A staurosporine-sensitive mutation (stt1) in yeast has been found in the PKC1 gene that encodes a protein kinase C homologue (Yoshida, S., Ikeda, E., Uno, I., and Mitsuzawa, H. (1992) Mol. Gen. Genet. 231, 337–344). We report here another staurosporine-sensitive mutant, stt4, which shows very similar phenotypes to that of the stt1 mutant. The stt4 temperature-sensitive mutant arrests mostly in G2/M phase at 37°C, and the stt4 deletion mutant shows an osmoremedial phenotype. Staurosporine sensitivity of the stt4 mutant was suppressed by overexpression of PKC1/STT1, indicating genetic interaction between stt4 and pck1/stt1. The nucleotide sequence of STT4 predicts a hydrophilic protein composed of 1,900 amino acid residues, with 26% sequence identity to the yeast VPS34 gene product and 27% to the catalytic subunit of mammalian phosphatidylinositol (PI) 3-kinase, respectively. Cell homogenates of the stt4 deletion mutant show normal PI3-kinase activity but lack most of the PI4-kinase activity that is detected in the wild-type. We conclude that STT4 encodes a yeast PI4-kinase that functions in the PKC1 protein kinase pathway.

Eukaryotic cell proliferation is directed through many signal transduction systems responding to hormones, growth factors, and neurotransmitters. Receptor-mediated activation of inositol phospholipid metabolism is one of the typical signal transduction pathways (1, 2). Phosphatidylinositol (PI)1 kinase plays an important role as the first committed enzyme in this pathway. Historically, three forms of PI kinase (type I, II, and III) have been distinguished on the basis of inhibition by detergents, such as Triton X-100, and adenosine (3, 4). The type I PI kinase phosphorylates the D-3 position of the inositol ring, whereas the type II and III enzymes phosphorylate the D-4 position (5). All three products of the type I PI3-kinase (PI-3P, PI-3,4P2, and PI-3,4,5P3) appear to be resistant to cleavage by phospholipase C (6, 7).

In Saccharomyces cerevisiae, PI is the third major phospholipid in membranes (8) and is essential for cell growth (9, 10). The synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2) in yeast cells is regulated by glucose (11, 12) and by sterol (13). PI kinase, phosphatidylinositol 4-phosphate (PIP) kinase and protein kinase C (PKC) have been characterized biochemically in yeast (14–19), and the former two were shown to be regulated by the RAS/cAMP cascade (20). A gene homologous to mammalian PKCa, PKC1, has been isolated in the yeast S. cerevisiae and was shown to be essential for cell division cycle with cells arresting mostly in the G2/M phase (21). VPS34, a gene whose product is homologous to the catalytic subunit of mammalian PI3-kinase (22), has been identified and shown to function in the vacuolar protein sorting pathway (23).

Biochemically, 45-kDa (p45) and 55-kDa (p55) isoforms of PI4-kinase have been purified from the membrane fraction of S. cerevisiae (14, 16). The two isoforms have different physicochemical and enzymological properties. Another isoform of PI4-kinase (125 kDa) (p125) has been purified from the cytosol (17). On the other hand, PI3-kinase has not been purified, although its activity has been detected (15). In vitro assay of PI kinase showed that activity of PI4-kinase is approximately 5-fold higher than that of PI3-kinase, and in vivo labeling assay with [3H]inositol showed that PI-3P is as abundant as PI-4P, although PI-3P constitutes only 3–10% of the PIP in mammalian cells (15).

Staurosporine, isolated as an antifungal activity derived from Streptomyces sp., is one of the most specific inhibitors of PKC (24). It inhibits PKC, probably by direct binding to the enzyme (25, 26). To understand the physiological roles of the PKC pathway, we isolated staurosporine-sensitive mutants which were also temperature-sensitive (stt). One of these, stt1, has been shown to be allelic to pck1 (27). Together with the finding that the PKC1 gene confers staurosporine resistance to yeast cells in a dose-dependent manner (27), we have suggested that mutations in the PKC1 regulatory pathway result in the staurosporine-sensitive phenotype.

In this paper, we report a potential PI4-kinase gene, STT4. Genetic interaction between pck1/stt1 and stt4 strongly suggests involvement of PI4-kinase in regulation of PKC activity.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Conditions, and Transformations—** All strains used in this study were derivatives of wild-type strain YSS–3D or YSS–8D except those used for mapping of the location of STT4. Yeast cells were grown in YPD (1% yeast extract, 2% polypeptide, 2% glucose) (28). Synthetic minimal medium, SD, (28) supplemented with appropriate nutrients was used to select for plasmid maintenance and gene replacement. Transformation was performed using the lithium acetate method (29) and genetic manipulation was carried out as described (28).

Bacterial strains XL1-Blue (30) were used for the propagation of all plasmids and phagemids. Phagemids BLUESCRIPTII (KS+ and KS−) and the helper phage M13K07 (31) were used to generate single-stranded template DNA for sequence determination. Escherichia coli cells were cultured in Luria broth and transformed or infected with phagemids by standard methods (32).

The S. cerevisiae genomic DNA libraries were constructed on the shuttle vectors, YEp13 (33) and YCP50 (34), respectively.
DNA Manipulations—DNA was prepared from yeast strains by the method of Winston et al. (35). It was subjected to electrophoresis through agarose gels after digestion with restriction endonucleases and transferred to nitrocellulose filters by standard procedures (36). Plasmid DNA was prepared from E. coli using alkaline lysis (32) or from S. cerevisiae using glass beads (28). DNA sequence analysis was carried out by dideoxy chain termination (37) with a 370A DNA sequencer (Applied System) according to the supplier’s instructions. Both strands of the 8.6 kb BamHI/PstI fragment were sequenced (Fig. 2B).

**STT4 Gene Integration, Replacement, and Plasmids**—The 3.4-kb BamHI/HindIII fragment of plasmid pSTS4-14 was subcloned into the integrating vector pRS806 (38). This plasmid was digested with XhoI, and the linearized DNA was used to transform a wild-type strain RA1-11. Restriction mapping and hybridization analysis of genomic DNA from the resulting transformants were carried out to confirm that integration had occurred at the STT4 locus. One such transformant was mated with the stt4-I mutant strain (STT4-12C). The resulting diploid was subjected to sporulation and tetrad analysis.

A deletion mutant allele of STT4 was constructed by the method of Rothstein (39). The 4.1-kb XhoI/SpeI fragment of STT4 (Fig. 2A) was replaced on pUC119 by the 1.3-kb XhoI/XbaI fragment of HIS3 from pJ217 (40) in reverse transcriptional orientation. The resulting plasmid was digested with EcoRI to confirm that the 2.2-kb EcoRI fragment contained the entire stt4-I mutation. The remaining 4.1-kb XhoI/SpeI fragment of STT4 was subjected to sporulation and tetrad analysis.

**Fig. 1. Phenotype of the stt1 stt4 double mutant.** Plates were incubated at 25 °C for 3 days. A, YPD; B, YPD containing 0.05 μg/ml staurosporine; C, YPD containing 0.1 μg/ml staurosporine; D, YPD containing 1 μg/ml staurosporine.

**Fig. 2. Subcloning, disruption, and sequence strategy of the STT4 gene.** A, complementation by STT4 DNA fragment of the stt4-I mutation. All inserts are represented relative to the large fragment found in plasmid pES4-1. pSTS4-13 is a derivative of YCp50, pSTS4-14 of pRS115, and the remainder of YEp13. The complementing ability of the plasmid is shown in the column on the right (+, complementation; –, no complementation). Abbreviations for restriction sites are as follows: A, XhoI; B, BamHI; H, HindIII; O, Xhol; P, PstI; S, SpeI. J represents a junction between insert DNA and a BamHI cleavage site of the vector YEp13. B, sequence strategy of the STT4 locus. Arrows show the directions and extent of the sequence determined.
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was determined. The STT4 gene predicts to encode 1,900 amino acid residues (nucleotide positions, 659-6359).

FIG. 3. Nucleotide sequence and predicted amino acid sequence of the STT4 gene. The 8.6-kb BamHI-PstI fragment (total base; 14,612)}
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Fig. 3—continued
introduced into the E. coli strain XL1-Blue and the transformant was induced by isopropyl-β-D-thiogalactopyranoside to express the lac2-STT4 fusion protein product. This hybrid protein was purified as described by Nakano et al. (44) and used to immunize rabbits.

The cell homogenate samples were electrophoresed on 7.5% (w/v) SDS-polyacrylamide gels using Laemmli's buffer system (45). Immunoblotting was performed as described by Towbin et al. (46) with some modifications (47).

RESULTS

Genetic Interaction between pkcl/sttl and stt4—To identify mutations in the PKCl pathway, we focused on the stt mutations that were suppressed by overexpression of the PKCl/STT1 gene with respect to staurosporine sensitivity. Among nine stt mutations, the stt4 mutation was always cosegregated with each other; 19 asci all showed parental genotypes. Ten colonies grown at 37°C on YPD plates, but could grow not only on plates containing 1 M sorbitol but also on those containing 250 mM CaCl₂, MgCl₂, or inositol (27). However, temperature sensitivity of the stt4 mutants was not suppressed by the addition of 100 mM sorbitol, CaCl₂, MgCl₂, or inositol (data not shown).

The cell homogenate samples were electrophoresed on 7.5% (w/v) SDS-polyacrylamide gels using Laemmli's buffer system (45). Immunoblotting was performed as described by Towbin et al. (46) with some modifications (47).

Cloning and Nucleotide Sequence of the STT4 Gene—The STT4 gene was cloned by complementation of the stt4-1 mutation. The stt4-1 strain SYT44 was transformed with a yeast genomic library constructed on YEp13 with the LEU2 marker (33). Among 20,000 transformants screened, two clones (pES4-1 and pES4-2) and one clone (pCS4-1) that complemented both temperature and staurosporine sensitivity of the stt4-1 mutation were obtained from the YEp13 and the YCP50 libraries, respectively. The restriction map of the inserts in these clones showed that they contained an overlapping region (Fig. 2A). Subcloning and complementation analyses indicated that the complementing activity resided in the 8.6-kb BamHI/Psal fragment (pSTS4-14, Fig. 2A).

To confirm that this complementing ability is not due to extragenic suppressors, integration mapping was carried out. The 3.4-kb BamHI/HindIII fragment was cloned from pSTS4-14 into the integrating vector pRS306 with the URA3 marker. The resulting plasmid was linearized at the internal unique XhoI site. A wild-type strain RA1-1B was transformed with the stt4 deletion mutant was 300 min, which was much longer than that of wild-type (110 min) was only a little longer than that of wild-type (100 min) in the YPD medium at 25°C. This osmolarity-dependent phenotype is very similar to that of the pkcl disrupted strain (48).

Assay of PI Kinase Activity—As Stt4p has homology to the catalytic subunit of mammalian PI3-Kinase, p110—in order to gain insight into possible functions of the STT4 gene product, a homology search of the Stt4p amino acid sequence in the protein database (NBRF) was performed. This search showed that the Stt4p sequence had noticeable similarity to Vps34p, a hydrophilic protein of 875 amino acids from S. cerevisiae (23), and p110, the catalytic subunit of mammalian PI3-kinase consisting of 1,068 amino acids (22). The amino acid sequence identity with Vps34p and p110 is approximately 26 and 27% in 387 and 470 overlapping amino acid sequences, respectively. Vps34p is required for vacuolar protein sorting and vacuole segregation (23). The vps34 mutant cells form pink colonies with the genetic background of the ade2 mutation because they cannot accumulate red pigment in the vacuoles (49). However, the stt4 mutant cells harboring the ade2 mutation formed red colonies, suggesting that stt4 mutant is not defective in vacuolar functions.

Assay of PI Kinase Activity—As Stt4p has homology to the catalytic subunit of mammalian PI3-kinase, the possibility was examined that Stt4p is one of the yeast PI kinases. PI kinase activity of yeast cell homogenates was assayed as described under "Experimental Procedures." In the stt4 null mutant, PI-4P production was greatly reduced relative to that of wild-type, although PI-3P production was equivalent to that of wild-type (Fig. 5).
When expressed on a multicopy plasmid, STT4 had no effect on the PI kinase activity (Fig. 6A). Western blot analysis using an anti-Stt4p antibody showed that there was no difference in the amount of Stt4p between wild-type cells and the stt4 null mutant cells harboring an STT4 plasmid (stt4::HIS3/pSTT4). Rather, overexpression of STT4 appeared to cause degradation of excess Stt4p, since lower molecular weight cross-reacting material was detected in these extracts (Fig. 6B).

**DISCUSSION**

We have isolated the STT4 gene by complementation of the temperature-sensitive growth of the staurosporine-sensitive stt4 mutant. The STT4 gene product predicts a hydrophilic protein of 1,900 amino acids. The C-terminal region of Stt4p has homology to the C-terminal regions of the yeast Vps34p (26% identity in 387 amino acids) and the catalytic subunit of mammalian PI3-kinase (p110) (27% identity in 470 amino acids). PI4-kinase activity of the stt4 deletion mutant cells is about 15% of wild-type cells (Fig. 5). Therefore, Stt4p is likely one of several yeast PI4-kinases. Furthermore, previous biochemical analyses have shown that multiple PI kinases are present in yeast, i.e. p45, p56, and p125 PI4-kinases and PI3-kinase (14-17). Thus, it is not surprising that the stt4 null mutant cells have residual PI4-kinase activity.

Vps34p is a hydrophilic protein of 875 amino acids and is required for vacuolar protein sorting and vacuole segregation (23). PI3-kinase activity is not detected in yeast cells depleted for the VPS34 gene (50). This observation excludes the possibility that the Stt4p PI kinase has some residual PI3-kinase activity. Overexpression of STT4 had no effect on PI4-kinase activity (Fig. 6A). It is probable that another factor(s) is required for the stabilization or activation of Stt4p PI4-kinase like mammalian PI3-kinase, a complex of the p85 regulatory subunit and the p110 catalytic subunit (22). STT4 is essential for cell growth in YPD medium but is dispensable for growth in the presence of an osmotic stabilizer.

**REFERENCES**

1. Nishinari, Y. (1994) Science 255, 1365-1370
2. Nishinari, Y. (1994) Nature 368, 498-498
3. Endersson, G., Dunn, S. N., and Cantley, L. C. (1987) Biochemistry 26, 6845-6852
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4. Whitman, M., Kaplan, D. R., Robert, T. M., and Cantley, L. C. (1987) Biochem. J. 247, 165-174
5. Whitman, M., Downes, C. P., Keeler, M., Kellet, T., and Cantley, L. C. (1988) Nature 333, 644-646
6. Lipt, D. L., Majerus, P. W., Gorga, F. R., Young, A. T., and Benjamin, T. L. (1989) J. Biol. Chem. 264, 8759-8763
7. Serunian, L. A., Haber, M. T., Poku, K., Kim, J. W., Bbee, S. G., Lowenstein, J. M., and Cantley, L. C. (1989) J. Biol. Chem. 264, 17809-17815
8. Henry, S. A. (1982) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern J. N., Jones, E. W., Broach, J. R., eds) pp. 101-118, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Becker, G. W., and Lester, R. L. (1977) J. Biol. Chem. 252, 8684-8690
10. Nakata, J., Kodaki, T., and Yamashita, S. (1987) J. Biol. Chem. 262, 4876-4881
11. Kato, K., Miyajima, A., Araki, K., and Matsumoto, K. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8172-8176
12. Hawkins, P. T., Stephens, L. R., and Piggott, J. R. (1983) J. Biol. Chem. 258, 3374-3383
13. Dahl, C., Biemann, H.-P., and Dahl, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4012-4016
14. Belinsky, D. J., Bae-Lee, M., Kelly, M. J., and Carman, G. M. (1988) J. Biol. Chem. 263, 18897-18903
15. Auger, K. R., Carpenter, C. L., Cantley, L. C., and Vartiovski, L. (1989) J. Biol. Chem. 264, 20151-20184
16. Nickels, J. T., Jr., Buxeda, R. J., and Carman, G. M. (1992) J. Biol. Chem. 267, 16302-16304
17. Flanagan, C. A., and Thorner, J. (1992) J. Biol. Chem. 267, 24117-24125
18. Ogita, K., Miyamoto, S., Kudo, H., Iwai, T., Oka, M., Ando, K., Kishimoto, A., Ikeda, K., Fukami, Y., and Nishizuka, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5011-5015
19. Simon, A. J., Milner, Y., Saville, S. P., Dvir, A., Mochly-Rosen, D., and Orr, E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3376-3380
20. Kato, M., Uno, I., Ishikawa, T., and Takensawa, T. (1989) J. Biol. Chem. 264, 4043-4045
21. Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., and Thorner, J. (1990) Cell 62, 213-224
22. Hikeshi, D., Otsu, M., Volvinis, S., Fry, M. J., Gett, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hauan, J. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1992) Cell 70, 419-429
23. Herman, P. K., and Emr, S. D. (1990) Mol. Cell. Biol. 10, 8743-8754
24. Omura, S., Iwai, Y., Hirano, A., Nakagawa, A., Awaya, J., Tsuji, Y., Takahashi, Y., and Masuda, R. (1977) J. Antibiot. (Tokyo) 30, 275-282
25. Takamaki, T., Nishino, H., Takahashi, I., Katayose, Y., Tomizato, M., and Tomita, K. (1986) Biochem. Biophys. Res. Commun. 138, 397-402
26. Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuu, Y., and Iba, H. (1987) J. Antibiot. (Tokyo) 40, 706-708
27. Yoshihara, S., Ikeda, E., Uno, I., and Mitozawa, H. (1992) Mol. Genet. 231, 337-344
28. Rose, M. D., Winston, F., and Hester, P. (1990) Methods in Yeast Genetics: a Laboratory Course Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Ito, H., Fukuda, Y., Murata, M., and Kuroda, A. (1983) J. Bacteriol. 153, 163-168
30. Bullock, W. O., Fernandez, J. M., and Short, J. M. (1987) BioTechniques 5, 376-379
31. Vieira, J., and Mesang, G. (1987) Methods Enzymol. 153, 3-11
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Yoshii, T., and Anraku, Y. (1989) Biochem. Biophys. Res. Commun. 163, 908-915
34. Morishita, T., and Uno, I. (1991) J. Bacteriol. 173, 2406-2408
35. Winston, F., Chumley, F., and Fink, G. R. (1985) Methods Enzymol. 104, 211-228
36. Southern, E. M. (1975) J. Mol. Biol. 98, 503-517
37. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 83, 5463-5467
38. Silko, S. R., and Hester, P. (1989) Genetics 122, 19-27
39. Rothstein, R. (1983) Methods Enzymol. 101, 202-211
40. Jones, J. R., and Prakash, L. (1990) Nature 346, 263-266
41. Vollrath, D., Davis, R. W., Connelly, C., and Hester, P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6027-6031
42. Walsh, J. P., Caldwell, R. K., and Majerus, P. W. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9184-9187
43. Rutter, U., and Muller-Hill, B. (1983) EMBO J. 2, 1791-1794
44. Nakano, A., Brada, D., and Schekman, R. (1988) J. Cell Biol. 107, 851-863
45. Laemmli, U. K. (1970) Nature 227, 680-685
46. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
47. Nishikawa, S., Umemoto, N., Ohsaki, Y., Nakano, A., and Anraku, Y. (1990) J. Biol. Chem. 265, 7440-7445
48. Levin, D. E., and Bartlett, H. F. (1990) J. Cell Biol. 114, 1221-1229
49. Wada, Y., Ohsaki, Y., and Anraku, Y. (1992) J. Biol. Chem. 267, 18655-18670
50. Herman, P. K., Stack, J. H., and Emr, S. D. (1992) Trends Cell Biol. 2, 369-374
51. Herman, P. K., Stack, J. H., DeMedema, J. A., and Emr, S. D. (1991) Cell 64, 425-437
52. Perkins, D. D. (1949) Genetics 34, 607-626
53. Mortimer, R. K., and Schild, D. (1985) Microbiol. Rev. 49, 181-212