BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 87–92 (1998)
ARTICLE NO. RC988914

Disruption of a Gene Encoding Phosphatidic Acid Phosphatase Causes Abnormal Phenotypes in Cell Growth and Abnormal Cytokinesis in *Saccharomyces cerevisiae*

Takeshi Katagiri*,† and Kazuo Shinozaki*,†,[1]

**Laboratory of Plant Molecular Biology, The Institute of Physical and Chemical Research (RIKEN), Tsukuba Life Science Center, 3-1-1, Koyadai, Tsukuba, Ibaraki, 305-0074, Japan; and †Institute of Biological Sciences, University of Tsukuba, Tennohdai, Tsukuba, Ibaraki, 305-0006, Japan*

Received June 1, 1998

Phosphatidic acid phosphatase (PAP) is an enzyme involved in lipid metabolism. Diacylglycerol (DG) and phosphatidic acid (PA) are a substrate and a product of PAP, respectively, and function as second messengers in several signal transduction pathways in animals. To investigate the function of PAP in *Saccharomyces cerevisiae,* we analyzed changes in cellular phenotypes of a mutant that has a disrupted PAP gene. Two putative genes for PAP (*ScPAP1* and *ScPAP2*) are in the *S. cerevisiae* genome. We generated a *ScPAP1*-null mutant and observed its cellular phenotypic changes. The *ScPAP1*-null mutant cells aggregated in liquid culture, and microscopical analyses showed that these mutant cells have an abnormal cell shape and abnormal cytokinesis during cell division. The *ScPAP1* is possibly involved in cell growth and cytokinesis in *S. cerevisiae.* Yeast phosphatidic acid phosphatase is possibly involved in cell growth and cytokinesis. © 1998 Academic Press

The phosphatidylinositol (PI) turnover system has important roles in various signal transduction pathways in animals (1). Phosphatidylinositol 4, 5-bisphosphate (PIP_2) is digested by phosphoinositide-specific phospholipase C (PI-PLC) to produce two well-known second messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DG). The IP_3 triggers the release of Ca^{2+} from internal stores to the cytoplasm, and the DG remains in the membrane and activates protein kinase C (PKC) (2). Recently, many phospholipid-related molecules have been shown to be involved in various signal transduction pathways in animals (3). Phosphatidic acid (PA), the phosphorylation product of DG, is one such molecule; it regulates PLC, phosphatidylinositol 4-phosphate 5-kinase (PIP5K) and the GTPase-activating protein, GAP (4-6). Phosphatidic acid phosphatase (PAP) produces DG by dephosphorylation of PA, while, in an opposite reaction, diacylglycerol kinase (DGK) phosphorylates DG to produce PA. The *Drosophila* gene Wunen encodes PAP and functions in the guidance of germ cells early in their migration from the lumen of the developing gut towards the overlying mesoderm in embryonic development (7). Partial purification of PAP has been reported in *Saccharomyces cerevisiae*, but the physiological functions of PA are still unknown (8, 9). In the *S. cerevisiae* genome, we found two putative genes for PAP. In this study, we report the characterization of one putative gene of PAP, *ScPAP1*, in yeast cells based on analyses of its gene disruption mutant (10). The *ScPAP1* gene disruptant showed abnormal cell aggregation and partial failure in cytokinesis during cell division. The possible functions of one of PAP in *S. cerevisiae* are discussed.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cultures were grown in a YPD medium (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose), and a SD medium (6.7% yeast nitrogen w/o amino acids (Difco), 2% glucose), supplemented with appropriate nutrients, was used to select cells with plasmids. Yeast cells were transformed by

[1] To whom correspondence and reprints should be addressed. Fax: 81-298-36-9060. E-mail: sinozaki@rtc.riken.go.jp.

Abbreviations used: PAP, phosphatidic acid phosphatase; DGK, diacylglycerol kinase; PA, phosphatidic acid; DG, diacylglycerol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4, 5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; IP_3, inositol 1, 4, 5-triphosphate; PKC, protein kinase C; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; GAP, GTPase-activating protein; PCR, polymerase chain reaction.

0006-291X/98 $25.00
Copyright © 1998 by Academic Press
All rights of reproduction in any form reserved.

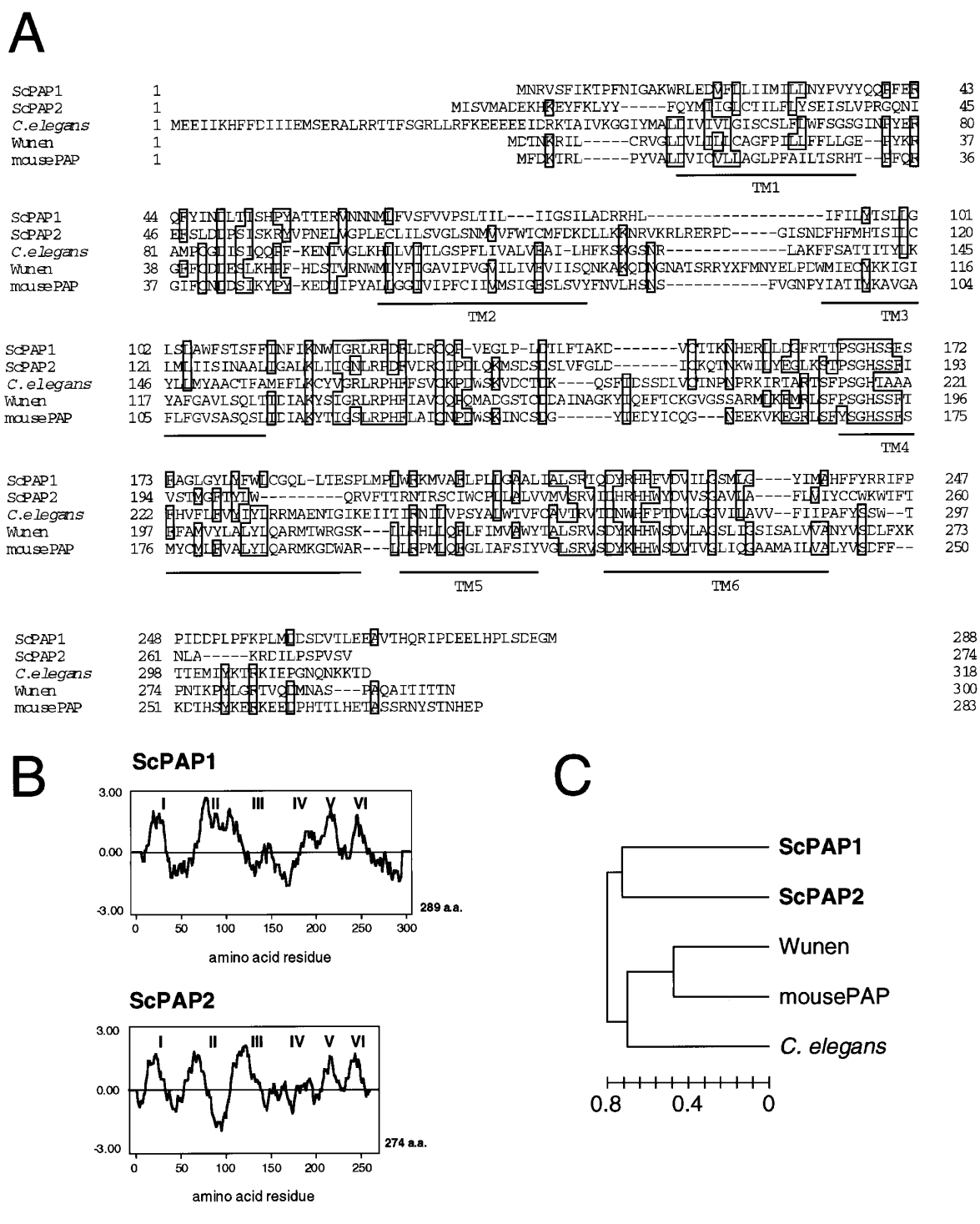


FIG. 1. (A) Comparison of the deduced amino acid sequence of putative PAPs from the PAPs from *S. cerevisiae* (*ScPAP1* and *ScPAP2;* accession numbers are U51031 and U33051, respectively), *C. elegans* (Accession number Z68105), *Drosophila* (7) and the mouse (16). Putative transmembrane domains are indicated as TM. The alignment was calculated using WUGCG. (B) Hydropathy plot of the ScPAP1 and ScPAP2 proteins. The hydropathy of the proteins was analyzed by the method of Kyte and Doolittle (22). (C) A phylogenetic tree of PAP proteins among *S. cerevisiae*, *C. elegans*, *Wunen* (*Drosophila*) and the mouse was constructed from the matrix of sequence similarities calculated with the UPGMA program (GENETYX). Numbers above the horizontal lines indicate the evolutional distance between one protein and another.

the lithium acetate method. General genetic manipulations were carried out as described by Guthrie *et al.* (11).

DNA sequence analysis. We used the GENETYX (Software Development, Tokyo) and UWGCG (GCG, Wisconsin, USA) program to analyze the DNA and amino acid sequences, and we used the UPGMA program for the phylogenetic analysis.

Cloning of the ScPAP1 gene. A putative *S. cerevisiae* PAP gene (name *ScPAP1*) was cloned by the polymerase chain reaction (PCR). Oligonucleotide primers 5′-CCGAATTCCGACCAAAATGACCAGAG-3′ (designated pYP1) and 5′-CCGAATTCCATACCTTCATCGGACA-3′ (designated pYP2) were synthesized according to accession number U51031. The PCR was carried out using these primers, *S. cerevisiae* genomic DNA as a template and Ampli Taq Gold DNA polymerase (Perkin-Elmer). The PCR-amplified fragments were digested with *Eco*RI, subcloned into the *Eco*RI site of pBluescript SK⁻II (Stratagene) and checked by sequencing.

Gene disruption and complementation. The gene disruption mutant ScPAP1 was obtained by a novel efficient gene disruption method using the *Candida glabirata HIS3* gene, which was established by Kitada *et al.* (10). In the gene disruption, a PCR fragment was used for homologous recombination. The PCR primers were designed from both ends, including a 40 mer in the coding region of *ScPAP1* and a 17 mer in the coding region of *C. glabirata HIS3* gene. Synthetic oligonucleotides were 5′-CAGAGTTTCGTTTATTAAAACGCCTTTCAACATAGGGGCGCGatcaacgtacagtg-3′ (designated pGD1) and 5′-CATACCTTCATCGGACAAAGGATGTAATTCCTCATCCGGGcaatctggcagctcgct-3′ (designated pGD2). The nucleotide sequence shown by capital letters indicates the *ScPAP1* sequence. The nucleotide sequence shown by small letters indicates the *C. glabirata HIS3* gene. A PCR was performed using *C. glabirata* genomic DNA as a template. The PCR product was introduced into an isogenic diploid *S. cerevisiae* strain RAY3A-D (12). This diploid strain was induced to sporulate, and both His^+ and His^- haploid colonies were stored. We confirmed by PCR that His^+ cells contained the disruptant *ScPAP1* gene and we named the strains the SGD11 and SGD12 (Table 1).

The *ScPAP1* DNA fragment was cloned into the *Eco*RI site of the YEpGAP vector (13). The plasmids were used to transform the SGD11 and SGD12 yeast cells. The SGD11-transformed YEp-*ScPAP1* and YEp-vectors were named DYS11 and DYV11, respectively (Table 1).

Cell sedimentation test. We used a simple method named the "cell sedimentation test" to measure the level of cell aggregation. The turbidity of each suspension culture was measured at A_{600}, and was adjusted to A_{600}=0.005 in 2 ml YPD. After 18 hours of shaking at 30°C, the cell-culture tubes were kept still in a tube rack. The turbidity at A_{600} of the upper 0.1 ml of each cell culture was measured at 10, 30, and 60 seconds.

PAP Assay of bacterially-expressed protein. The *ScPAP1* DNA fragment was cloned into the pGEX vector and was transformed into *E. coli* JM109 cells. The expression of the recombinant protein was carried out as described previously by Urao *et al.* (14). The harvested cells were resuspended in MTPBS (150 mM NaCl, 15 mM Na_2HPO_4, 4 mM NaH_2PO_4), and were sonicated for 10 seconds twice on ice. Then the sample was centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant and pellet were used as the soluble and membrane fractions for the PAP enzyme assay, respectively. The PAP enzyme assay was performed by the method of Kanoh *et al.* (15).

RESULTS

Primary Structure of Putative PAP Genes in S. cerevisiae

We found two genes for PAP (*ScPAP1* and *ScPAP2*) in the *S. cerevisiae* genome sequence. The amino acid sequences of the putative PAP proteins of *S. cerevisiae* (*ScPAP1* and *ScPAP2*) were derived from the genome sequence (accession numbers U51031 and U33051). Both *ScPAP1* and *ScPAP2* are on chromosome IV. Figure 1A shows their deduced amino acid sequences. The deduced molecular masses of the putative ScPAP1 and ScPAP2 proteins are 33.5 and 31.2 kDa, respectively. A hydropathy plot showed that the putative ScPAP1 and ScPAP2 proteins contained six transmembrane (TM) domains

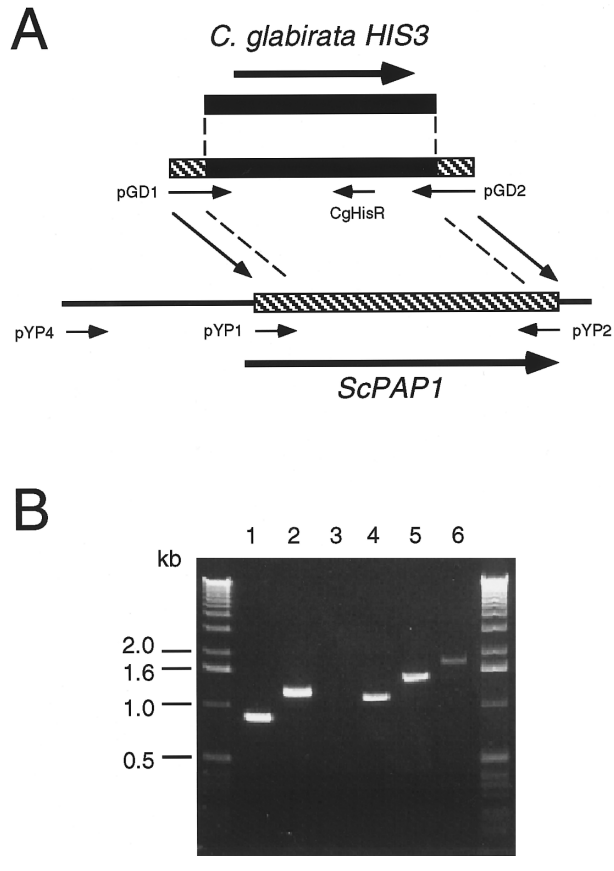


FIG. 2. Disruption the *ScPAP1* gene. (A) Construction of the disrupted *scpap1:: C. glabirata HIS3* gene. A PCR fragment for homologous recombination was used in the gene disruption. The oligonucleotide primers for PCR used pGD1 and pGD2 (See Materials and Methods). The PCR was performed using *C. glabirata* genomic DNA as a template. (B) PCR analysis of the disrupted *ScPAP1* gene. To examine whether the *ScPAP1* gene was disrupted by *C. glabirata HIS3* gene, PCR was performed using the pYP1, pYP2, pYP4 (derived from *ScPAP1*) and CgHisR (derived from *C. glabirata HIS3* gene) as primers. Used templates DNAs were 3A-D-1 genomic DNA (wild type) (lanes 1, 3 and 5) and *ScPAP1* null mutant genomic DNA (SDG11) (lanes 2, 4 and 6). When we used pYP4 and CgHisR as primers, the amplified band of about 1.2 kb was obtained with *ScPAP1* null mutant genomic DNA as a template (lane 4), but no band was detected with wild type genomic DNA (lane 3). When we used pYP1 and pYP2 (lanes 1 and 2), and pYP4 and pYP2 (lanes 5 and 6), longer DNA bands with the *HIS3* gene insertion were detected in lanes 2 and 6 with *ScPAP1* null mutant genomic DNA. PCR primer sequences: pYP4, 5′-ATTGTATCAGTCACAGG-3′; CgHisR, 5′-CTATGCTAGGACACCCTTAGTGG-3′. Other primers, see Materials and Methods.

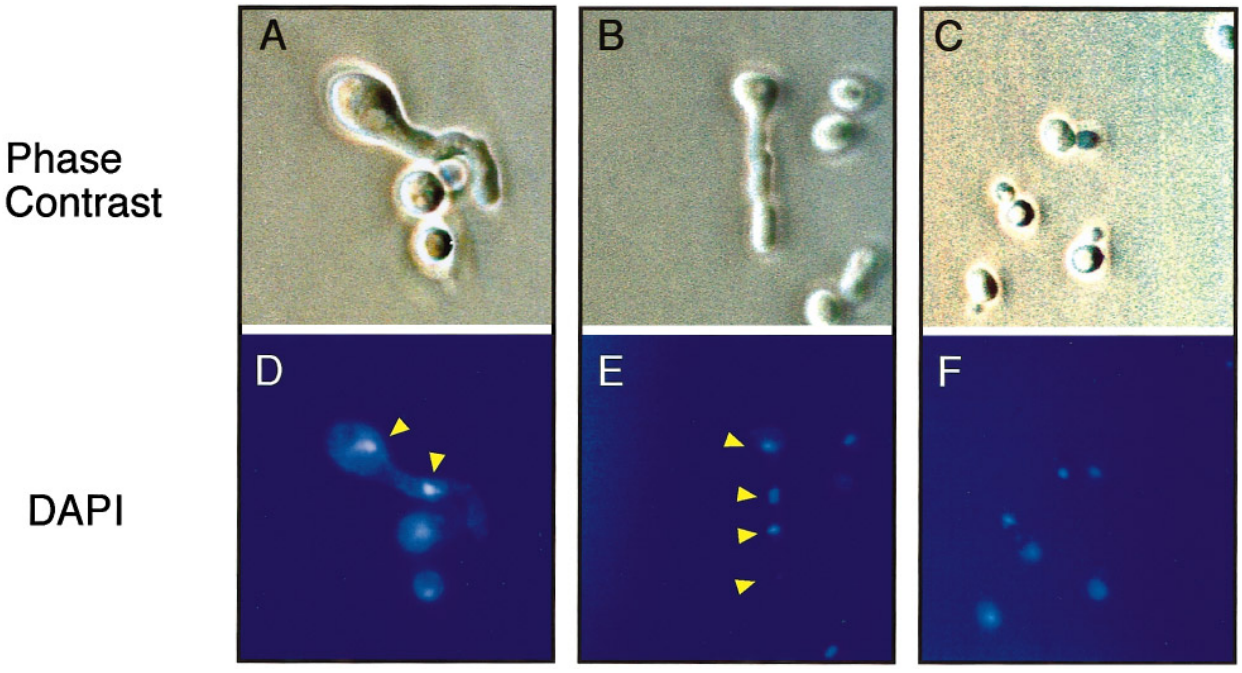


FIG. 3. Microscopical analysis of cultured cells of SGD11 (*ScPAP1* null mutant) (A, B, D and E) and 3A-D-1 (wild type) (C and F). SGD11 failed in cytokinesis, whereas their nuclear division proceeded normally. There are two nuclei in one cell (A and D) and four nuclei in one cell (B and E). Yellow arrowheads indicate nuclei. The cell outlines are visible by phase contrast (A–C). The cells were fixed and stained for fluorescence microscopy with DAPI for nuclear staining (D–F).

(Fig. 1B). The PAP activity has been detected in *S. cerevisiae*, and the molecular masses of the partially purified PAPs were 45 kDa and 104 kDa (8). The difference in molecular weight may be due to their properties as membrane proteins and dimerization in solution.

Figure 1A compares the sequences of PAPs from *S. cerevisiae*, *Caenorhabtis elegans*, *Drosophila* (7), and the mouse (16). Highly conserved regions are between TM domains 3 and 4, and in TM domains 4 and 6. Phylogenetic analysis showed a low sequence similarity between the two yeast PAPs, and a high sequence similarity between the *Drosophila* and mouse PAPs (Fig. 1C).

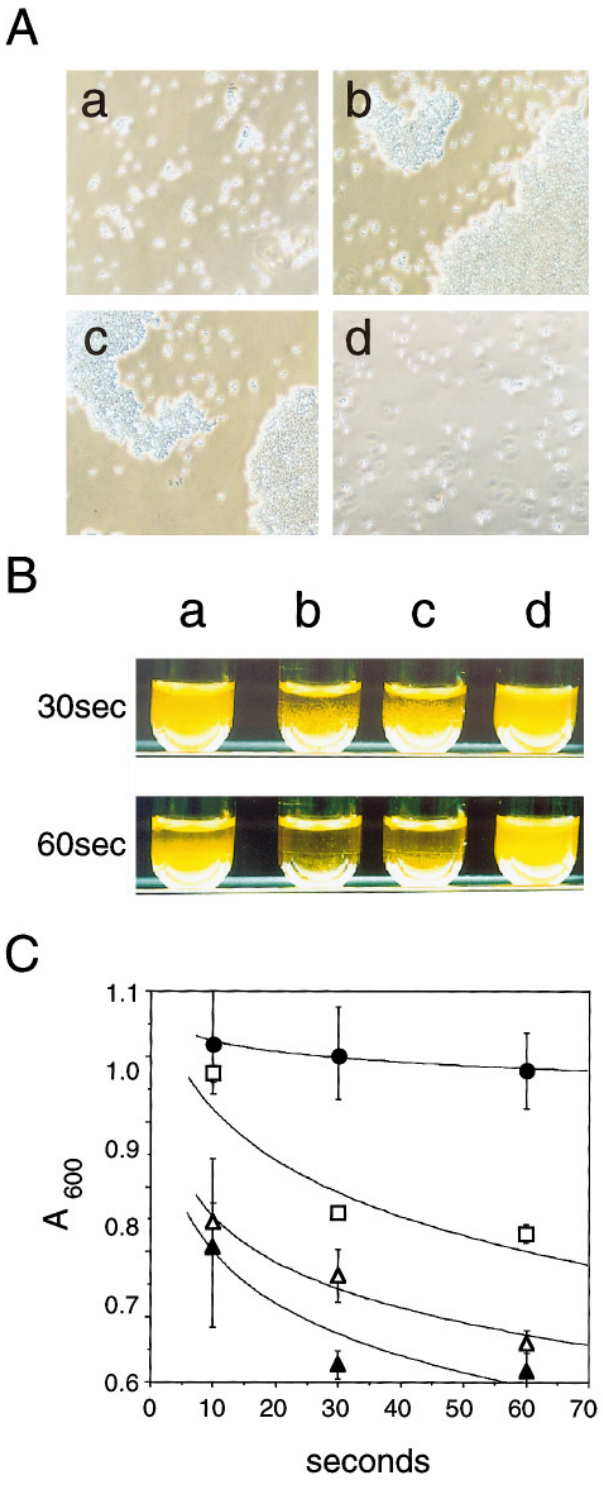
Phenotypes of the ScPAP1 Null Mutant

To analyze the function of the PAP gene, *ScPAP1*, in *S. cerevisiae*, we isolated a gene disruptant mutant using homologous recombination. We used a PCR fragment that has a fusion gene with the 5′ and 3′ coding regions of the *ScPAP1* gene at both borders and has the *Candida glabirata HIS3* gene inside (Fig. 2A). The *ScPAP1* gene disruptant was isolated from the transformant of RAY3A-D cells with a PCR fragment using tetrad analysis. The *ScPAP1* null mutant SGD11 (Table 1) showed abnormal cell aggregation in liquid culture at 30°C (Fig. 3). Microscopic observation of *ScPAP1* null mutant cells

TABLE 1

S. cerevisiae Strains Used in This Study

Strain	Relevant characteristics	Source
RAY3A-D	*Mat***a**/*Matα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3*	Tanaka *et al.* (12).
3A-D-1	*Mat***a** *ura3 leu2 trp1 his3*	This study
3A-D-2	*Matα ura3 leu2 trp1 his3*	This study
SDG11	*Mat***a** *ura3 leu2 trp1 his3 scpap1::C. glabirata HIS3*	This study
SDG12	*Matα ura3 leu2 trp1 his3 scpap1::C. glabirata HIS3*	This study
DYS11	*Mat***a** *ura3 leu2 trp1 his3 scpap1::C. glabirata HIS3* (YEp-TRP1-ScPAP1)	This study
DYS12	*Matα ura3 leu2 trp1 his3 scpap1::C. glabirata HIS3* (YEp-TRP1-ScPAP1)	This study
DYV11	*Mat***a** *ura3 leu2 trp1 his3 scpap1::C. glabirata HIS3* (YEp-TRP1-vector)	This study
DYV12	*Matα ura3 leu2 trp1 his3 scpap1::C. glabirata HIS3* (YEp-TRP1-vector)	This study
DHNY101	*Mat***a** *ura3 leu2 trp1 his3 ade2 rho1::HIS3* (YCp-LEU2-GAL1-Rho1)	Nonaka *et al.* (17).



revealed their abnormal cell shapes. The *ScPAP1* null mutant cells had irregular, pear-like and stick-like shapes. These irregular cell shapes were found to be more severe in aggregated cells. Based on microscopic analysis of cells stained with DAPI, we found that the *ScPAP1* mutant cells failed in cytokinesis whereas their nuclear division proceeded normally (Fig. 3). The *ScPAP1* null mutant cells aggregated and easily sunk to the bottom of the liquid medium in the culture tubes (Fig. 4). To investigate the growth rate of the *ScPAP1* null mutant cells, two-day cultured wild type haploid (3A-D-1) and *ScPAP1* null mutant (SGD11) cells were centrifuged and their total proteins were measured. The growth rate of the *ScPAP1* null mutant cells was the same as that of wild-type cells (data not shown).

Transformation of ScPAP1 Partially Suppressed the Abnormality of the ScPAP1 Null Mutant

To study if the *ScPAP1* gene can suppress abnormal phenotypes of the *ScPAP1* null mutant cells, we transformed the *ScPAP1* gene in *ScPAP1* null mutant cells using the YEpGAP vector. The transformant with the *ScPAP1* gene (DYS11), the transformant with YEp vector (DYV11), the *ScPAP1* null mutant (SGD11) and wild type cells (3A-D-1) were cultured in YPD medium for 12 h. The level of cell aggregation was reduced in the DYS11 (a) compared with SGD11 (b) and the DYV11 (c) (Fig. 4A). To analyze the level of cell aggregation quantitatively, we measured the rate of cell sedimentation of each cell (named the cell sedimentation assay). Severely aggregating cells sedimented much faster than normal cells (Figures 4B and C). The DYS11 cells (a), the transformant with the *ScPAP1* gene, sedimented more slowly than the SGD11 cells (b). The DYV11 cells (c), the transformant with the YEp vector, sedimented as fast as the SGD11 cells. The 3A-D-1 wild type cells (d) sedimented slowly. These results suggest that the *ScPAP1* gene on the YEp vector partially suppressed abnormal cell aggregation of the *ScPAP1* null mutant SGD11.

PAP Activity of the Recombinant ScPAP1 Protein Expressed in Escherichia coli

To confirm that the ScPAP1 protein has PAP activity, the *ScPAP1* cDNA was cloned into the pGEX expression vector. The *E. coli* cells that contained pGEX-*ScPAP1* were washed, were sonicated to extract proteins from the cells, and were separated into two fractions: membrane and soluble fractions. We detected

FIG. 4. Complementation of the *ScPAP1* null mutant by transformation with *ScPAP1* on a YEpGAP vector. (A) Microscopical analysis of yeast cells DYS11 (a), DYV11 (b), SGD11 (c), 3A-D-1 (d). (B) Sedimentation of yeast cells by aggregation. (a)–(d) are the same as described in (A). (C) The sedimentation rate of yeast cells: 3A-D-1 (circle), DYS11 (square), SGD11 (opened triangle), and DYV11 (closed triangle). Each yeast culture was adjusted to A_{600}=0.005 and the culture started at 30°C and 180 rpm. After 12 hr of incubation the yeast cells were observed under a light microscope (phase contrast) (A). After 18 hr of incubation the culture tubes were observed at 30 and 60 seconds without shaking (B). The upper 0.1-ml of the culture medium at 10, 30 and 60 seconds without shaking was measured at A_{600} (C). The results represent the averages of the three independent experiments, with the error bars representing the standard errors of the means.

higher PAP activity in the membrane fraction of *E. coli* cells with pGEX-*ScPAP1* (0.76±0.07 U/mg protein) than with the pGEX vector alone (0.41±0.05 U/mg protein). The soluble fraction of *E. coli* cells with pGEX-*ScPAP1* had no PAP activity. The data are means ± se (n=3). These results indicate that the *ScPAP1* gene encodes a functional PAP enzyme.

DISCUSSION

The PI turnover system has important roles in various signal transduction pathways in animals (1). Physiological roles of some of the phospholipid-related molecules, such as DG and PA, have not been well studied. To understand the biological functions of these molecules, we used *S. cerevisiae* for our experiments as the first step. Because *S. cerevisiae* has two advantages to accomplish our purpose. First, sequence information of the whole genome is available. Second, we can easily disrupt genes of our interest.

We found two genes named *ScPAP1* and *ScPAP2* in *S. cerevisiae* which have sequence homology to PAPs from *Drosophila* and mouse. These two PAP genes have low sequence similarity, suggesting they may have different functions. Moreover, the *ScPAP1* null mutant cells revealed two clear mutant phenotypes. In this paper, we focused on characterization of the function of the one of the PAP genes, *ScPAP1*, using its mutant. The *ScPAP1* null mutant cells showed two clear phenotypes, abnormal cell shapes and aggregation in liquid culture (Fig. 3 and 4). The DAPI staining indicated that the *ScPAP1* null mutant cells failed in cytokinesis whereas their nuclear division proceeded normally (Fig. 3). We think these mutant phenotypes are caused by some defects in cytoskeleton and/or cell wall. The null mutant cells may fail to form so-called contractile ring in animal cells during cytokinesis. Our results obtained from complementation analysis (Fig. 3 and 4) and biochemical analysis of the recombinant ScPAP1 protein indicate that the *ScPAP1* encodes a functional PAP and is involved in abnormal cell aggregation.

We also found that the *Rho1* null mutant (DHNY101) cells have a similar phenotype of cell aggregation in YPD liquid culture (17 and Katagiri, T., Nonaka, H., Tanaka, K., Takai, Y., and Shinozaki, K. unpublished data). The *Rho1* gene encodes a small GTP-binding protein of the Rho family (18). The Rho1 is involved in bud formation and is thought to function in signaling during cytokinesis (19). More recently, Ras GAP has been shown to be involved in cytokinesis in Dictyostelium (20). The interaction between Ras-related proteins and GAP molecules is thought to be regulated by PA (6, 21). These observations suggest that ScPAP1 and Rho1 may be involved in a common or similar signaling process(es) during cytokinesis. We are analyzing the possibility of the functional interaction between ScPAP1 and Rho1.

ACKNOWLEDGMENTS

We thank Ms. Akiko Shiratori and Dr. Yasufumi Murakami of the Laboratory of Cell Physiology, RIKEN. We thank Dr. Fumio Sakane of the Department of Biochemistry, Sapporo Medical College for helpful discussion about PAP assay. We also thank Dr. Tsuyoshi Mizoguchi of the Laboratory of Plant Molecular Biology, RIKEN for fruitful suggestions and discussions. This work was supported in part by the Special Coordination Fund of the Science and Technology Agency of the Japanese Government, a grant-in-aid from the Ministry of Education, Science and Culture of Japan, and the Program for Promotion of Basic Research Activities for Innovative Biosciences to KS. TK was supported by JSPS Research Fellowships for Young Scientists.

REFERENCES

1. Ettlinger, C., and Lehle, L. (1988) *Nature* **331,** 176–178.
2. Nishizuka, Y. (1984) *Nature* **309,** 693–698.
3. Divecha, N., and Irvine, R. (1995) *Cell* **80,** 269–278.
4. Jones, G. A., and Carpenter, G. (1993) *J. Biol. Chem.* **268,** 20845–20850.
5. Moritz, A., Graan, P. N. D., Gispen, W. H., and Wirtz, K. W. (1992) *J. Biol. Chem.* **267,** 7207–7210.
6. Tsai, M. H., Yu, C. L., Wei, F. S., and Stacey, D. W. (1989) *Science* **243,** 522–525.
7. Zhang, N., Zhang, J., Purcell, K. J., Cheng, Y., and Howard, K. (1997) *Nature* **385,** 64–67.
8. Morlock, K., McLaughlin, J., Lin, Y., and Carman, G. (1991) *J. Biol. Chem.* **266,** 3586–3593.
9. Dillon, D., Chen, X., Zeimetz, G., Wu, W., Waggoner, D., Dewald, J., Brindley, D., and Carman, G. (1997) *J. Biol. Chem.* **272,** 10361–10366.
10. Kitada, K., Yamaguchi, E., and Arisawa, M. (1995) *Gene* **165,** 203–206.
11. Guthrie, C., and Fink, G. R. (1991) *Methods in Enzymology.*
12. Tanaka, K., Matsumoto, K., and Toh-e, A. (1988) *EMBO J.* **7,** 495–502.
13. Banno, H., Hirano, K., Nakamura, T., Irie, K., Nomoto, S., Matsumoto, K., and Machida, Y. (1993) *Mol. Cell. Biol.* **13,** 4745–4752.
14. Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N., and Shinozaki, K. (1994) *Mol. Gen. Genet.* **244,** 331–340.
15. Kanoh, H., Imai, S., Yamada, K., and Sakane, F. (1992) *J. Biol. Chem.* **267,** 25309–25314.
16. Kai, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1996) *J. Biol. Chem.* **271,** 18931–18938.
17. Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A., and Takai, Y. (1995) *EMBO J.* **14,** 5931–5938.
18. Madaule, P., Axel, R., and Am, A. M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 779–783.
19. Yamochi, W., Tanaka, K., Nonaka, H., Maeda, A., Musha, T., and Takai, Y. (1994) *J. Cell. Biol.* **125,** 1077–1093.
20. Lee, S., Escalante, R., and Firtel, R. (1997) *Development* **124,** 983–996.
21. Tsai, M., Hall, A., and Stacey, D. (1989) *Mol. Cell. Biol.* **9,** 5260–5264.
22. Kyte, J., and Doolittle, R. (1982) *J. Mol. Biol.* **157,** 105–132.