-Pkc1 interaction was crucial for Rho1-regulated actin polarization, and a deficiency in *CHO1* impaired the repolarization of actin under heat shock-stressed conditions. Our results suggest that the PS-mediated interaction between Rho1 and Pkc1 is crucial for actin polarization.

Results and discussion

Importance of the C1 domain of Pkc1 for the suppression of the phenotype of the rho1 mutant

Rho1 plays important roles in polarized cell growth and actin polarization in *S. cerevisiae*.[1,2,5] A genetic interaction between *RHO1* and *PKC1*, i.e., a constitutively active allele of *PKC1* (*PKC1*^{R398P}), was found to suppress the growth defect of temperature-sensitive *rho1* mutants (*rho1-2* and *rho1-5*) at non-permissive temperatures.[7] In addition, the overexpression of the wild type allele of *PKC1* was shown to suppress the temperature-sensitive growth defect of the *rho1-5* mutant.[14] We recently demonstrated a direct interaction between the C1 domain of Pkc1 and Rho1 in co-immunoprecipitation experiments.[13] Furthermore, the substitution of 4 cysteine residues (Cys^{442}, Cys^{445}, Cys^{512}, and Cys^{515}), which contribute to the formation of zinc fingers in the C1 domain, with serines (*PKC1*$^{4C/S}$)[15,16] impaired the physical interaction with Rho1.[13] These results imply that the C1 domain of Pkc1 plays a pivotal role in Rho1-mediated cellular function. To investigate this possibility, we examined whether the overexpression of *PKC1*$^{4C/S}$ suppresses the phenotypes of the *rho1-2* mutant exhibited at non-permissive temperatures. As shown in Fig. 1A, the overexpression of wild type *PKC1* suppressed the growth defect of the *rho1-2* mutant at 37°C; however, this was not the case in *Pkc1*$^{4C/S}$. To verify whether the substitution of Cys residues in the C1 domain of Pkc1 with Ser affect the expression or stability of Pkc1$^{4C/S}$ protein in *rho1-2* cells, we conducted the Western blotting to examine the Pkc1 protein level. As shown in Fig. 1B, Pkc1$^{4C/S}$ protein levels were slightly lower than those of wild type Pkc1. We have previously reported that TOR (target of rapamycin) complex 2 phosphorylated Pkc1 at Thr^{1125} and Ser^{1143}, and phosphorylation levels of Thr^{1125} affected the stability of Pkc1 protein.[16] We also reported that the Cys-to-Ser substitution in the C1 domain of Pkc1 reduced the phosphorylation levels of Thr^{1125}.[16] Therefore, it is conceivable that decreased protein levels of Pkc1$^{4C/S}$ are due to the stability of it.

We also investigated whether the Cys-to-Ser substitution (4C/S) in the C1 domain of Pkc1 affected the polarization of actin in *rho1-2* cells at non-permissive temperatures (Fig. 1C). In wild type *RHO1* cells, the polarization of actin was maintained after 3 h at 37°C (proportion of cells with depolarized actin: 25°C, 14%; 37°C, 18%). On the other hand, the polarization of actin in *rho1-2* cells was impaired at 37°C (proportion of cells with depolarized actin, 91%), and this was suppressed by the overexpression of wild type *PKC1* (proportion of cells with depolarized actin, 26%). However, actin polarization was not fully retained in *rho1-2* cells overexpressing *PKC1*$^{4C/S}$ at 37°C (proportion of cells with depolarized actin, 50%) (Fig. 1C). These results indicate that the physical interaction between Rho1 and Pkc1 via its C1 domain influences the function of Rho1, such as polarized cell growth and actin polarization.

Role of the C1 domain of Pkc1 in the repolarization of actin patches in adaptation to heat shock stress

We showed that the overexpression of C1 domain-mutated Pkc1 (Pkc1$^{4C/S}$) failed to suppress the functional deficiency in Rho1 in the *rho1-2* mutant. We then confirmed whether the C1 domain is crucial for the function of Pkc1 itself. The *PKC1* deletion mutant will not grow without sorbitol, an osmoprotectant,[5] because cell wall synthesis is severely defective in the *pkc1* deletion mutant, which leads to cell lysis without an osmoprotectant. A mutant carrying Pkc1$^{4C/S}$ as the sole Pkc1 was found to grow without sorbitol,[15,16] which suggests that Pkc1$^{4C/S}$ mutant cells synthesize cell walls to survive under normal osmotic conditions; however, this mutant is hypersensitive to cell wall-perturbing agents, such as Calcofluor White, caffeine, and SDS.[15] In the present study, we showed that the Pkc1$^{4C/S}$ mutant exhibited a temperature-sensitive phenotype at 38°C (Fig. 2A). In addition, overexpression of the C1 domain in cells carrying the wild type Pkc1 caused a moderate temperature-sensitive phenotype. Since the Pkc1$^{4C/S}$ mutant was

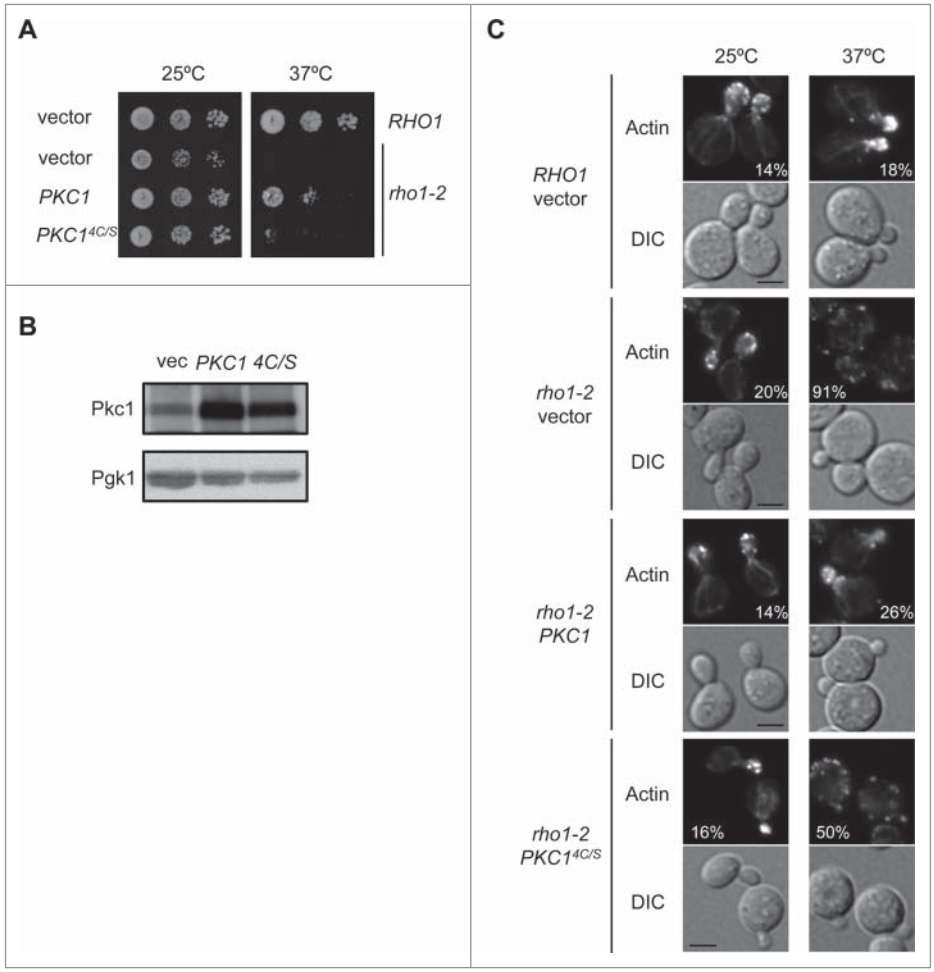

Figure 1. Importance of the C1 domain of Pkc1 for the suppression of *rho1-2* phenotypes (A) *RHO1* (YOC764) and *rho1-2* (YOC752) cells carrying the vector (YEp352), YEp352-*PKC1*, or YEp352-*PKC1*$^{4C/S}$ were cultured in SD medium at 25°C until $A_{610} = 0.3$, 4 μl of each cell suspension was spotted onto SD agar plates, and cells were incubated at 25°C or 37°C for 3 d. (B) *rho1-2* (YOC752) cells carrying the vector (YEp352), YEp352-*PKC1*, or YEp352-*PKC1*$^{4C/S}$ were cultured in SD medium at 25°C until $A_{610} = 0.3$. Cell extracts were subjected to SDS-PAGE following by Western blotting to determine the protein levels of Pkc1 using anti-Pkc1 antibodies. As the loading control, Pgk1 protein levels were determined using an anti-Pgk1 monoclonal antibody. (C) *RHO1* (YOC764) and *rho1-2* (YOC752) cells carrying the vector (YEp352), YEp352-*PKC1*, or YEp352-*PKC1*$^{4C/S}$ were cultured in SD medium at 25°C until $A_{610} = 0.3$, and were then shifted to 37°C. After an incubation for 3 h, cells were stained for actin with rhodamine-phalloidin followed by observations under a fluorescence microscope. The proportion of cells with depolarized actin was assessed by counting the cells in which actin had not accumulated in the bud. More than approximately 250 cells were counted in each experiment. Bar, 2.5 μm.

sensitive to higher temperatures, further effect in terms of temperature sensitivity was not observed when the C1 domain was overexpressed (Fig. 2A). High temperatures are known to cause cell wall stress.[5,17] These results suggest that the physical interaction between Rho1 and Pkc1 via its C1 domain is not necessarily required for the maintenance of CWI under non-stressed conditions; however, a mutation in the C1 domain defective in the interaction with Rho1 makes cells labile to cell wall stress.

We then examined whether the 4C/S mutation in Pkc1 affects its function in actin polarization. As shown in Fig. 2B, actin repolarization during adaptation to heat shock stress was impaired in the Pkc1$^{4C/S}$ mutant (proportion of cells with depolarized actin after 3 h at 38°C: wild type Pkc1, 20%; Pkc1$^{4C/S}$, 43%). These results suggest that the physical interaction between Rho1 and Pkc1 via its C1 domain is important for the polarization of actin patches.

PS is important for actin polarization by regulating the physical interaction between Rho1 and Pkc1

Our recent studies revealed that PS plays important roles in tethering Pkc1 to the plasma membrane as well as in the physical interaction between Rho1 and Pkc1 via its C1 domain.[13] In the present study, we demonstrated that the C1 domain of Pkc1 was important for the

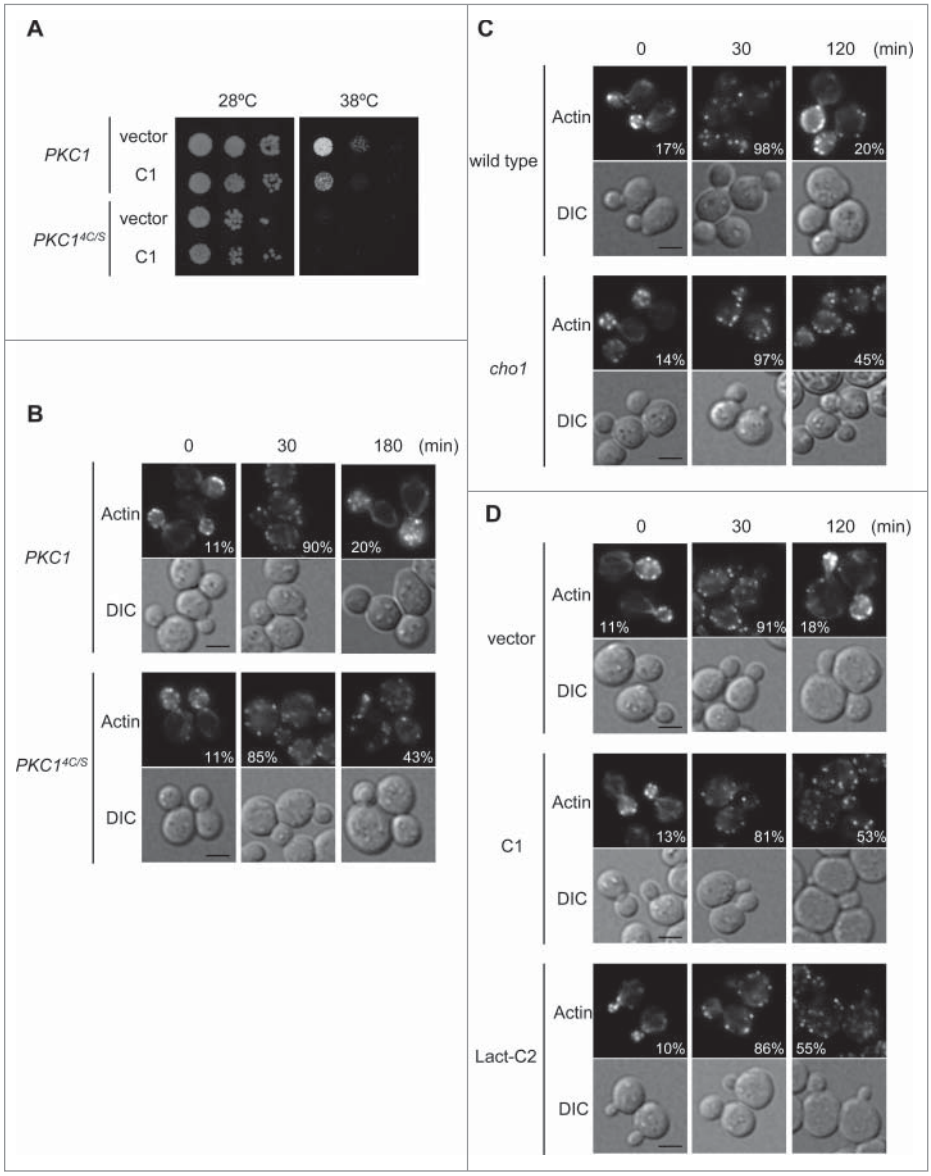

Figure 2. Involvement of PS-related Rho1-Pkc1 in actin repolarization (A) *pkc1*Δ (DL376) cells carrying YCp50-*PKC1* or YCp50-*PKC1*$^{4C/S}$ and vector (pRS424) or pRS424-ADH1p-PKC1C1 were cultured in SD medium at 28°C until $A_{610} = 0.3$, 4 μl of each cell suspension was spotted onto SD agar plates, and cells were incubated at 28°C or 38°C for 3 d. (B) *pkc1*Δ (DL376) cells carrying YCp50-*PKC1* or YCp50-*PKC1*$^{4C/S}$ were cultured in SD medium at 25°C until $A_{610} = 0.3$, and were then shifted to 38°C. After an incubation for the period indicated, cells were stained for actin as described in Fig. 1C. (C) Wild type (YPH250) and *cho1* cells were cultured in SD medium containing 1 mM ethanolamine at 25°C until $A_{610} = 0.3$, and were then shifted to 37°C. After an incubation for the period indicated, cells were stained for actin, as described in Fig. 1C. (D) Wild type (YPH250) cells carrying the vector (YEp352), ADH1p-PKC1C1, or YEp352-ADH1p-3HA-Lact-C2 were cultured in SD medium at 25°C until $A_{610} = 0.3$, and were then shifted to 37°C. After an incubation for the times indicated, cells were stained for actin, as described in Fig. 1C.

repolarization of actin patches to bud during adaptation to heat shock stress. Therefore, we examined whether PS was also involved in this event using a *cho1* mutant defective in PS synthase. As shown in Fig. 2C, the repolarization of actin patches in buds was impaired in *cho1* cells after a shift to a higher (37°C) temperature for 2 h (proportion of cells with depolarized actin: wild type, 20%; *cho1*, 45%). We reported that the overexpression of the C1 domain of Pkc1 or C2 domain of lactadherin (Lact-C2), which specifically binds to PS,[18] interfered with the physical interaction between Rho1 and Pkc1 via its C1 domain, thereby reducing the activity of the Mpk1 MAP kinase signaling network downstream of Pkc1.[13] We then investigated whether the competitive perturbation of the Rho1-Pkc1 interaction by the overexpression of the C1 domain or Lact-C2 had the same effects in terms of actin repolarization during adaptation to heat shock stress, as observed for the *cho1* mutant. The results

obtained showed that the overexpression of the C1 domain or Lact-C2 inhibited the repolarization of actin patches in buds (proportion of cells with depolarized actin after 2 h at 37°C: vector, 18%; C1, 53%; Lact-C2, 55%) (Fig. 2D).

The repolarization of actin patches under heat shock-induced conditions may be dependent on the activation of the Mpk1 MAP kinase cascade.[8,10] We recently reported that PS was necessary for the activation of the Mpk1 MAP kinase cascade by regulating the physical interaction between Rho1 and Pkc1.[13] Furthermore, the overexpression of the C1 domain of Pkc1 or Lact-C2 inhibited the heat shock-induced phosphorylation of Mpk1, which appeared to interfere with the interaction between Rho1 and Pkc1 via its C1 domain, which was capable of binding to PS.[13] Hatakeyama et al.[19] very recently reported that the C terminus polybasic sequence of Rho1 weakly bound to PS, while the cellular localization of Rho1 in *cho1* cells was unaffected under non-stressed conditions. However, cortical Rho1 localization was impaired under hypotonic conditions. In addition, the repolarization of actin patches was impaired in *cho1* cells during adaptation to hypotonic stress. Hypo-osmotic stress has been shown to activate the Mpk1 MAP kinase cascade.[20,21] We conclude that PS is involved in the repolarization of actin under stressed conditions, in which activation of the Mpk1 MAP kinase cascade is likely to play a role, and the process of which PS mediates the physical interaction between Pkc1 and the small GTPase Rho1.

Materials and methods

Medium and reagents

The medium used was SD (2% glucose, 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases being added where necessary. When required, 1 mM ethanolamine was supplemented to support the growth of a *cho1* mutant.

Strains

The *S. cerevisiae* strains used were DL376 (*MAT***a** *leu2-3, 112 trp1-1 ura3-52 his4 can1 pkc1Δ*::*LEU2*),[22] YPH250 (*MAT***a** *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52*) and its isogenic *cho1*::*TRP1* mutant,[13] YOC764 (*MATα ade2 his3 leu2 lys2 trp1 ura3 rho1*::*HIS3 ade3*::(*pRHO1-RHO1*::*LEU2*)), and YOC752 (*MATα ade2 his3 leu2 lys2 trp1 ura3 rho1*::*HIS3 ade3*::(*pRHO1-rho1-2*::*LEU2*)).[4]

Plasmids

The plasmids YCp50-*PKC1*, YEp352-ADH1p-3HA-Lact-C2, and ADH1p-PKC1C1 were described previously in detail.[13,16] YEp352-*PKC1* (pFR22) was kindly provided by Dr. Thorner.[23]

To create YCp50-$PKC1^{4C/S}$ and YEp352-$PKC1^{4C/S}$, the 1.2-kb fragment containing the C1 region of $PKC1^{4C/S}$ obtained by the digestion of pRS416-*MET25p*-$PKC1^{4C/S13}$ with ClaI was introduced into the ClaI site of YCp50-*PKC1* or YEp352-*PKC1* to replace the C1 domain of each plasmid with $PKC1^{4C/S}$ and yield YCp50-$PKC1^{4C/S}$ and YEp352-$PKC1^{4C/S}$.

To create pRS424-ADH1p-PKC1C1, the fragment obtained by the digestion of ADH1p-PKC1C1[16] with SacI and KpnI was introduced into the SacI and KpnI sites of pRS424.

Western blotting

Procedures for the detection of Pkc1 were described previously.[16] An anti-Pkc1 antibodies[16] and anti-Pgk1 monoclonal antibody (#A6457; Molecular Probes) were used as primary antibodies. Immunoreactive bands were visualized with an Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore) using a LAS-4000 mini-imaging system (Fujifilm).

Spot assay

Cells were cultured in SD medium until the early log phase of growth, and then diluted to $A_{610} = 0.1$ with sterilized 0.85% NaCl solution. Cell suspensions were diluted serially (1:10) with the sterilized 0.85% NaCl solution and spotted (4 μl) onto SD agar plates.

Actin staining

Cells were fixed with formaldehyde (final concentration, 4%) at room temperature for 1 h. After fixation, cells were harvested, washed twice with phosphate-buffered saline (PBS; pH 7.4), and suspended in 30 μl of PBS. Rhodamine-phalloidin (Molecular Probes) was added to the cell suspension to a final concentration of 33 units/ml (1.1 μM), and the cell suspension was then incubated at 4°C in the dark overnight. Cells were collected by centrifugation and washed twice with PBS, and the distribution of actin was subsequently observed using a fluorescence microscope (BX51, OLYMPUS), which was equipped with a digital camera DP70 (OLYMPUS).

Abbreviations

MAP mitogen-activated protein
PKC protein kinase C
PS phosphatidylserine
SD synthetic dextrose

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

W.N. and Y. I. designed experiments, and W.N. performed experiments. W.N. and Y. I. analyzed the data and wrote the manuscript.

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