Protein Farnesylation Is Critical for Maintaining Normal Cell Morphology and Canavanine Resistance in Schizosaccharomyces pombe*

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Protein farnesyltransferase (FTase) plays important roles in the growth and differentiation of eukaryotic cells. In this paper, we report the identification of the Schizosaccharomyces pombe gene cpp1 encoding the β-subunit of FTase. The predicted amino acid sequence of the cpp1 gene product shares significant similarity with FTase β-subunits from a variety of organisms. S. pombe FTase purified from E. coli exhibits high enzymatic activity toward the CAAX farnesylation motif substrates (where C represents cysteine, A represents an aliphatic amino acid, and X is preferentially methionine, cysteine, serine, alanine, or glutamine) while showing little preference for CAAL geranylgeranylation motif substrates (where L represents leucine or phenylalanine). Cpp1 is not essential for growth as shown by gene disruption; however, mutant cells exhibit rounded or irregular cell morphology. Expression of a geranylgeranylated mutant form, Ras1-CVIL, which can bypass farnesylation, rescues these morphological defects. We also identify a novel phenotype of cpp1 mutants, hypersensitivity to canavanine. This appears to be due to a 3-4-fold increase in the rate of arginine uptake as compared with wild-type cells. Expression of the geranylgeranylated mutant form of a novel farnesylated small GTPase, SpRheb, is able to suppress the elevated arginine uptake rate. These results demonstrate that protein farnesylation is critical for maintaining normal cell morphology through Ras1 and canavanine resistance through SpRheb.

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The abbreviations used are: FTase, protein farnesyltransferase; GGTase I and II (GGTase I and GGT-II) (1, 2). FTase recognizes the C-terminal CAAX motif (where C represents cysteine, A is an aliphatic amino acid, and X is preferentially methionine, cysteine, serine, alanine, or glutamine) present in substrate proteins such as Ras and the γ-subunits of yeast heterotrimeric G-protein and transducin. GGTase I recognizes the CAAL motif (similar to the CAAX motif except the C-terminal residue is leucine or phenylalanine) present in proteins such as Rho1 and Cdc42. FTase and GGTase I are both heterodimers of α- and β-subunits and share a common α-subunit. Unlike FTase and GGTase I, GGTase II contains a third subunit in addition to the α/β heterodimer and prenylates proteins such as Rab family small GTPases, which end with CC or CXC. The three-dimensional structures of the rat FTase as well as FTase complexed with its substrates have recently been solved by x-ray crystallography (3–5).

FTase has been identified from a wide range of organisms including Saccharomyces cerevisiae, plants, and mammalian cells (1, 2, 6–8); and protein farnesylation has been shown to play critical roles in a variety of cellular processes in these organisms. In S. cerevisiae, farnesylation is necessary for processing of the a-factor mating pheromone and regulation of the Ras/cAMP pathway (1), while in A. thaliana, FTase is important in abscisic acid signal transduction (7, 9). In mammalian systems, FTase is directly involved in Ras/mitogen-activated protein kinase signaling pathways (1, 10). Recent demonstrations that inhibitors of FTase (FTIs) block a wide range of human tumors point to the importance of farnesylation in growth and transformed phenotypes of cancer cells (1, 11–14). In addition, these inhibitors were also shown to affect cell cycle and induce apoptosis in transformed cell lines (14–16). Many of these tumors or cell lines do not contain mutant Ras, pointing to the intriguing possibility that the real target(s) of FTIs is not Ras but rather a novel farnesylated protein(s) involved in cell proliferation, cell cycle control, or apoptosis (17, 18). Therefore, identifying phenotypic consequences of the loss of FTase is important for gaining insights into FTase substrates.

We sought to exploit Schizosaccharomyces pombe to study farnesylation and to identify physiologically relevant substrates of FTase because it is genetically amenable and many aspects of S. pombe cellular functions are similar to those in multicellular organisms (19). Therefore, S. pombe provides a useful model for studying farnesylation with respect to mammalian cells. As a first step, we attempted to identify genes encoding subunits of FTase in S. pombe. Previously, we reported cloning of the α-subunit of GGTase I, cpp1 (1), which was identified in a yeast two-hybrid screen for proteins that interact with Cwg2 (20), the β-subunit of GGTase I (21). Since FTase and GGTase I share a common α-subunit, we have therefore have polymerase chain reaction; FPP, farnesyl diphosphate; MES, 4-morpholineethanesulfonic acid.
also identified the α-subunit of FTase. In this paper, we describe the β-subunit of FTase, Cpp1, which, when co-purified with the α-subunit, exhibits high farnesyltransferase activity toward CAAX-ending proteins. Disruption of cpp1 results in abnormal cell morphology, which is mainly due to the lack of farnesylation of the small GTPase Ras1. We also show that cpp1-mutated yeast cannot grow in the absence of arginine, which is due to the loss of function of the novel small GTPase SpRheb.

These results implicate the involvement of SpRheb in the regulation of arginine uptake in S. pombe.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Growth Conditions**—S. pombe strains SP224 (h ura4 leu1-32 ade6) and SP224 (h ura4 leu1-32 ade6) were both provided by Dr. D. Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). cpp1 mutant strains WY17A, WY21A, and WY21B were all derived from SP224. The haploid S. cerevisiae strain KMY200-aggNO2-12A (MATa ura3 his3 ade8 trpl1::URA3) (22) contains a disruption cassette. S. pombe cells were grown in complete medium (YES) or minimal medium plus necessary supplements (EMMS) at 26, 30, or 35 °C. EMMS-L indicates EMMS lacking leucine. S. cerevisiae cells were cultured in rich (YPD) or synthetic complete (SC) medium. All yeast transformations were carried out according to standard procedures (23, 24).

**Sequence Analysis and Searches**—BLAST (25) searches were performed at the Sanger Center (Hinxton, UK) on the site Worldwide Web. Sequence alignments were created using the CLUSTAL program in PC/GENE (IntelliGenetics, Inc.). Sequence accession numbers are Z99162 (Cpp1), M22753 (Dpr1), U83708 (tFtp), L10414 (hFtp), and AI034382 (SpRheb).

**Construction of Plasmids**—The open reading frame of the cpp1 gene was obtained by PCR amplification from a S. pombe cDNA library cloned in the pACT vector (20), using primers that contain Nde1 and BamHI restriction sites and high fidelity Vent polymerase (New England Biolabs). The resulting cpp1 fragment was cloned into the corresponding site of pWHa, a S. cerevisiae 2 μm vector that contains a hemagglutinin (HA) epitope, to create pWHa-CPP1. The cpp1 cDNA was then sequenced to ensure no mutations were introduced. pYD-DPR1 is a 2 μm plasmid carrying the Dpr1 gene under its own promoter. To generate pREP1HA-CPP1 and pREP81HA-CPP1, a blunt-ended EcoRI to BamHI fragment of HA-CPP1 was inserted into the blunt-ended Nde1 and BamHI sites of the pREP1 and pREP81 thiamine-repressible vector, respectively (26, 27). SpRheb plasmids were designated as pREPSpRHEB, pREPSpRHEB-SVIA, and pREPSpRHEB-CVIL, respectively.

Recombinant FTase and Substrate Purification—Recombinant S. pombe FTase was purified as a Cpp1/Cwp1 heterodimer from the E. coli strain DH5α. To express recombinant FTase in DH5α, two plasmids were constructed. One of these plasmids, pGEX-CPP1, which carries the cpp1 gene fused in frame to the glutathione S-transferase (GST) encoding gene, was constructed by subcloning a Ndel–SalI fragment of ptet1::rpm1 (isolated from pGEXX-3 (Amerham Pharmacia Biotech). The other plasmid, pBC-CW1, which contains the cpp1 gene, was inserted into a BamHI site of ptet-BS + (Stratagene), which carries the chloramphenicol resistance gene. Both plasmids were co-transformed into DH5α. Cells were grown to A600 = 0.5 at 26 °C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4–8 h. After centrifugation, cells were suspended in a sonication buffer (2 mM phosphate buffer, pH 7.5 Ci mmol−1) (NEN Life Science Products) was used as prenyl donor. Incubations were carried out at 30 °C for varying times with 0–4 μM purified protein substrates, 50–100 μM enzyme, and 1 μM [3H]FPP.

To confirm the loss of FTase function in cpp1 strains, soluble cell extracts prepared from cpp1 and SP224 strains were used in the above assay as the source of FTase enzyme. Extracts were prepared from yeast cultures grown to midlog phase. After centrifugation, cells were suspended in buffer A (100 mM MES, sodium (pH 6.5), 0.1 mM MgCl2, 0.1 mM EGTA, 1 mg/ml bovine serum albumin, 1% Lubrol, 0.15 mg/ml dithiothreitol, 0.15 mg/ml lysozyme) plus protease inhibitor mixture (COMPLETE; Roche Molecular Biochemicals), broken by sonication, and centrifuged to remove insoluble material. The supernatant was then incubated with 50% glutathione-agarose slurry (Sigma) for 2 h at 4 °C. Bound FTase was eluted using 2 ml of 10 mM reduced glutathione. Protein concentration was quantitated by the Bradford method. Substrates for FTase assays (28) were purified similarly to S. pombe FTase.

**Farnesyltransferase Assays**—FTase assays were carried out as described (29). Bacterially purified S. pombe FTase was used as the enzyme. Fusion proteins GST-CIIS, GST-CIIL, GST-Rho3 (S. cerevisiae), maltose-binding protein-RhoA, and GST-Cdc42 (human) were used as substrates. The prenylation substrates [3H]FPP (3 Ci mmol−1) (NEN Life Science Products) was used as prenyl donor. Incubations were carried out at 30 °C for varying times with 0–4 μM purified protein substrates, 50–100 μM enzyme, and 1 μM [3H]FPP.

To the cell extracts, 200000 g for 1 h to generate soluble cell extracts. FTase assays were performed at 30 °C for 20 min using 50 μg of extracts, protein substrate GST-CII, and prenyl donor [3H]FPP. Reaction mixtures were then separated by SDS-polyacrylamide gel electrophoresis. The gel was fixed, treated with Amplify (Amerham Pharmacia Biotech), dried, and subjected to autoradiography.

**Gene Disruption and Integration**—The entire cpp1 gene was deleted from the genome of the haploid strain SP224 and replaced by the S. pombe urad4 gene. 500-base pair fragments of the 5′- and 3′-flanking regions of cpp1 were obtained by PCR from genomic S. pombe DNA. PCR primers were designed so that short sequences of the S. pombe urad4 gene were added onto the 3′-end of the 5′-flanking region and the 5′-end of the 3′-flanking region. A 1.7-kilobase pair urad4 gene fragment was also obtained by PCR from the SpRheb plasmid (26). The cpp1-1flanking regions were then fused with the urad4 gene in a single PCR. The resulting 2.7-kilobase pair PCR product was purified and used to transform SP224. Stable urad4 colonies were isolated and screened by whole cell PCR (30). Using primers that anneal to genomic sequences outside of the disruption cassette and primers internal to the urad4 gene, we confirmed that containing the cpp1 disruption were identified. Three such disruptants were obtained and designated as WY17A, WY21A, and WY21B.

**Arginine Uptake Assays**—Arginine uptake assays were performed as reported by Urano et al. with minor modifications. Satulated cultures grown in EMMS-L were diluted in the same medium and grown at least three generations to midlog phase (A600 = 0.2–0.5) at 26 or 30 °C. Cells were then centrifuged and suspended in EMMS-L to A600 = 2. To initiate the uptake assay, cold L-arginine was added to a final concentration of 100 μM, and i-3H]Larginine (38.5 Ci/mmol, Sigma; 40.0 Ci/ mmol, ARC) was added to a final concentration of 4.16 μCi/ml. For each time point, 200 μl of cells were diluted in 5 ml of deionized water, filtered immediately, and washed twice with 5 ml of water using a vacuum manifold. Fisherbrand G4 or Whatman GPC glass microfiber filters were used for filtration. Filters were then dried and immersed in 2 J. Urano, A. P. Tabancay, W. Yang, and F. Tamanoi, submitted for publication.
Ecoscint A (National Diagnostics). The radioactive amino acid remaining was counted using a Beckman LS6500 Scintillation counter.

RESULTS

cpp1 Encodes the Putative β-Subunit of S. pombe FTase—We previously reported the cloning of the GTase I α-subunit gene in S. pombe, cpp1 (20). Using the yeast two-hybrid system, cpp1 was found to be an interacting partner for cwp1, the β-subunit gene of GTase I (21). Since FTase and GTase I share a common α-subunit, we next sought to identify the β-subunit of FTase in order to characterize FTase from this organism. A BLAST program search using DPR1, the gene encoding the β-subunit of S. cerevisiae FTase (31), against the S. pombe genome sequence at the Sanger Center revealed an open reading frame exhibiting significant similarity to DPR1. We termed the gene cpp1 (for cwp1 partner). As shown in Fig. 1, the cpp1 gene encodes a protein of 382 amino acids that shows a 33.8% identity and a 65.3% overall similarity with the Dpr1 protein. Similar scores are obtained when compared with the human FTase β-subunit (33.4% identity and 63.9% overall similarity) as well as with other mammalian and tomato β-subunits. Highly conserved stretches are found in the β-subunits, which also exist in the cpp1 gene product (i.e. residues 202–210 and residues 249–257). We have previously described residues Tyr362 and Tyr366 of S. cerevisiae Dpr1 to be critical for recognition of its protein substrates (29). These residues are conserved in Cpp1 as well.

cpp1 Complements the S. cerevisiae dpr1 Mutation—In order to provide genetic evidence that cpp1 encodes the FTase β-subunit, we examined whether it can complement the S. cerevisiae dpr1 mutation. Disruption of DPR1 in S. cerevisiae results in temperature-sensitive growth at 35 °C (32); consequently, we determined if expression of cpp1 rescues growth at 35 °C (A) or 26 °C (B).
at this temperature. The cpp1+ gene was cloned into the 2μ yeast vector pWHA under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter. The resulting construct (pWHA-CPP1) was transformed into the dpr1 mutant strain KMY200-Δsgp-No2–12A. A 2μ vector carrying DPR1 under the DPR1 promoter as well as vector alone were also transformed into KMY200-Δsgp-No2–12A. As can be seen in Fig. 2B, temperature-sensitive growth of KMY200-Δsgp-No2–12A was suppressed at 35 °C by expression of cpp1+ as well as by DPR1 but not by vector alone. This suppression is consistent with the observation that farnesylated proteins are almost fully processed in these cells. We have previously demonstrated that proteins prepared from dpr1 mutant cells can be farnesylated in vitro, while proteins from wild-type cells cannot be modified. This difference is presumably due to accumulation of unmodified proteins in dpr1 mutant cells. As with wild-type cells, proteins prepared from dpr1 cells expressing cpp1+ could not be farnesylated by purified FTase in vitro (data not shown). This result suggests that majority of CAAX-ending proteins were modified in vivo. The complementation of the temperature sensitivity of dpr1 mutants by cpp1+ requires cpp1+ to be

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overexpressed, since expression from a centromeric vector had minimal effect (data not shown).

Recombinant Cwp1/Cpp1 Heterodimer Exhibits High FTase Activity and Preferentially Farnesylates CAAX-ending Proteins—To obtain biochemical evidence that the ccpp1 mutant genes encode subunits of FTase, we constructed a GST fusion of ccpp1 in an ampicillin selection plasmid, pGEX5X-3. The ccpp1 gene, on the other hand, was cloned into a chloramphenicol selection plasmid, pBC-KS(+) (+). These constructs were co-transformed into E. coli, and the GST-Cpp1/Cwp1 complex was purified to near homogeneity using a glutathione column (Fig. 3A). Fig. 3B shows the incorporation of [3H]farnesyl as a function of protein substrate concentration. This enzyme preparation exhibited potent FTase activity toward the CAAX substrate GST-CII, which was comparable with that of purified S. cerevisiae FTase (data not shown). In contrast, lower incorporation was observed with the CAAL substrate GST-CII. K_m values for protein substrates were calculated from Lineweaver-Burke plots of S. pombe FTase assays carried out in the presence of saturating amounts of farnesyl diphosphate. S. pombe FTase exhibited an apparent K_m of 370 ± 60 nm toward GST-CII and a K_m of 990 ± 90 nm for GST-CIII. These values are similar to those of S. cerevisiae and the human enzymes, both of which have lower K_m values for GST-CIII than for GST-CII (250 nm versus 1440 nm for S. cerevisiae and 380 nm versus 2050 nm for human) (29, 33). In addition to GST-CII, the S. pombe FTase also efficiently farnesylated another CAAX protein, S. cerevisiae Rho3, while prenylating two different CAAL proteins, Cdc42 and RhoA, with very low efficiency (Fig. 3C). These results indicate that S. pombe FTase preferentially farnesylates CAAX-ending proteins. However, our results are not in agreement with those of Danjoh and Fujiyama (34), who reported that S. pombe FTase shows similar affinities toward both CAAX and CAAL substrates. Differences in enzyme preparations utilized may account for this discrepancy. We used recombinant FTase from Escherichia coli, while Danjoh and Fujiyama used partially purified FTase from S. pombe cell extracts.

Disruption of ccpp1 Mutant Results in Distinct Phenotypes Including Morphological Changes—To gain insight into the physiological function of FTase in S. pombe, we disrupted the ccpp1 gene by homologous recombination. The entire open reading frame of ccpp1 was replaced with the ura4+ gene in the haploid strain SP224, using the one-step gene replacement method, and the disruption was verified by PCR (see “Experimental Procedures”). To ensure that FTase activity is indeed abolished in ccpp1 cells, we performed FTase assays using ccpp1 and wild-type cell extracts as the source of FTase. Assay samples were separated by SDS-polyacrylamide gel electrophoresis, and FTase activity was assessed by the appearance of a radioactive band indicating farnesylated GST-CII. No FTase activity was observed in the ccpp1 mutant cell extracts (WY17A, WY17B, WY21A) as compared with extracts of ccpp1 cells (SP224 and WY25A) (Fig. 4).

Although deletion of ccpp1 was not lethal, the mutants did exhibit phenotypes similar to those of S. cerevisiae dpr1 mutants including slow growth rate at 26 °C, with a doubling rate three times that of the wild-type (data not shown). The mutants were also temperature-sensitive for growth. As shown in Fig. 5A, the three ccpp1 mutants (WY17A, WY21A, WY21B) did not grow at 35 °C in contrast to the parental strain, SP224. The temperature-sensitive defect was complemented by expression of ccpp1 under the low expression thiamine-repressible nmt81 promoter but not by vector alone (Fig. 5B). Another phenotype we observed is that ccpp1 mutants are defective in mating (data not shown). When ccpp1 mutant cells (P-mating type) were mated with the M-mating type strain SP223, no apparent conjugation was observed, nor were zygotic asci formed. On the other hand, the cross between SP223 and the ccpp1 control strain, SP224, efficiently produced zygotic asci.

One dramatic phenotype of ccpp1 disruption was rounded or irregularly shaped cells in contrast to the elongated morphology of wild-type cells (Fig. 6, A, B, and C). This phenotype was not rescued under cell wall stabilizing conditions of 1.2 mM sorbitol (data not shown). However, when the ccpp1 gene was expressed in WY17A (ccpp1) cells under the control of the nmt81 promoter, morphology was restored to that of the wild type (Fig. 6D). This confirmed that the morphological changes are due to the loss of FTase function.

Expression of a Geranylgeranylated Mutant Form of Ras1 Restores Normal Morphology to ccpp1 Mutant Cells—The
small GTPases Ras and Rho are thought to have important functions in cell morphogenesis in *S. pombe* (35–37). Moreover, these proteins contain a C-terminal CAAX or CAAL motif that is prenylated by FTase or GGTase I, respectively. *S. pombe* Ras1 plays a role in cell morphogenesis by regulating a protein complex consisting of a Rho-related protein Cdc42sp and its effectors (36, 37). *ras1* disruption results in swollen cells much like *cpp1* mutants (38). In addition to Ras1, two Rho proteins, Rho2 and Rho3, also have CAAX farnesylation signals (39). To examine whether the *cpp1* morphology is due to the lack of farnesylation of Ras1, Rho2, or Rho3, we tested if overexpression of these proteins complements the *cpp1* morphological defects. In FTase-deficient cells, some normally farnesylated proteins can be prenylated, albeit to a much lesser extent, by GGTase I (40). This low level of prenylation, in addition to
overexpression, may result in the production of enough functional protein to suppress the morphological defects. HA-tagged Ras1, Rho2, or Rho3 was expressed in WY17A (cpp1Δ) and SP224 (wild type) under the thiamine-repressible nmt1 promoter. Cells were grown in medium containing thiamine and then shifted to thiamine-free medium to induce expression. Overexpression of Ras1 partially converted the round cell shape of WY17A to the rodlike cell morphology similar to wild type (Fig. 7, compare C with A and B). In contrast, overexpression of Rho2 or Rho3 did not affect WY17A morphology (Fig. 7, D and E). Further evidence for Ras1 involvement in the morphological changes was obtained using a geranylgeranylated mutant form of Ras1 (Ras1-CVIL), which bypasses the farnesylation requirement. The C-terminal residue was mutated from cysteine to leucine to create the C motif. As shown in Fig. 7F, WY17A cells expressing Ras1-CVIL under the control of the nmt81 promoter, which is much weaker than nmt1, became elongated several hours after thiamine withdrawal; furthermore, the suppression was better than that seen using wild-type Ras1. This result suggests that geranylgeranylated Ras1 can function as well as farnesylated Ras1. In contrast to Ras1-CVIL, Rho3-CIIIL did not rescue the morphological defects of cpp1Δ mutants (data not shown). Rho3-CIIIL was probably functional, since it suppressed the altered morphology of rho3Δ null cells exhibited at elevated temperatures (data not shown). Similar experiments using WY21A produced comparable results (data not shown). Expressions of all the proteins were confirmed by Western analysis (data not shown). The effect of geranylgeranylated Rho2 expression was not evaluated, since loss of rho2Δ function has minor effects on cell shape (39). Taken together, these results suggest that Ras1 is a major farnesylated protein regulating cell morphogenesis.

Since Ras1 is also involved in the mating pathway in S. pombe (36, 38), we examined if Ras1 expression would accordingly complement the cpp1Δ mating defect. WY17A expressing Ras1-CVIL was mated with a wild-type strain of the opposite mating type (SP223) and allowed to sporulate. As expected, zygotic asci were observed after 3 days (data not shown). In contrast, Rho3-CIIIL did not complement the mating deficiency of cpp1Δ mutants (data not shown). Thus, S. pombe FTase also regulates mating through the Ras1 pathway.

Disruption of cpp1Δ Results in Hypersensitivity to Canavanine—Another dramatic change we observed with cpp1Δ mutants is hypersensitivity to canavanine, a toxic arginine analogue. WY21A (cpp1Δ) cells transformed with either vector alone or the wild-type cpp1Δ gene were grown to saturation in EMMS-L medium. 2-fold serial dilutions were made, and 5 μl of cells were then spotted onto the indicated plates and allowed to grow at 26 °C for several days. Sensitivity was determined by assessing the A600 at which cell growth is no longer observed. As shown in Fig. 8A, both vector and cpp1Δ transformants grew well on canavanine-free plates. The growth difference between the two strains is presumably due to the slower growth rate of cpp1Δ mutant cells. When cells were plated on 1 mg/liter canavanine, cpp1Δ transformants showed slight sensitivity at A600 = 0.0625 whereas vector transformants showed minimal or no growth even at A600 = 2. Therefore, cpp1Δ mutants exhibited at least a 32-fold higher canavanine sensitivity than cpp1Δ wild-type cells.

cpp1Δ Mutants Exhibit Increased Arginine Uptake Due to the Loss of Farnesylation of the Small GTPase SpRheb—One explanation for the canavanine hypersensitivity is that cpp1Δ mutants are able to take up more canavanine, thus increasing the intracellular canavanine concentration to lethal levels. Canavanine is transported into the cell via the arginine permease Can1p (41). To test the above possibility, we examined the arginine uptake rate of cpp1Δ mutants (WY21A) harboring either vector or the cpp1Δ gene. Cells were grown in EMMS-L to midlog phase and harvested. Arginine uptake was followed over time by the addition of [3H]arginine to the culture. To
minimize possible effects due to arginine metabolism during prolonged incubation periods, a short time course was performed. As depicted in Fig. 9, vector-transformed cpp1− mutants exhibited a 3–4-fold increase in the initial rate of arginine uptake compared with cpp1− mutant cells bearing a wild-type cpp1− gene. The uptake rate was linear up to at least 5 min of incubation. These data suggest that farnesylation is important for the regulation of arginine uptake and implicate a farnesylated protein(s) to be involved in this process.

To rule out the possibility that Ras1 or Rho3 is the target responsible for arginine uptake, we expressed Ras1 and Rho3 under the control of the nmt1 promoter as well as Ras1-CVIL and Rho3-CIIIL under the control of the nmt81 promoter. Neither Ras1 nor Ras1-CVIL was able to decrease the arginine uptake rate of cpp1− mutant cells to the wild-type level (Fig. 10A). Similar results were obtained for Rho3 and Rho3-CIIIL (data not shown). These results indicate that Ras1 and Rho3 are not involved in the regulation of arginine uptake in S. pombe.

ScRheb, the S. cerevisiae homologue of the mammalian small GTPase Rheb, is farnesylated in vitro and in vivo.2 Gene disruption of ScRHEB also confers canavanine hypersensitivity, which can be rescued by expression of the S. pombe Rheb homologue (SpRheb).2 We therefore investigated whether expression of SpRheb suppresses the arginine uptake defect of cpp1− mutants. cpp1− mutants (WY21A) transformed with various SpRheb constructs under the control of the SpRheb promoter were used to perform arginine uptake assays. As shown in Fig. 10B, expression of wild-type SpRheb as well as a mutant defective in prenylation, SpRheb-SVIA, took up arginine at rates similar to cpp1− mutants. In contrast, the geranylgeranylated mutant, SpRheb-CVIL, restored uptake rates to the level in cpp1−-transformed cells (Fig. 10B).

We next tested the ability of SpRheb to suppress the canavanine hypersensitivity of cpp1− mutants. In this experiment, various forms of the SpRheb protein were expressed in cpp1− mutant cells (Fig. 8B). As expected, SpRheb-CVIL was able to suppress the canavanine hypersensitivity of cpp1− mutants. On the other hand, wild-type SpRheb and prenylation-defective SpRheb-SVIA only exerted minimal effect. Taken together, these results suggest that FTase affects the regulation of arginine uptake by farnesylating SpRheb in S. pombe.

**DISCUSSION**

Farnesyltransferase plays important physiological roles in the growth and differentiation of eukaryotic cells. FTase has been identified in several organisms including the budding yeast, plants, mammals (1, 2, 6–8), and recently, parasites (42). In this paper, we identify the β-subunit (Cpp1) of FTase in the fission yeast S. pombe. Overall, Cpp1 shows significant sequence similarity to other FTase β-subunits. Furthermore, cpp1− is able to complement the S. cerevisiae FTase β-subunit mutation (dpr1). Biochemical characterization of purified S. pombe FTase demonstrated that it preferentially farnesylates substrates ending with the CAAX motif, whereas the CAAL motif, much like enzymes from S. cerevisiae and human. Together with our previous identification of the α-subunit Cwp1 (20), both subunits of S. pombe FTase have now been identified. cpp1− is not an essential gene in S. pombe; however, the disruption results in a number of interesting phenotypes. They include slow growth at low temperatures, inability to grow at high temperatures, dramatic morphological changes, mating deficiency of P-type cells, and hypersensitivity to canavanine. Clearly, loss of farnesylation in S. pombe has significant physiological effects. Of these effects the growth defects were the only phenotypes that are not complemented by the small GTPases tested,3 suggesting that these phenotypes are due to other farnesylated proteins. Further studies are needed to identify these proteins. Although not examined in this study, it is also likely that mating of S. pombe M-type cpp1− mutants is affected. In addition to the loss of Ras1 signaling, the M mating factor ends with the CAAX motif, whose modification is required for pheromone response by P-type cells (43).

Of the many phenotypes cpp1− mutants display, the morphological changes are of particular interest. Instead of elongated rod-shaped cells characteristic of wild-type strains, cpp1− mutants form round or misshapen cells, a phenotype observed even at low temperatures. This phenotype contrasts with S. cerevisiae dpr1 mutants, which show no significant morphological alterations at low temperatures despite slow growth (32). Although three GTPases, Ras1, Rho2, and Rho3, all contain the CAAX farnesylation signal, our analysis revealed that Ras1 is the primary protein responsible for the morphological changes observed with cpp1− mutants. The effect of Ras1 and Ras1-CVIL expression could be explained by their interactions with a protein complex including Cdc42p; Scd1/Ral1, the putative Cdc42p guanine nucleotide exchange factor; and Scd2, a Src homology 3 domain-containing protein (35–37). These interactions could then mediate downstream events, which ultimately lead to effects on cell morphology. Accordingly, an activated Cdc42p would be expected to reverse the altered morphology of cpp1− mutant cells. However, this is
difficult to demonstrate, since expression of activated Cdc42ep itself causes similar morphological changes (44).

Although Rho2 and Rho3 do not appear to be primarily involved in the morphological changes observed with cpp1− mutant cells, they do appear to play roles in the morphology of S. pombe cells. Gene disruption of rho3− causes cell rounding, enlargement, and cytokinesis defects at elevated temperatures (45). The tips of rho3− mutants become enlarged, forming bowling pin-like shapes; some cells also become lemon-shaped. These morphologies, however, are different from that of cpp1− mutants. Thus, Rho3 appears to be involved in a morphological pathway distinct from Ras1, and the lack of protein farnesylation primarily affects the Ras1 pathway. In fact, rho3− morphology at elevated temperatures resembles that of S. cerevisiae rho3 mutants, and S. pombe Rho3 complements the S. cerevisiae rho3 mutant phenotype. Recently, S. cerevisiae Rho3 has been shown to interact with components of the exocytosis machinery (46); consequently, S. pombe Rho3 may affect morphology as a result of its effect on exocytosis. Rho2, which was recently reported to interact with PCK homologues (Pck1p and Pck2p) involved in cell wall synthesis and cell integrity (47), may have minor effects on cell morphology (39).

Another important consequence of FTase deficiency is hypersensitivity to canavanine. This appears to be the result of an increased rate of canavanine uptake, since cpp1− mutants show a 3−4-fold increase in the rate of arginine uptake and canavanine is transported mainly via the arginine permease Can1p. Consistent with cpp1− mutant cells, S. cerevisiae FTase mutants also exhibit canavanine hypersensitivity and elevated arginine uptake. Thus, increased arginine uptake may be a general phenotype of FTase-deficient cells. Further analysis showed that this phenotype is unrelated to phenotypes caused by Ras1 or Rho3, since the increased arginine uptake cannot be suppressed by the expression of geranylgeranylated Ras1 or Rho3. Instead, the elevated arginine uptake is due to loss of farnesylation of another small GTPase, SpRheb, which was recently identified as a homologue of the mammalian Rho protein. These proteins are members of a novel class of the Ras superfamily of GTPases. In addition to S. pombe and mammals, other potential class members were identified in S. cerevisiae, the fruit fly (Drosophila melanogaster), the Zebrafish (Danio rerio), and the sea squirt (Ciona intestinalis) through homology searches. Members of this class share similar effector domains and a conserved arginine residue at the position corresponding to the 12th amino acid in Ras, which is a glycine. This arginine appears to be important for maintaining full activity of the S. cerevisiae Rho protein. Moreover, all of the members of the Rho class of proteins for which we could obtain full sequence contain the CAAX motif, suggesting that they are farnesylated proteins. However, only mammalian and S. cerevisiae Rho proteins have been demonstrated to be farnesylated (48). Further work is needed to discern how farnesylation of SpRheb influences arginine uptake. Results obtained with ScRheb have provided some insights into this question. Disruption of ScRHEB also results in increased arginine uptake, which appears to be due to effects of ScRheb on the arginine specific permease, Can1p (49, 50). Although the mechanism of this regulation remains to be elucidated, the effect on arginine uptake seems to be mediated through interactions of GTP-bound ScRheb via its effector domain. Since SpRheb is capable of suppressing the increased arginine uptake in srcreb disruptants, the mode of SpRheb action is probably similar to that of ScRheb. More studies are needed to validate this point.

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REFERENCES
1. Sattler, I. and Tamanoi, F. (1996) in Regulation of the RAS Signaling Network (Maruta, H., and Akiyama, A., eds) pp. 95–137, R. G. Landes, Austin, TX
2. Zhang, F. L., and Casey, P. J. (1996) Annu. Rev. Biochem. 65, 241–269
3. Park, H. W., Boduburu, S. R., Moomaw, J. F., Casey, P. J., and Beece, L. S. (1997) Science 275, 1800–1804
4. Long, S. B., Casey, P. J., and Beece, L. S. (1989) Biochemistry 28, 9612–9618
5. Strickland, C. L., Windsor, W. T., Syto, R., Wang, L., Bond, R., Wu, Z., Schwartz, J., Le, H. V., Beece, L. S., and Weber, P. C. (1989) Biochemistry 28, 16601–16611
6. Yalovsky, S., Trueblood, C. E., Callan, K. L., Narita, J. O., Jenkins, S. M., Rine, J., and Gruissem, W. (1997) Mol. Cell. Biol. 17, 1986–1994
7. Cutler, S., Ghasssemian, M., Bonetta, D., Conney, S., and McCourt, P. (1996) Science 273, 1295–1291
8. Yang, Z., Cramer, C. L., and Watson, J. C. (1993) Plant Physiol. 101, 667–674
9. Pei, Z. M., Ghasssemian, M., Kwak, C. M., McCourt, P., and Schroeder, J. I. (1990) Science 250, 287–290
10. Campbell, S. L., Khorosavi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1413
11. Gibbs, J. B., and Oliff, A. (1996) Annu. Rev. Pharmacol. Toxicol. 37, 143–166
12. Shimizu-Lacritz, L. M., Oz, Z., Banda, E., Kohl, N. E., Gibbs, J. B., Oliff, A., and Rosen, N. (1995) Cancer Res. 55, 5302–5309
13. Sun, J., Qian, Y., Hamilton, A. D., and Sebti, S. M. (1995) Cancer Res. 55, 4243–4247
14. Barrington, R. E., Subler, M. A., Rands, E., Omer, C. A., Miller, P. J., Hundleby, J. E., Koester, S. K., Trower, D. A., Beards, D. J., Conner, M. W., Gibbs, J. B., Hamilton, K., Kohlan, K. S., Mosser, D. D., O’Neill, T. J., Schaber, M. D., Senderak, E. T., Windle, J. J., Oliff, A., and Kohl, N. E. (1996) Mol. Cell. Biol. 16, 85–92
15. Suzuki, N., Urano, J., and Tamanoi, F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15356–15361
16. Lebowitz, P., Sakamura, D., and Prendergast, G. C. (1997) Cancer Res. 57, 708–713
17. Gibbs, J. B., Graham, S. L., Hartman, G. D., Kohlan, K. S., Kohl, N. E., Omer, C. A., and Oliff, A. (1997) Curr. Opin. Chem. Biol. 1, 197–203
18. Cox, A. D., and Der, C. J. (1997) Biochim. Biophys. Acta 1333, F1–F71
19. Zhao, Y., and Lieberman, H. B. (1995) DNA Cell Biol. 14, 359–371
20. Arellano, M., Coll, P. M., Yang, W., Duran, A., Tamanoi, F., and Perez, P. (1998) Mol. Microbiol. 29, 1357–1367
21. Diaz, M., Sanchez, Y., Bennett, T., Sun, C. R., Godoy, C., Tamanoi, F., Duran, A., and Perez, P. (1993) EMBO J. 12, 5245–5254
22. Nakayama, N., Arai, K., and Matsumoto, K. (1988) Mol. Cell. Biol. 8, 5410–5416
23. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Mol. Cell. Biol. 12, 1357–1367
24. Okazaki, K., Okazaki, N., Kame, K., Jinno, S., Tanaka, K., and Okawaya, H. (1990) Nucleic Acids Res. 18, 6485–6489
25. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
26. Maundrell, K. (1993) Gene (Amst.) 123, 127–130
27. Basi, G., Schmid, E., and Maundrell, K. (1993) Gene (Amst.) 123, 131–136
28. Finegold, A. A., Johnson, D. I., Farnsworth, C. C., Gelb, M. H., Judd, S. R.,
S. pombe FTase Regulates Morphology and Arginine Uptake

29. Del Villar, K., Mitsuzawa, H., Yang, W., Sattler, I., and Tamanoi, F. (1997) *J. Biol. Chem.* **272**, 680–687

30. Wang, H., Kohalni, S. E., and Cutler, A. J. (1996) *Anal. Biochem.* **237**, 145–146

31. Goodman, L. E., Perou, C. M., Fujiyama, A., and Tamanoi, F. (1998) *Yeast* **4**, 271–281

32. Goodman, L. E., Judd, S. R., Farnsworth, C. C., Powers, S., Gelb, M. H., Glomset, J. A., and Tamanoi, F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9665–9669

33. Del Villar, K., Urano, J., Guo, L., and Tamanoi, F. (1999) *J. Biol. Chem.* **274**, 27010–27017

34. Danjoh, I., and Fujiyama, A. (1996) *Eur. J. Biochem.* **236**, 847–851

35. Chang, E. C., Barr, M., Wang, Y., Jung, V., Xu, H. P., and Wigler, M. H. (1994) *Cell* **79**, 131–141

36. Verde, F. (1996) *Curr. Opin. Microbiol.* **1**, 712–718

37. Hugh, D. A. (1995) *Semin. Cell Biol.* **6**, 89–94

38. Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T., and Yamamoto, M. (1986) *Cell* **44**, 329–336

39. Hirata, D., Nakano, K., Fukui, M., Takenaka, H., Miyakawa, T., and Mabuchi, I. (1998) *J. Cell Sci.* **111**, 149–159

40. Trueblood, C. E., Ohya, Y., and Rine, J. (1993) *Mol. Cell. Biol.* **13**, 4260–4275

41. Fant, E. A., and Creanor, J. (1984) *J. Gen. Microbiol.* **130**, 3265–3273

42. Yokoyama, K., Trobridge, P., Buckner, F. S., Van Voorhis, W. C., Stuart, K. D., and Gelb, M. H. (1998) *J. Biol. Chem.* **273**, 26497–26505

43. Nielsen, O., and Davey, J. (1995) *Semin. Cell Biol.* **6**, 95–104

44. Miller, P. J., and Johnson, D. I. (1994) *Mol. Cell. Biol.* **14**, 1075–1083

45. Nakano, K., Imai, J., Arai, R., Matsui, Y., Toh-e, A., and Mabuchi, I. (1998) *Cell Struct. Funct.* **23**, 118

46. Robinson, N. G. G., Guo, L., Imai, J., Toh-e, A., Matsui, Y., and Tamanoi, F. (1999) *Mol. Cell. Biol.* **19**, 3580–3587

47. Arellano, M., Valdivieso, M. H., Cadenas, T. M., Coll, P. M., Duran, A., and Perez, P. (1999) *J. Cell Sci.* **112**, 3568–3578

48. Clark, G. J., Kinch, M. S., Rogers-Graham, K., Sehti, S. M., Hamilton, A. D., and Der, C. J. (1997) *J. Biol. Chem.* **272**, 10608–10615

49. Grenson, M., Mousset, M., Wiaman, J. M., and Bechet, J. (1966) *Biochim. Biophys. Acta* **127**, 325–338

50. Ahmad, M., and Bussey, H. (1986) *Curr. Genet.* **10**, 587–592

51. Caldwell, G. A., Wang, S. H., Naider, F., and Becker, J. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1275–1279

52. Whiteway, M. S., and Thomas, D. Y. (1994) *Genetics* **137**, 967–976

53. Gelb, M. H., Scholten, J. D., and Sebolt-Leopold, J. S. (1998) *Curr. Opin. Chem. Biol.* **2**, 40–48