BTS1 Encodes a Geranylgeranyl Diphosphate Synthase in
Saccharomyces cerevisiae*

Yu Jiang‡, Philip Proteau§, Dale Poulter§, and Susan Ferro-Novick†‡

From the ‡Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06536 and the §Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

Protein prenylation utilizes different types of isoprenoids groups, namely farnesyl and geranylgeranyl, to modify proteins. These lipophilic moieties attach to carboxyl-terminal cysteine residues to promote the association of soluble proteins to membranes. Most prenylated proteins are geranylgeranylated. Geranylgeranylation is catalyzed by two different prenyltransferases, the type I and type II geranylgeranyl transferases, both of which utilize geranylgeranyl diphosphate as a lipid donor. In the yeast Saccharomyces cerevisiae, the BET2 gene encodes the β-subunit of the type II geranylgeranyl transferase. Mutations in this gene cause a defect in the geranylgeranylation of small GTP-binding proteins that mediate vesicular traffic. In an attempt to analyze those genes whose products may interact with Bet2, we isolated a suppressor of the bet2-1 mutant. This suppressor gene, called BTS1, encodes the yeast geranylgeranyl diphosphate synthase. BTS1 is not essential for the vegetative growth of cells; however, disrupting it impedes the geranylgeranylation of many cellular proteins and renders cells cold sensitive for growth. Our findings imply that BTS1 suppresses the bet2-1 mutant by increasing the intracellular pool of geranylgeranyl diphosphate.

Protein prenylation is a post-translational lipid modification that involves the covalent attachment of isoprenoid groups onto cysteine residues at or near the carboxyl termini (Casey, 1992; Schafer and Rine, 1992; Sinensky and Lutz, 1992). The attachment of a lipophilic isoprenoid group to proteins is believed to increase their hydrophobicity, allowing otherwise hydrophilic proteins to associate with membranes. Up to 0.5% of total cellular proteins are estimated to be prenylated (Epstein et al., 1991). Known prenylated proteins include small GTP-binding proteins of the Ras superfamily, nuclear lamins, the yeast mating pheromone α-factor, and trimeric G proteins (Casey, 1992; Schafer and Rine, 1992; Sinensky and Lutz, 1992). These proteins are engaged in a variety of cellular processes, which include the control of cell growth, signal transduction, cytoskeleton, and intracellular membrane traffic (Balch, 1990; Barbacid, 1987).

Two different isoprenoid groups, farnesyl (15 carbons) and geranylgeranyl (20 carbons), are post-translationally attached to proteins (Epstein et al., 1991). Farnesyl is added to proteins that terminate in a CAAX motif (where C is cysteine, A is an aliphatic amino acid, and X can be methionine, cysteine, alanine, glutamine, phenylalanyline, or serine), while geranylgeranyl is transferred onto proteins that end in CAAL (where L is leucine), CC, or CXC motifs (X is any amino acid) (Reiss et al., 1990, 1991, 1992; Seabra et al., 1991, 1992). Most known prenylated proteins are geranylgeranylated (Epstein et al., 1991).

Farnesyl and geranylgeranyl groups are attached to proteins from all-trans farnesyl diphosphate (FPP)1 and all-trans geranylgeranyl diphosphate (GGPP), respectively (Casey, 1992). These lipid precursors are intermediates in the isoprenoid biosynthetic pathway (Goldstein and Brown, 1990). This pathway consists of a series of reactions by which mevalonate is converted into a diverse family of lipophilic molecules that contain a repetitive five-carbon structure. The isoprenoids are subsequently incorporated into a large number of end products, which includes: sterols, ubiquinones, dolichols, tRNAs, and prenylated proteins (Goldstein and Brown, 1990).

FPP is the product of the farnesyl diphosphate synthase. This enzyme, which is the most abundant and widely occurring prenyltransferase, catalyzes the formation of FPP by the sequential addition of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP), and geranyl diphosphate (GPP) (Anderson et al., 1989; Bartlett et al., 1985; Sheares et al., 1989). In some organisms, GGPP is synthesized by a GGPP synthase that catalyzes stepwise additions of IPP to DMAPP, GPP, and FPP. This type of GGPP synthase activity has been detected in mammalian tissue. However, eukaryotic geranylgeranyl diphosphate synthases are known that synthesize GGPP by the addition of a single molecule of IPP to FPP (McCaskill and Croteau, 1993; Sagami et al., 1992, 1993, 1994). But, due to its low activity and the problems in separating this enzyme from FPP synthase, its purification has proven to be difficult (Runquist et al., 1992; Sagami et al., 1993, 1994, 1995).

GGPP is the substrate for two different protein prenyltransferases, the type I (GGTase-I) and type II (GGTase-II) geranylgeranyl transferases (Jiang and Ferro-Novick, 1994; Seabra et al., 1991, 1992). GGTase-I catalyzes the transfer of a geranylgeranyl group from GGPP onto proteins that terminate in a CAAL motif, while GGTase-II attaches geranylgeranyl to terminal CC or CXC residues. Its protein substrates include members of the Rab family of small GTP-binding proteins (Jiang and Ferro-Novick, 1994; Seabra et al., 1992). In the yeast Saccharomyces cerevisiae, the GGTase-II is composed of three subunits, which are encoded by BET2, BET4 (formerly called MAD2), and MR56, respectively (Rossi et al., 1991; Jiang and Ferro-Novick, 1994).

The abbreviations used are: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; HPLC, high pressure liquid chromatography; kb, kilobase(s).

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† To whom correspondence should be addressed: Howard Hughes Medical Institute, Yale University School of Medicine, Boyer Center for Molecular Medicine, 295 Congress Ave., New Haven, CT 06536-0812. Tel.: 203-737-5207; Fax: 203-787-5394.

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Ferro-Novick, 1994; Jiang et al., 1993; Li et al., 1993). The BET2 gene product binds to the product of the BET4 gene to form the catalytic component of this enzyme. MR56 encodes the accessory protein that binds the protein substrate. Mutations in these genes abolish the geranylgeranylation of Ypt1p and Sec4p, two small GTP-binding proteins that mediate intracellular membrane trafficking (Jiang and Ferro-Novick, 1994; Li et al., 1993; Rossi et al., 1991). The bet2–1 gene is a recessive temperature-sensitive mutant allele that fails to grow at 37 °C (Newman and Ferro-Novick, 1987). In this mutant, a failure to geranylgeranylate Ypt1p and Sec4p leads to a defect in the membrane association of these proteins. This deficiency results in a block in intracellular membrane trafficking (Rossi et al., 1991). In an attempt to identify new genes that may interact genetically with BET2, BET4, or MR56, we isolated a suppressor of the bet2–1 mutant. This suppressor gene, named BTS1, suppresses the growth defect of bet2–1 when expressed on a low (CEN) or high (2 μm) copy vector. Sequence analysis revealed a significant homology between BTS1 and the geranylgeranyl diphosphate synthase from Neurospora crassa, suggesting that BTS1 encodes the homologue of this gene in S. cerevisiae. In accordance with this proposal, the BET1 gene product was found to be required for the membrane attachment of Ypt1p and Sec4p, a process that is known to require geranylgeranylation. When BTS1 was expressed in bacterial cells, it generated an activity that was able to convert FPP to GGPP, thereby conclusively demonstrating that the BET1 gene product is the yeast geranylgeranyl diphosphate synthase. This enzyme is a previously unidentified component of the yeast isoprenoid biosynthetic pathway.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions—The following strains were used in this study: ANY119 (MATa/α, bet2–1, ura3–52, his4–619, NY648 (MAT α/α, leu2–3, 112/leu2–3, 112, ura3–52/ura3–52), NY180 (MATα, ura3–52, leu2–3, 112), SFNY26–6A (MATα, his4–619), and SFNY368 (MATα, ura3–52, leu2–3, 112, URA3:BTS1). Yeast strains were grown at 25 or 37 °C in either YP or selective minimal medium that was supplemented with 2% glucose.

Isolation of BTS1—The yeast genomic DNA library used in this study was prepared by ligation of genomic DNA that was prepared from ANY119. This DNA was partially digested with Sau3A and inserted into the BamHI site of pRS316 (CEN, URA3). The library was used to transform the bet2–1 mutant (ANY119), and the transformants were (1 × 105) selected on minimal medium lacking uracil. After a 3-day incubation at 25 °C, the cells were stamp onto YPD plates and incubated for 5 min, washed, and lysed in 1.4 ml of ice-cold lysis buffer (0.8 M sorbitol, 10 mM triethanolamine (pH 7.2), 1 mM EDTA) as described before (Rossi et al., 1991). Cell debris was removed after a 3-min spin at 450 g for 5 min. After a 1-h incubation at 25 °C, the spheroplasts were harvested by centrifugation in a clinical centrifuge during a spin at 1400 rpm for 5 min. The radioactivity in each fraction was determined by liquid scintillation spectrometry. Products were analyzed using HPLC. For the product analysis, bovine serum albumin was omitted from the standard assay mixture, but 10 μM nucleotide fluoride was present to suppress phosphatase activity. After a 1-h incubation at 37 °C, the reaction was terminated by the addition of EDTA (125 μM, final concentration). Unlabeled GGPP (25 μM) was added, and 150 μl of the reaction mixture was injected onto a Shodex Asahipak ODP-50 column (4.6 mm inner diameter) × 250 mm). 2-min fractions were collected, and the radioactivity in each fraction was determined by liquid scintillation counter after the addition of 15 ml of CytoScint-ES.

Preparation of Yeast Extracts and Protein Prenylation Assay—Yeast cells were grown in YPD medium at 25 °C to late log phase. The cells were washed twice with ice-cold 0.7 M sorbitol, 1 mM MgCl2, and resuspended in 0.2 ml Lysis buffer (0.8 M sorbitol, 10 mM dithiothreitol, 25 μM of extract, 0.4 μM of recombinant Ypt1p, and varying concentrations of [1H]GGPP (American Research Lab, 17,500 dpm/μmol). The reaction mixture was incubated at 30 °C for 30 min before it was terminated with 1 ml of 1 M HCl in ethanol (1 ml) and filtered on a Whatman GF/A filter as described before (Jiang et al., 1993).

RESULTS

Isolation of Suppressors of the bet2–1 Mutant—bet2–1 is a temperature-sensitive mutant that grows at 25 °C (permissive temperature) but dies at 37 °C. To isolate genes whose products may interact with the Bet2 protein (Bet2p), we screened a yeast genomic library that was prepared from the bet2–1 mutant for plasmids that conferred growth at 37 °C. After screening 1 × 106 transformants, 11 positive colonies were obtained and retested. The growth of mutant cells containing six of these plasmids (group A) was indistinguishable from that of wild type at 37 °C (data not shown). The other five (group B), however, did not suppress as well. Restriction analysis indicated that the plasmids in group A contained the BET2 structural gene. Since the genomic library was prepared from bet2–1
mutant cells, the restoration of growth observed at 37 °C is not true complementation. Plasmids in group B contained an overlapping 2.0-kb region of DNA. Therefore, the gene that suppresses the bet2-1 mutant is located within this 2.0-kb fragment.

The smallest group B plasmid (pS8) that we isolated contained a 2.8 kb insert (Fig. 1b). To analyze the ability of this insert to suppress bet2-1, we cloned this fragment into a high copy URA3 vector (pRS426) to generate pS8-2. When pS8-2 was transformed into bet2-1 mutant cells, suppression was significantly enhanced (Fig. 1, compare a and b). In fact, growth of the mutant was restored to that of wild type (Fig. 1, compare c and d), suggesting that suppression was gene dosage dependent.

Plasmid pS8-2 increases the membrane-bound pool of Ypt1p and Sec4p in bet2-1 mutant cells—Previous studies have shown that the membrane association of Ypt1p and Sec4p is defective in bet2-1 mutant cells (Rossi et al., 1991). This defect is a consequence of the failure to geranylgeranylate these proteins (Jiang et al., 1993; Rossi et al., 1991). Thus, the lethal phenotype of the bet2-1 mutant is likely to be a consequence of the inability of these proteins to attach to membranes. Since plasmid pS8-2 suppresses the growth defect of the bet2-1 mutant at 37 °C, it may also cure the membrane attachment defect observed in these cells. To address this possibility, we transformed pS8-2 into bet2-1. When the distribution of Ypt1p and Sec4p was examined in these transformants and compared to the mutant and wild type, pS8-2 was found to enhance the membrane association of these small GTP-binding proteins (Fig. 2, compare the amount in the lysate (T) to the supernatant (S) and pellet (P) fractions). The presence of pS8-2 did not lead to an increase in the residual GGTase-II activity that can be measured in bet2 mutant cells (Jiang et al., 1993). Thus, the restoration of the membrane association of Ypt1p and Sec4p is not a consequence of increasing GGTase-II activity.

Cloning and Sequencing the Suppressor Gene—To localize the suppressor within the 2.8-kb genomic fragment described above, subclones of pS8-2 were constructed and inserted into pRS316 (URA3, CEN). Suppression studies revealed that the Sac site contained within this fragment is critical for its activity. The smallest region of DNA capable of suppressing bet2-1 was found to be a 1.6-kb SspI-NruI fragment. This region of DNA was sequenced in both directions using the strategy shown in Fig. 3. An open reading frame of 1005 base pairs that spans the Sac site was identified. We called the gene that encodes this open reading frame Bts1p (Bet Two Suppressor). Bts1p is predicted to encode a protein of 335 amino acids with a calculated molecular mass of 38,627 daltons (Fig. 4). Overall, the amino acid composition of the Bts1p is hydrophilic, and no significant hydrophobic stretches were observed.

Bts1p Is Homologous to Known Prenyltransferases—Comparison of the predicted Bts1p amino acid sequence with the Swiss-Prot protein sequence database revealed a significant similarity between Bts1p and the N. crassa albino-3 gene product (Carattoli et al., 1991). These proteins are 40% identical at the amino acid level with the most conserved region localized to the middle of these proteins (Fig. 5). The albino-3 gene encodes a geranylgeranyl diphosphate synthase in the carotenoid biosynthetic pathway of N. crassa (Nelson et al., 1989). Bts1p also contains five conserved regions found in other FPP and GGPP synthases (Chen and Poulter, 1994). These comparisons suggest that Bts1p encodes GGPP synthase, an unidentified prenyltransferase of S. cerevisiae.

Disruption of the BTS1 Gene—To investigate if BTS1 is required for the vegetative growth of yeast cells, we disrupted one copy of this gene in diploid cells and performed tetrad analysis. This disruption was constructed by exchanging a 0.65-kb segment of the coding region with the URA3 gene. The disrupted gene was then transformed into NY648 to replace one of the chromosomal copies. The Ura+ transformants were selected, sporulated, and analyzed. In all of the 48 tetrads examined, four viable spores were obtained. However, two of the colonies in each of the tetrads displayed a growth defect at 25 °C. The small colonies were Ura+, indicating that they contained the disrupted BTS1 gene. This was confirmed by Southern blot analysis using BTS1 as a probe (see "Materials and Methods"). Thus, BTS1 is not essential for the vegetative growth of yeast cells. But in its absence, growth is impaired. The growth of the disrupted strain (SFNY368 or BTS1 disruptant) was examined further at different temperatures. As shown in Fig. 6, BTS1 cells (Fig. 6, a and d) grew as well as wild type at 30 °C (Fig. 6, b and c). However, at lower temperatures (25 and 14 °C) a growth defect emerged. Only small colonies appeared
after 3 days at 25°C (Fig. 6, a and d), while at 14°C, the cells did not survive (Fig. 6, a and d). This result clearly demonstrated that SFNY368 is cold sensitive for growth.

The BTS1 Gene Product Is Required for the Membrane Attachment of Ypt1p and Sec4p—Ypt1p and Sec4p are two small GTP-binding proteins that regulate intracellular membrane traffic (Ferro-Novick and Novick, 1993). Like many small GTP-binding proteins, they are synthesized in the cytosol but be-
The ability of Ypt1p and Sec4p to bind to membranes is conferred by the addition of the 20-carbon, geranylgeranyl moiety (Jiang et al., 1993). The geranylgeranylation of these proteins is catalyzed by a protein prenyltransferase that utilizes GGPP as a lipid donor. If \( BTS1 \) encodes GGPP synthase, disruption of this gene should result in the depletion of GGPP. Consequently, the geranylgeranylation of Ypt1p and Sec4p will be abolished. To test this hypothesis, we examined the membrane association of these proteins in the \( DBTS1 \) strain.

**Prenyltransferase Activity of Crude Extracts**

To test the hypothesis that \( BTS1 \) encodes a geranylgeranyl diphosphate synthase, we cloned the gene into a pUC118 vector to express it in *E. coli*. Crude extracts of *E. coli* containing pUC118 (control) or pUC118/\( BTS1 \) were assayed for prenyltransferase activity in the presence of [1-14C]IPP, using DMAPP or FPP as the allylic substrate, and the reaction mixture was analyzed by HPLC. The prenyltransferase activity observed was dependent...
upon the presence of FPP, since no counts were obtained when the pUC118/BTS1 extract was assayed in the absence of FPP (not shown). The radioactive product of this incubation co-eluted with unlabeled synthetic GGPP, indicating that it is GGPP (Fig. 8). No conversion of FPP to GGPP was seen with the pUC118 control. Both extracts also showed low levels of activity in the conversion of DMAPP to an acid-labile product. However, because the extent of conversion was the same for both samples, this activity could not be due to Bts1p (not shown). These findings confirm that BTS1 encodes a geranylgeranyl diphosphate synthase.

bet2-1 Mutant Extracts Have a Lower Affinity for GGPP—We next investigated the mechanism by which the overexpression of BTS1 suppresses the lethality of the bet2-1 mutant. One possibility is that BTS1 suppresses by increasing the intracellular pool of GGPP, thereby compensating for a mutant GGTase-II that has a lower affinity for GGPP. To test this hypothesis, we measured the GGTase-II activity of wild type and bet2-1 mutant extracts in the presence of varying concentrations of GGPP. As a control, we also assessed the activity of bet4-2 mutant extracts. BET4 encodes the \( \alpha \)-subunit of this enzyme complex, forms a complex with Bet4p, the \( \beta \)-subunit of this enzyme complex, and other small GTP-binding proteins. In an attempt to identify new genes whose products may interact with Bet2p, we isolated a suppressor of the bet2-1 mutant. Our data demonstrates that this suppressor gene, called BTS1, encodes a geranylgeranyl diphosphate synthase, an unidentified prenyltransferase of the yeast isoprenoid biosynthetic pathway. The BTS1 gene product functions on this pathway to convert FPP to GGPP.

DISCUSSION

Previously, we have shown that the yeast GGTase-II is composed of three subunits (BET2, BET4, and MR56). Bet2p, the \( \beta \)-subunit of this enzyme complex, forms a complex with Bet4p, the \( \alpha \)-subunit (Jiang et al., 1993). Mr56p is an escort protein that presents protein substrate to the Bet2p-Bet4p complex (Jiang et al., 1994). During geranylgeranylation, the Bet2p-Bet4p complex binds to and transfers GGPP to Ypt1p, Sec4p, and other small GTP-binding proteins. In an attempt to identify new genes whose products may interact with Bet2p, we isolated a suppressor of the bet2-1 mutant. Our data demonstrates that this suppressor gene, called BTS1, encodes a geranylgeranyl diphosphate synthase, an unidentified prenyltransferase of the yeast isoprenoid biosynthetic pathway. The BTS1 gene product functions on this pathway to convert FPP to GGPP.
in binding and catalysis (Ashby and Edwards, 1990; Joly and Edwards, 1993; Song and Poulter, 1994). This finding suggested that BTS1 encodes the yeast GGPP synthase. To confirm this hypothesis, we expressed the BTS1 gene in bacteria. Bacterial lysates that express Bts1p were found to contain an activity that synthesizes GGPP from IPP and FPP.

The suppression of the bet2-1 mutant by BTS1 could be explained in several ways. The BTS1 gene product may itself have GGTTase-II activity, or it could directly interact with GGTTase-II to stimulate its activity. In either situation, the overexpression of BTS1 would be expected to increase GGTTase-II activity. However, this was not observed. Alternatively, suppression may simply be a consequence of increasing the intracellular pool of GGPP. Since the BTS1 gene is not essential for the growth of yeast cells, the BTS1 product may itself explain why the failure to prenylate these essential proteins.

Since BTS1 is not essential for the growth of yeast cells, the synthase gene may be duplicated. Preliminary DNA hybridization experiments, however, argue against this possibility. Another explanation for the dispensability of BTS1 is that GGTTase-II might utilize FPP as an alternate substrate. However, since GGTTase-II cannot transfer FPP to Ypt1p, this possibility seems unlikely (Jiang et al., 1993). Furthermore, extracts prepared from ΔBTS1 cells do not support the transfer of [3H]FPP onto Ypt1p. Thus, it is more likely that another prenyltransferase, such as hexaprenyl diphosphate synthase, might produce small amounts of GGPP as an intermediate product during the elongation of FPP to longer polyprenoid chains. In the ΔBTS1 strain, GGPP may be formed in this way, enabling yeast cells to survive at certain temperatures in the absence of the geranylgeranyl synthase.

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