Protein Farnesyltransferase in Plants: Molecular Characterization and Involvement in Cell Cycle Control

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Farnesylation is required for membrane targeting, protein–protein interactions, and the biological activity of key regulatory proteins, such as Ras small GTPases and protein kinases in a wide range of eukaryotes. In this report, we describe the molecular identification of a plant protein farnesyltransferase (FTase) and evidence for its role in the control of the cell cycle in plants. A pea gene encoding a homolog of the FTase subunit was previously cloned using a polymerase chain reaction-based strategy. A similar approach was used to clone a pea gene encoding a homolog of the FTase subunit. The biochemical function of the pea FTase homologs was demonstrated by the reconstitution of FTase enzyme activity using FTase fusion proteins coexpressed in Escherichia coli. RNA gel blot analyses showed that levels of FTase mRNAs are generally higher in tissues, such as those of nodules, that are active in cell division. The relationship of FTase to cell division was further analyzed during the growth of suspension-cultured tobacco BY-2 cells. A biphasic fluctuation of FTase enzyme activity preceded corresponding changes in mitotic activity at the early log phase of cell growth. Moreover, manumycin, a specific inhibitor of FTase, was effective in inhibiting mitosis and growth in these cells. Using synchronized BY-2 cells, manumycin completely blocked mitosis when added at the early S phase but not when added at the G2 phase. These data suggest that FTase is required for the plant cell cycle, perhaps by modulating the progression through the S phase and the transition from G1 to the S phase.

INTRODUCTION

Isoprenylation is required for membrane association and cellular activity of many eukaryotic regulatory proteins. This post-translational modification involves covalent attachment of an isoprenyl moiety (either 15-carbon farnesyl or 20-carbon geranylgeranyl) to the cysteine residue of a short motif (CXXX) at the C terminus of proteins (Farnsworth et al., 1990; Glomset et al., 1990; Maltese, 1990; Rilling et al., 1990; Hancock and Marshall, 1993; Casey, 1994). The isoprenyl moiety also plays a critical role in the specific interaction of isoprenylated proteins with other signaling proteins (Fukada et al., 1990; Marshall, 1993; Kisselev et al., 1994; Piorfiri et al., 1994). Three distinct enzymes catalyzing isoprenylation reactions have been identified in yeast and mammals, each modifying proteins with unique C-terminal motifs (Kohl et al., 1991; Maltese et al., 1991; Khosravi-Far et al., 1991; Moores et al., 1991; Kinsella and Maltese, 1992). Among the three enzymes, the structure and function of FTase is most extensively studied in yeast and mammalian systems. FTases are composed of two nonidentical subunits, α and β (Chen et al., 1991a, 1991b; He et al., 1991; Reiss et al., 1991a), and genes encoding both subunits have been cloned from yeast and mammals (Chen et al., 1991a, 1991b; He et al., 1991). The two subunits form a stable complex, and both are required for catalytic activity (Chen et al., 1991a, 1991b; He et al., 1991; Reiss et al., 1991a). Each subunit contains five conserved internal sequence repeats that are thought to be critical for enzymatic activity (Boguski, 1992; Andres et al., 1993). The function of the α subunit is not clear, although it has been suggested that it binds isoprenyl pyrophosphate (Reiss et al., 1991a, 1992). The β subunit binds the substrate protein in a Zn2+-dependent manner (Reiss et al., 1991a, 1992). Nonetheless, the mechanisms underlying subunit interaction and regulation of enzyme activity remain essentially unknown.

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Proteins known to be farnesylated include the Ras small GTPases, fungal mating factors, the γ subunit of heterotrimeric G proteins, prelamins A and B, protein kinases, and molecular chaperones (Hancock et al., 1989; Inglese et al., 1992b; Zhu et al., 1993; Sinensky et al., 1994; Ong et al., 1995). Thus, protein farnesylation is involved in the modulation of many important processes, including signal transduction, cell cycle control, protein export, and development (Sepp-Lorenzino et al., 1991; Cox et al., 1992; Inglese et al., 1992a, 1992b; Kato et al., 1992; Hancock and Marshall, 1993; Marshall, 1993; Kisselev et al., 1994; Hara and Han, 1995; Kauffmann et al., 1995; Scheer and Gierschik, 1995). One of the most important functions of FTase appears to be its involvement in the control of cell cycle and mitogenic signaling. Differential farnesylation of Ras at the G1 phase and of nuclear lamins at the early S phase was shown in synchronized human cells (Sepp-Lorenzino et al., 1991). FTase mRNA and enzyme activity levels are higher in Ras-induced human skin carcinomas than in normal skin cells (Khan et al., 1996). Moreover, farnesylation is required for the function of Ras in the mitogenic signaling that controls the G1/S to S transition in animal systems (Cox et al., 1992; Kato et al., 1992; Bokoch and Der, 1993; Itha et al., 1993; Kauffmann et al., 1995; McCormick, 1995).

Recently, an oncogenic human protein tyrosine phosphatase lacking the pea gene encoding the FTase subunit homolog (PsFTa) and demonstrated functional identity of FTase by reconstituting FTase activity from the pea α and β (PsFTb) subunits coexpressed in Escherichia coli. Further analyses of FTase enzyme activity and FTase inhibitor-induced inhibition of cell division strongly support a role for FTase in plant cell cycle control.

RESULTS

Cloning and Characterization of a Pea Gene Encoding a Homolog of the FTase α Subunit

Two degenerate oligonucleotides (FTa3 and FTa4) corresponding to repeats 2 and 3 of the rat FTase α subunit (Chen et al., 1991a; Andres et al., 1993) were used to amplify a product of ~130 bp from a pea root tip cDNA library. Sequence analysis revealed that this fragment encodes a deduced polypeptide of 44 amino acids with strong sequence similarity to the FTase α subunits from rat and yeast (61% identical to rat and 39% identical to yeast) (Chen et al., 1991a; He et al., 1991). DNA gel blot hybridization analysis showed that this fragment hybridized with a single HindIII fragment in pea genomic DNA (data not shown).

The 132-bp polymerase chain reaction (PCR) fragment was used to screen the pea root tip cDNA library, and three clones (designated PsFTa1, PsFTa2, and PsFTa3) containing the longest inserts were analyzed further. As shown in Figure 1, PsFTa1 and PsFTa2 have identical nucleotide sequences, except that PsFTa1 (138 bp) is shorter at both ends than is PsFTa2 (1285 bp). PsFTa1 starts with nucleotide +14 and ends with the polyadenylated tail at nucleotide 1151, whereas PsFTa2 starts with nucleotide +6 and ends with the polyadenylated tail at nucleotide 1291. The difference in length at the 3' untranslated region is most likely due to use of alternative polyadenylation sites. The third clone, PsFTa3, is a chimera between a 354-bp sequence overlapping 5' regions of PsFTa1 and PsFTa2 and a promiscuous sequence. This chimeric sequence is probably the result of recombination during in vivo excision of plasmid cDNA from phage. Nonetheless, PsFTa3 is 5 bp longer at the 5' end than is PsFTa2 and includes a possible ATG initiation codon that establishes an open reading frame encoding a predicted polypeptide of 333 amino acids.

To determine whether this ATG is indeed a translation initiation codon, we used an EcoRI fragment at the 5' end of the clone PsFTa1 to isolate four additional clones from a pea genomic library. All four clones are identical in sequence and contain a stop codon at nucleotides −18 to −16 upstream of the first ATG codon in PsFTa3 (Figure 1). Furthermore, there is no obvious consensus splicing acceptor sequence ([T/C]GNC/TAG) between this stop codon and the first ATG (Mount, 1982), and there are several pairs of AG dinucleotides ((GATA) between this stop codon and the first ATG (Mount, 1982), and there are several pairs of AG dinucleotides between the stop and ATG codons. These AG pairs are rarely seen in the −15 through −5 region of an acceptor (Seif et al., 1979). Sequences surrounding ATG (GAAATGGGC) conform to the plant consensus translation initiation sequence AAC-
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AATGGCC (Lutcke et al., 1987). In addition, the longest cDNA clone is close to the length of the PsFTa transcript (~1.4 kb) as revealed by RNA gel blot hybridization (see below). It is therefore very likely that the 999 nucleotides represent the entire coding region of the pea FTase α subunit.

The PsFTa polypeptide predicted from the open reading frame has an estimated molecular mass of 38.5 kD. As shown in Figure 2, PsFTa exhibits strong sequence similarity to the FTase α subunits from rat and yeast (Chen et al., 1991a; He et al., 1991). PsFTa contains the five tandem sequence repeats present in all FTase α subunits from other organisms (Boguski, 1992; Andres et al., 1993; Feng and Kung, 1993). The positions of four conserved residues, glutamate, arginine, tryptophane, and arginine, are invariant in all five repeats, except for the glutamate residue in the first repeat. Like the β subunit homolog (Yang et al., 1993), PsFTa exhibits greater overall sequence similarity to the mammalian α subunit (37% identity to rat FTase α subunit) than to the yeast α subunit (25% identity). Both N- and C-terminal regions are less conserved. Like the yeast FTase α subunit, PsFTa is ~50 amino acids shorter at the N terminus than is the mammalian FTase α subunit. Only 12 of 99 residues at the C terminus of PsFTa are identical to the yeast or mammalian α subunit.

To assess PsFTa gene copy number, we performed genomic DNA gel blot hybridization analysis. As shown in Figure 3, a single EcoRI or HindIII fragment was detected under both low- and high-stringency conditions. The probe cross-hybridized with two EcoRI fragments because the PsFTa cDNA contains an EcoRI site. These results suggest that PsFTa is encoded by a single gene in pea.

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Figure 1. Nucleotide and Predicted Amino Acid Sequences of the cDNA Encoding the Pea FTase α Subunit.

shown is a DNA sequence composite of three cDNA clones (PsFTa1, PsFTa2, and PsFTa3) and a genomic clone. The starting position of each cDNA clone is indicated. Nucleotide position +1 indicates the putative translation initiation codon. The nucleotide sequence is numbered at left; the amino acid sequence is numbered at right. The first in-frame stop codon upstream of the putative initiation codon and the stop codon at the end of the open reading frame are marked with asterisks. The nucleotide sequence has been submitted to GenBank with accession number U63258.

To demonstrate that the pea homologs of FTase subunits (PsFTa and PsFTb) form a functional FTase enzyme, we attempted to reconstitute FTase activity by using PsFTa and PsFTb fusion proteins expressed in E. coli. PsFTa and PsFTb coding sequences were each fused to a glutathione S-transferase (GST) gene in a pGEX-based vector, and novel polypeptides of expected molecular sizes were detected (Figure 4A). Each GST fusion protein was affinity-purified using glutathione-conjugated Sepharose, and a mixture of an equal amount of each protein was used for FTase activity assays. The fusion proteins resulted in no significant FTase activity (data not shown). Similar phenomena were observed with independently derived subunits of fungal and mammalian FTase (He et al., 1991). This was presumably the result of incorrect folding or assembly of independently expressed fusion proteins.

To circumvent this problem, we constructed a bicistronic plasmid (pGEX-FTa/b shown in Figure 5) to direct coexpression of the two polypeptides in the same E. coli cell. The expression of the two fusion proteins was confirmed by protein gel analysis, as shown in Figure 4B. Extracts from this E. coli strain were used for the FTase activity assay, using tritiated farnesyl pyrophosphate (3H-FPP) and a yeast recombinant Ras protein (Ras–CAIM) as substrates (Randall et al., 1993). As shown in Figure 4C, 3H-FPP was incorporated into Ras–CAIM in the reaction containing protein extracts from cells coexpressing PsFTa and PsFTb fusion proteins or the yeast FTase subunits (He et al., 1991). The labeled Ras proteins appeared as double bands, probably as a result of protein degradation or different protein conformations. No 3H-FPP was incorporated into Ras–CAIM in the reactions containing PsFTa or PsFTb fusion protein alone. The recombinant protein Ras–SVLS, which cannot be farnesylated by the fungal or mammalian FTase, was not labeled (Figure 4C). These results demonstrate that PsFTa and PsFTb are functional pea FTases.
The deduced amino acid sequences of the FTase α subunit from rat (Chen et al., 1991a) and yeast (He et al., 1991) were aligned to PsFTa with Megalign (DNASTAR, Inc., Madison, WI). Numbers at right indicate residue positions of the predicted amino acid sequence. Consensus residues are indicated by black rectangles. Gaps introduced into the alignments are indicated by dashes. Five internal amino acid sequence repeats are underlined. Four invariable residues (E, N, R, and W) within the repeats are marked with asterisks.

Under our assay conditions (2 hr at 30°C), $^3$H-FPP showed weak binding to a 15-kD E. coli protein, regardless of which fusion proteins were expressed in E. coli, indicating that the labeling of this E. coli protein was not the result of FTase activity (see Figure 4C). In addition, $^3$H-FPP appeared to bind to proteins corresponding to the size of GST-PsFTa (62 kD) or the yeast α subunit (38 kD) whenever these proteins were present. This result supports the hypothesis that the FTase α subunit plays a role in binding isoprenyl substrates (Reiss et al., 1991a, 1992).

Coordinate and Differential Regulation of Gene Expression for FTase Subunits during Plant Development

To gain clues to the physiological function of FTase in plants, we studied expression patterns for genes encoding the pea FTase α and β subunits by using RNA gel blot hybridization analyses. As shown in Figure 6A, a PsFTa transcript of ~1400 nucleotides was detected in most parts of pea plants. Transcript levels were highest in nodules in which plant cells presumably divide actively. Moderate levels of PsFTa transcripts were found in roots and floral buds, whereas transcript levels were very low or barely detectable in other parts, such as open flowers, leaves, and stems. Because the roots used for RNA isolation were infected with Rhizobium spp and may have contained microscopic nodules, these tissues might have contributed significantly to the transcript levels detected in roots. Rehybridization of the RNA blot with a probe for the pea 23S RNA gene (Yang and Watson, 1993) showed that most samples contained equivalent amounts of RNA; slightly lower amounts of RNA were loaded for young leaves, large nodules, and stems.

Figure 3. Genomic DNA Gel Blot Analysis of PsFTa.

Five micrograms of pea genomic DNA was digested with the indicated restriction enzymes and hybridized with the PsFTa cDNA, as described in the text. Molecular length markers are indicated at left in kilobases. Numbers at right indicate lengths in kilobases of genomic DNA fragments that hybridized with the probe.
The expression pattern of PsFTb was similar to that of PsFTa in all parts of pea plants, except for floral buds (Figure 6B). In floral buds, PsFTa transcript levels were relatively high, whereas the PsFTb transcript was barely detectable. The PsFTb transcript detected in roots consistently showed somewhat reduced mobility; the molecular basis of this observation remains to be determined.

These results indicate that although the expression of PsFTa and PsFTb genes is coordinately regulated in most tissues, there is differential regulation for these two genes in certain parts of pea plants. Furthermore, these results show that FTase gene expression is highly regulated during the plant developmental processes. In general, greater amounts of FTase mRNA are found in tissues of nodules and floral buds undergoing active cell division, suggesting a role for plant FTase in cell cycle regulation analogous to that found for the mammalian FTase.

Changes in FTase Activity Are Correlated with Cell Division in Suspension-Cultured BY-2 Cells

The relationship of FTase activity to cell division was directly assessed in the well-characterized tobacco BY-2 suspension-cultured cell system (Nagata et al., 1992). BY-2 cells were harvested at different times after the transfer of stationary cells to fresh medium, and cell fresh weight, mitotic index, and FTase enzyme activity were determined.

No significant increase in cell fresh weight was observed until 3 days after subculture, and growth continued through day 7 (Figure 7A). The mitotic activity (assessed as a percentage of cells with condensed chromatin) followed an apparent biphasic change (Figure 7B). A rapid increase in the mitotic index occurred 24 hr after subculture. After reaching the first peak within 36 hr, the mitotic index leveled off by 48 hr. This was followed by a second major peak between days 3 and 4.

(B) Coexpression of pea FTase subunit proteins in E. coli by using the bicistronic plasmid shown in Figure 5. Protein extracts from E. coli cells expressing GST (pGEX) and GST-PsFTa fusion (pGEX-FTa) or coexpressing GST-PsFTa fusion and PsFTb (pGEX-FTa/b) were separated on a 10% SDS–polyacrylamide gel and stained with Coomassie blue.

(C) FTase enzyme assays for subunit proteins expressed in E. coli. Enzyme assays were conducted using [3H-FPP and recombinant Ras proteins (Ras-CAIM and Ras-SVLS), as described in text. Designations are as given in (A) and (B). pBC16-113, the plasmid expressing the yeast FTase α and β subunits (Caplin and Marshall, 1995). The 62-kD band is the GST-PsFTa fusion, the 38-kD band is the yeast α subunit, the 26-kD band is the yeast Ras recombinant protein, and the 15-kD band is a nonspecific FPP binding protein from E. coli.

In (A) and (B), numbers at left indicate protein molecular mass markers in kilodaltons.
and a steady decline to the low activity of stationary cells by day 7.

The kinetics of FTase enzyme activity also followed a biphasic fluctuation that precedes the fluctuation of mitotic activity (Figure 7C). Enzyme activity increased within 6 hr after subculture and reached the first peak by 12 hr, before any observed increase in mitotic activity. Enzyme activity declined between 24 and 36 hr, which is coincident with the first rapid increase in mitotic index. FTase activity reached a maximum by day 3 and decreased by day 4, again preceding changes in mitotic activity. These results provide further evidence for the potential involvement of FTase in regulation of the plant cell cycle.

A Competitive Inhibitor of FTase Blocks Cell Cycle Progression in BY-2 Cells

The role of farnesylation in cell cycle control was directly tested using manumycin, a specific inhibitor of protein FTase (Hara et al., 1993; Tamanoi, 1993; Gibbs et al., 1994; Hara and Han, 1995; Nagase et al., 1996). This cell-permeable inhibitor was shown to be selective for FTase, but not for a related enzyme GGTase I or enzymes involved in the biosynthesis of isoprenoid intermediates, and thus has been widely used to study the function of FTase in yeast and animals (Hara et al., 1993; Tamanoi, 1993; Gibbs et al., 1994; Hara and Han, 1995; Nagase et al., 1996).

To test whether manumycin also functions as a selective FTase inhibitor in plant cells, we investigated the effect of manumycin on in vivo protein isoprenylation in BY-2 cells. Cells were initially treated with mevinolin (a hydroxymethylglutaryl CoA reductase inhibitor) to block the endogenous production of isoprenoids (e.g., FPP, geranylgeranyl pyrophosphate [GGPP]); these cells were then incubated with H-FPP or H-GGPP in the presence of manumycin. As shown in Figure 8, manumycin at a concentration as low as 2.5 μM completely blocked in vivo protein farnesylation but not geranylgeranylation. These results are consistent with the effect of manumycin in animal and yeast systems, in which it was shown that IC_{50} (concentration that results in 50% inhibition) for the inhibition of FTase by manumycin is ~2.5 μM (Hara et al., 1993; Tamanoi, 1993; Gibbs et al., 1994; Nagase et al., 1996).

To study its effect on cell division, we added manumycin to suspension-cultured BY-2 cells at various times after subculture in fresh medium, and mitotic indices were determined every 24 hr. As shown in Figure 9A, manumycin strongly inhibited cell division when added 1 or 2 days after subculture. Manumycin treatment on day 4, by which time FTase activity had dropped and mitotic activity had leveled off in untreated cultures (see Figure 7), had no significant effect on cell division. Because of the time intervals (36-hr time point was not taken), the biphasic mitotic activity was not observed in these experiments.
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Days after subculture

Figure 7. Changes in FTase Enzyme Activity and Mitotic Indices during Tobacco BY-2 Cell Suspension Culture.

Seven-day-old cells were transferred to fresh Murashige and Skoog medium. At various times after transfer, cells were harvested, weighed, and aliquoted for FTase assays, and the mitotic index was determined as described in the text. The data shown are the mean of two replicates. The standard error is indicated where the value exceeded the size of the symbols used.

(A) Fresh weights of BY-2 cells from 50 mL of suspension-cultured cells collected on filter paper.
(B) Mitotic index determined from scoring 1000 cells stained with 1% orcein and calculated as the percentage of cells with condensed chromatin.
(C) FTase enzyme activity presented as total amount of \(^{3}H\)-FPP incorporation into Ras proteins in one standard reaction, as described in the text.

To assess whether the effect of manumycin on cell division simply resulted from cell growth inhibition, we determined fresh weights at the end of the culture cycle (day 7). As shown in Figure 9B, cell fresh weights were significantly lower in cultures treated with manumycin on day 1 and day 2, and thus were correlated with the inhibition of cell division. However, cell fresh weights for cultures treated with manumycin on day 4 were comparable to untreated cells. Because logarithmic cell growth continues throughout the culture cycle (see Figure 7A), these results indicate that manumycin does not have a direct effect on cell growth. The addition of farnesyl pyrophosphate to manumycin-treated cells rescued cell division to a large extent, providing further evidence for the specific effect of manumycin on tobacco FTase (data not shown).

To analyze further the effect of manumycin on cell cycle progression, we used BY-2 cells that were synchronized by the addition of aphidicolin, a specific inhibitor of DNA polymerase. Stationary phase BY-2 cells were transferred to fresh medium and arrested in the early S phase with aphidicolin (Nagata et al., 1992). Aphidicolin inhibition was released by washing the cells, and manumycin was added at the time of aphidicolin release (0 hr) or at the G\(_2\) phase (6 hr after aphidicolin release; Nagata et al., 1992). As shown in Figure 10, manumycin treatment at the early S phase (0 hr) completely blocked mitotic activity. In contrast, treatment at the G\(_2\) phase (6 hr) did not inhibit mitosis. These results suggest that manumycin blocks cell cycle progression through the S phase but has no effect on the G\(_2\)-to-M transition.

DISCUSSION

In a previous study, we cloned a gene encoding a pea homolog of the FTase \(\beta\) subunit (PsFT\(\beta\)) (Yang et al., 1993). In this

Figure 8. Inhibition of in Vivo Protein Farnesylation by Manumycin.

Mevinolin-treated BY-2 cells were incubated with \(^{3}H\)-FPP or \(^{3}H\)-GGPP in the absence (-) or presence (+) of 2.5 \(\mu\)M manumycin (Man.). Cell-free protein extracts were separated by SDS-PAGE, and farnesylated proteins were visualized by fluorography, as described in the text. Numbers at left indicate protein molecular mass markers in kilodaltons.
Two hundred microliters of 7-day-old cells was transferred to 20 mL of fresh medium. Manumycin was added to cultures at the time indicated by arrowheads, and the mitotic index was determined every 24 hr, as described in Figure 7. Control indicates no addition of manumycin. Data are the mean from two independent experiments. For mitotic indices, standard errors were <10% in all samples; for fresh weights, standard errors are indicated. (A) Mitotic indices. (B) Fresh weights.

**Figure 9.** Inhibition of Cell Division by Manumycin in BY-2 Cell Suspension Cultures.

Two hundred microliters of 7-day-old cells was transferred to 20 mL of fresh medium. Manumycin was added to cultures at the time indicated by arrowheads, and the mitotic index was determined every 24 hr, as described in Figure 7. Fresh weights were measured on day 7. Control indicates no addition of manumycin. Data are the mean from two independent experiments. For mitotic indices, standard errors were <10% in all samples; for fresh weights, standard errors are indicated. (A) Mitotic indices. (B) Fresh weights.

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In our studies, both the enzyme activity and mitotic index followed an apparent biphasic pattern of fluctuation during the growth of suspension-cultured BY-2 cells (Figure 7). The first peak may reflect the synchronization of a small population of BY-2 cells after the transfer of stationary cells to fresh medium, whereas the second major peak is likely to represent nonsynchronous division for the majority of cells. Because the degree of BY-2 cell synchronization is highly dependent upon growth conditions,

were shown to be essential for enzyme catalytic activity of the mammalian FTase (Andres et al., 1993). Although the pea FTase subunits show regions that are distinct from previously characterized FTases, our data definitively demonstrate their molecular identities based on reconstitution of FTase activity in E. coli coexpressing the PsFTa and PsFTb gene products.

Our data on the expression of PsFTa and PsFTb genes and the regulation of FTase activity support the hypothesis that FTase is involved in the control of cell cycle progression in plants. First, RNA gel blot hybridization analyses indicated that the expression of FTase genes is associated with tissues undergoing rapid cell division. In addition, there is a close correlation between changes in FTase activity and mitotic activity in suspension-cultured tobacco cells. In our studies, both the enzyme activity and mitotic index followed an apparent biphasic pattern of fluctuation during the growth of suspension-cultured BY-2 cells (Figure 7). The first peak may reflect the synchronization of a small population of BY-2 cells after the transfer of stationary cells to fresh medium, whereas the second major peak is likely to represent nonsynchronous division for the majority of cells. Because the degree of BY-2 cell synchronization is highly dependent upon growth conditions,
subtle differences in our conditions could explain why the biphasic fluctuation of the mitotic index was not observed in previous investigations (Nagata et al., 1992). Interestingly, our data demonstrate that the changes in FTase activity precede the changes in mitotic activity in both phases by at least 6 hr. Because there is a lag period of ~6 hr between the S and M phases of the cell cycle in synchronized BY-2 cells (Nagata et al., 1992), these results are consistent with farnesylation being involved in the early events of the cell cycle such as the G0/G1-to-S transition.

The role for FTase in cell cycle control in plants is directly supported by experiments involving the FTase competitive inhibitor manumycin. Manumycin strongly inhibited cell division only when added 1 or 2 days after transfer to fresh medium (Figure 9A). Manumycin had no effect on cell growth or division when added on day 4, by which time FTase activity dropped and mitotic activity started to decline while logarithmic cell growth continued (see Figure 7). This indicates that the inhibition of cell division by manumycin is not related to cell growth per se. Moreover, manumycin blocks cell cycle progression in synchronized BY-2 cells when added at early S phase but not at G2. These results agree with those of Morehead et al. (1995), who used general isoprenylation inhibitors. The treatment of suspension-cultured tobacco BY-2 cells with the hydroxymethylglutaryl coA reductase inhibitor mevinolin or the protein isoprenylation inhibitor perillyl alcohol inhibited growth of suspension-cultured BY-2 cells, but only when it was added within the first 2 days after transfer to fresh medium (Morehead et al., 1995).

Several lines of evidence strongly suggest that manumycin blocks cell cycle progression in BY-2 cells by specifically inhibiting FTase activity. First, we showed that manumycin at a concentration as low as 2.5 μM (IC50 value for fungal and mammalian FTase) effectively inhibited protein farnesylation but not geranylgeranylation in BY-2 cells. Second, the inhibition of cell division by manumycin can be rescued by exogenously supplied FPP. Third, the timing of sensitivity to manumycin treatments in suspension-cultured BY-2 cells is similar to that for mevinolin and perillyl alcohol (Morehead et al., 1995), suggesting that manumycin is most likely to affect only the processes that involve protein isoprenylation. Moreover, manumycin was shown to have no effects on the biosynthesis of isoprenoid products in mammalian cells (Nagase et al., 1996). Finally, the timing of effective inhibition of cell division by manumycin is in accord with the change in FTase activity and mitotic activity in suspension-cultured cells, further supporting the selectivity of this inhibitor for FTase.

Our data imply a role for FTase in the regulation of S phase progression and the G1/G0-to-S transition in plants. In mammalian cells, specific farnesylation of several proteins has been linked to cell cycle progression: laminas are farnesylated between the G1 and S phases and Ras is farnesylated in the G1 phase (Sepp-Lorenzino et al., 1991). Nuclear laminas have been implicated in DNA replication in Xenopus oocytes (Moir et al., 1995). Plant proteins related to mammalian nuclear laminas have been described previously (Frederick et al., 1992; McNulty and Saunders, 1992; Tong et al., 1993); however, their farnesylation and role in DNA replication remain to be determined. In mammalian cells, farnesylation is required for the activation of Ras GTPases involved in the mitogenic signaling that controls the G0/G1-to-S transition (Cox et al., 1992; McCormick, 1995). A Ras homolog has not been identified in plants to date. Nonetheless, incorporation of [3H-FPP into tobacco proteins of similar molecular sizes as mammalian lamins (55 to 60 kD) and Ras proteins (20 to 26 kD) supports the notion that farnesylated lamin and Ras homologs are present in plants (see Figure 8). Identification of proteins that are differentially farnesylated at the G1 and S phases will further define the role of protein farnesylation in the control of the cell cycle in plants.

Apart from cell cycle control, plant FTase may be involved in other aspects of plant growth and development. Homologs of the chaperone DnaJ and heavy metal binding proteins were found to be farnesylated in plants, suggesting a potential role for FTase in stress responses (Zhu et al., 1993; Randall et al., 1996). Recently, several Arabidopsis mutants that exhibit hypersensitivity to abscisic acid (ABA) were identified (Cutler et al., 1996). Surprisingly, one of these mutants was characterized as a T-DNA insertion mutation in the gene encoding an FTase β subunit homolog. This finding suggests a critical role for FTase in ABA signaling and ABA-mediated growth regulation.

PsFTa mRNA but not PsFTb mRNA accumulates to significant levels in floral buds, although the accumulation of these two transcripts is coordinately regulated in most pea organs or tissues (see Figure 6). There is evidence that both FTase and GGTase I share the same α subunit in yeast and mammalian cells (Seabra et al., 1991). If this also holds true for plant isoprenyltransferases, the more abundant floral PsFTa transcript may be required for GGTase I activity. It is also possible that there are multiple isoforms of the FTase β subunit that share the same PsFTα. This notion is supported by our DNA gel blot hybridization analysis indicating that the pea genome contains a small gene family encoding the β subunit (D. Qian and Z. Yang, unpublished results), whereas PsFTα is encoded by a single gene (Figure 3). Additional research will address whether plants indeed have multiple FTase isoforms and whether each plays a distinct role in modulating one of the potential FTase-dependent processes described above.

The mechanism for the regulation of FTase activity is likely to involve divergent regions of the subunit proteins. The pea FTase subunits have distinct structural features. PsFTb contains a region of ~50 amino acids near the C terminus, which is absent in the yeast and rat counterparts (Yang et al., 1993). Both the pea and yeast FTase α subunits (He et al., 1991; see Figure 2) lack an N-terminal proline-rich domain of ~50 amino acids in length that is present in the rat FTase α subunit (Chen et al., 1991a). This domain is not essential for enzyme catalytic activity (Andres et al., 1993). The C-terminal regions among the plant, yeast, and mammalian α subunits are also quite variable (see Figure 2). Recent studies show that the mammalian FTase α subunit binds to and is phosphorylated by a TGF-β receptor, suggesting that FTase may be directly regulated by other signaling proteins (Kawabata et al., 1995; Wang et al.,
METHODS

Plant Materials

Pea (Pisum sativum cv Alaska) seedlings were germinated and grown on moist paper towels in darkness for 4 days before being transferred to a growth chamber. After 1 day in the growth chamber under constant light at 22°C, leaves were harvested for DNA isolation. For RNA extraction, different parts, including small nodules (<2 mm), large nodules (>2 mm), roots without visible nodules, fully expanded mature leaves, rapidly expanding young leaves, tendrils, stems, floral buds, and mature open flowers, were harvested from mature plants grown in a growth room at 22°C under constant white light.

Polymerase Chain Reaction Amplification and cDNA Cloning and Sequencing

Two degenerate oligonucleotides, FTa3 (5'-CAAGGCCTAAAGAATTCCTAGCTCAGTTGA-3') and F Tb4 (5'-CCAAGCGATGGATAGATTGATCT/ TTGCAAGTCGTC-3'), corresponding to amino acid residues 162 to 169 (OPKMYQW) and 196 to 203 (DAKNYHAW), respectively, of the rat farnesyltransferase (FTase) a subunit (Chen et al., 1991a), were used as primers for polymerase chain reactions (PCR). Phage DNA from a pea root tip cDNA library constructed in λZAP (T. Jacob, University of Illinois, Urbana-Champaign) was isolated by using the method of Sambrook et al. (1989). PCR amplifications were performed in a 100-µl reaction mixture containing 100 ng of phage DNA, 400 pmol of each primer, and 5 units of Taq DNA polymerase (Perkin-Elmer Cetus). Thirty-five amplification cycles were conducted at 94°C for 1 min (denaturation), 45°C for 30 sec (annealing), and 72°C for 30 sec (synthesis). Ends of amplified PCR products were filled in using Escherichia coli DNA polymerase I and phosphorylated using T4 polynucleotide kinase and ATP. Blunt-ended fragments were cloned into the SmaI site of pBluescript SK- (Stratagene) and sequenced using Sequenase Version 2.0 (United States Biochemical). Three clones were sequenced and found to contain identical 132-bp fragments, which encode a 44-amino acid polypeptide exhibiting strong sequence similarity to both mammalian and yeast a subunits.

The 132-bp fragment, labeled with 32P-dCTP by using a random primer labeling system (Gibco BRL), was used to screen the pea root tip cDNA library. Duplicate filters were hybridized in a solution containing 6 x SSPE (1 x SSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, 10 mM EDTA), 5 x Denhardt's solution (5 x Denhardt's solution is 0.4% [w/v] Ficoll, 0.4% [w/v] polyvinylpyrrolidone 0.4% [w/v] BSA), 40% formamide, and 100 µg/ml sonicated single-stranded salmon sperm DNA at 42°C. Of 2 x 10⁶ plaques screened, >30 positive clones were identified. Selected clones were excised in vivo, according to the instructions of the manufacturer (Stratagene). Three clones containing inserts of ~1.3 kb were sequenced on both strands, as described above. Because all of these cDNA clones appear to lack 5' ends, genomic clones were isolated by screening ~2 x 10⁶ plaques of a pea genomic library from S. Gaunt (University of Minnesota, St. Paul) by using an EcoRI fragment from the 5' end of the cDNA clone, PsFTa1 (see Figure 1). Five positive clones were identified, subcloned into pBluescript SK-; and sequenced using a primer complementary to the 5' end of the PsFTa1 cDNA sequence (Figure 1, nucleotides +55 to 75).
and subcloned into SalI and SstI sites within the oligonucleotide downstream of the translation start codon. The resulting bicistronic plasmid, pGEX-FTalb, was transformed into E. coli DH5α. A single colony was inoculated into 5 mL of Luria-Bertani liquid medium. Bacterial cells were cultured at 37°C to an OD600 of 1.0. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.33 mM, and cultures were incubated for an additional 4 hr at room temperature.

The cells were harvested by centrifugation and resuspended in 250 μL of lysis buffer (50 mM Hepes, pH 7.5, 5 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin, 0.1 mM leupeptin, and 1 mg/50 mL lysozyme). The lysates were stored at -70°C. Immediately before the assay, frozen cell lysates were thawed on ice, 10 μg/mL DNase I was added, and lysates were centrifuged at 50,000g for 15 min using a TL100 ultracentrifuge (Beckman, Fullerton, CA). Protein extracts were used for FTase assays as described below.

Determination of Growth and Mitotic Index of Tobacco BY-2 Cells

Suspension-cultured tobacco BY-2 cells were incubated by shaking at room temperature in Murashige and Skoog liquid medium containing 4.3 g/L Murashige and Skoog salts (Sigma), 100 mg/L isonitol, 1 mg/L thiamine, 0.2 mg/L 2,4-D, 255 mg/L KH2PO4, pH 5.0, and 30 g/L sucrose. Routine cultures were maintained by transferring 1 mL of 7-day-old cells to 50 mL of fresh medium in a 250-mL flask every 7 days. For the kinetic study of FTase activity and mitotic activity, cells were harvested at different time points after the transfer to fresh medium. Cells were collected by filtering through P8 filter paper (Fisher Scientific, Pittsburgh, PA), and fresh weights were measured. Cells from each time point were aliquoted for FTase assay and mitotic index score. Cells for FTase assays were frozen in liquid nitrogen and kept at -70°C until use.

The mitotic index was scored at the time of harvest. To determine the mitotic index, we stained cells with a 1% orcein solution in 45% acetic acid. One thousand stained cells were examined for each sample, and the examination was repeated at least twice. The mitotic index is calculated as the percentage of cells with condensed chromatin (Katsuta et al., 1990).

In Vitro FTase Enzyme Assay

To determine FTase enzyme activity in suspension-cultured BY-2 cells, we prepared cell-free extracts by grinding frozen BY-2 cells in a precooled mortar with ice-cold extraction buffer (250 mM mannitol, 50 mM Hepes, 3 mM EGTA, 1 mM EDTA, pH 7.5, 10 μg/mL leupeptin, 5 μg/mL aprotinin, 5 μg/mL pepstatin, 5 μg/mL chymostatin). Homogenates were centrifuged at 50,000g for 15 min using a TL100 ultracentrifuge to remove cell debris. The protein concentration was determined by the Bradford method (Bio-Rad).

In vitro FTase activity was assayed as described previously (Randall et al., 1993). Briefly, a typical reaction (126 μL) contained 50 mM MgCl2, 50 μM ZnCl2, 5 mM EDTA, 50 mM Hepes, pH 7.5, 450 μg of cell-free protein extract, and 10 μg of Ras–CAIM (where A is alanine, I is isoleucine, and M is methionine) or Ras–SVLS (where S is serine, V is valine, and L is leucine) recombinant proteins. Recombinant Ras–CAIM and Ras–SVLS were kindly provided by D.N. Crowell (Indiana University–Purdue University at Indianapolis; Randall et al., 1993). This mixture was preequilibrated at 30°C for 2 min before the addition of 2.5 μCi of tritiated farnesyl pyrophosphate (3H-FPP) (60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO; or Du Pont–New England Nuclear, Boston, MA). The reaction mixture was incubated at 30°C for 40 min and terminated with 1 M HCl in 100% ethanol. Precipitated proteins were collected and washed with 15 mL of 100% ethanol on G6 glass fiber filters (Fisher Scientific). Air-dried filters were counted by a liquid scintillation counter. To confirm the incorporation of 3H-FPP into Ras substrates, we separated a 10-μL aliquot of reaction mixtures on a 14% SDS–polyacrylamide gel, and labeled proteins were detected by fluorography. Gels were fixed in isopropanol–water–acetic acid (25:65:10 [v/v/v]) for 30 min, incubated in Amplify fluorographic reagent (Amersham, Arlington Heights, IL) for 40 min, and dried onto filter paper before being exposed to x-ray film for 1 week at -70°C.

To determine FTase activity of E. coli–expressed FTase fusion proteins, we added either 18 μg of purified fusion proteins or 490 μg of extracts of E. coli cells expressing different fusion proteins as described above to reaction mixtures. As negative controls, extracts from E. coli cells containing pGEX, pGEX-FTh, or pGEX-FTb were used. For positive controls, an E. coli strain expressing the yeast FTase α and β subunits was used (He et al., 1991). This strain was provided by M. Marshall (Indiana University Medical Center, Indianapolis; Caplin and Marshall, 1995) with the permission of S. Powers (University of Dentistry and Medicine of New Jersey, Piscataway). The incorporation of 3H-FPP into the recombinant Ras protein was determined by the SDS-PAGE analysis and fluorography as described above.

Synchronization of BY-2 Cells

Cells were synchronized essentially as described by Nagata et al. (1992). Seven-day-old cells were diluted 50-fold in fresh Murashige and Skoog media containing aphidicolin (5 mg/L) and cultured with shaking for 24 hr. Aphidicolin inhibition was released by washing cells in fresh media at least three times. Washed cells were incubated in fresh medium with shaking, and an aliquot of cells was taken every 30 min or 1 hr for the determination of mitotic indices, as described above.

Manumycin Treatments

Manumycin was obtained from M. Hara (Kyowa Hakko Kogyo Co., Tokyo, Japan). A stock solution of 10 mM in DMSO was stored in darkness. At various times after subculture or synchronization, manumycin was added to BY-2 suspension cultures to a final concentration of 2.5 μM.

In Vivo Protein Farnesylation Assay

A stock solution (10 mM) of mevinolin (a gift to C.L. Cramer from Merck Sharp & Dohme Research Laboratories, Rahway, NJ) was prepared as described by Bach and Lichtenthaler (1982). Mevinolin was added to 2-day-old BY-2 cells to a final concentration of 10 μM. After 16 hr, treated cells were allowed to settle. An aliquot of 35 μL of settled cells was resuspended in an equal volume of fresh medium containing 5.0 μM manumycin and 0.5 μCi radioactive isoprenyl substrates (3H-FPP, 60 Ci/mmol or 3H geranylgeranyl pyrophosphate [3H-GGPP], 193 Ci/mmol). Cells were incubated for an additional 4 hr before being washed with 5 mL of Murashige and Skoog medium. Washed cells were homogenized in 30 μL of 1× protein loading buffer (50 mM Tris-
Cl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Cell lysates were centrifuged in a microcentrifuge, and 20 µL of the supernatants was subjected to electrophoresis and fluorography, as described above.

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