Plant Physiol. (1996) 110: 1349–1359

Specific Prenylation of Tomato Rab Proteins by Geranylgeranyl Type-II Transferase Requires a Conserved Cysteine-Cysteine Motif[1]

Shaul Yalovsky, Ann Elizabeth Loraine, and Wilhelm Gruissem*

Department of Plant Biology, 111 Koshland Hall, University of California, Berkeley, California 94720–3102

Posttranslational isoprenylation of some small GTP-binding proteins is required for their biological activity. Rab geranylgeranyl transferase (Rab GGTase) uses geranylgeranyl pyrophosphate to modify Rab proteins, its only known substrates. Geranylgeranylation of Rabs is believed to promote their association with target membranes and interaction with other proteins. Plants, like other eukaryotes, contain Rab-like proteins that are associated with intracellular membranes. However, to our knowledge, the geranylgeranylation of Rab proteins has not yet been characterized from any plant source. This report presents an activity assay that allows the characterization of prenylation of Rab-like proteins in vitro, by protein extracts prepared from plants. Tomato Rab1 proteins and mammalian Rab1a were modified by geranylgeranyl pyrophosphate but not by farnesyl pyrophosphate. This modification required a conserved cysteine-cysteine motif. A mutant form lacking the cysteine-cysteine motif could not be modified, but inhibited the geranylgeranylation of its wild-type homolog. The tomato Rab proteins were modified in vitro by protein extract prepared from yeast, but failed to become modified when the protein extract was prepared from a yeast strain containing a mutant allele for the α subunit of yeast Rab GGTase (*bet4 ts*). These results demonstrate that plant cells, like other eukaryotes, contain Rab GGTase-like activity.

Isoprenoid metabolism in plants produces a variety of compounds important for photosynthesis, cell growth, and disease resistance (for review, see Bach, 1995; Chappell, 1995). Some isoprenoid end products unique to plant cells include membrane phytosterols, photoprotective carotenoid pigments, the phytol moiety of Chl, pathogen-induced phytoalexins, and the three plant hormones GA_3, ABA, and cytokinin. This is in contrast to animals, in which the major isoprenoid end product is cholesterol, which is routinely imported by cells via a highly regulated pathway from distinct sites of synthesis (Goldstein and Brown, 1990). Except for the plant hormones, there is no evidence for transport and import of isoprenoids by plant cells. Therefore it is of central importance to understand how plant cells achieve a tight control between isoprenoid biosynthesis and cell growth and differentiation.

The discovery during the last few years of a small group of proteins in mammalian cells and yeast that are prenylated by C_{15}-farnesyl or C_{20}-geranylgeranyl intermediates of the sterol biosynthesis pathway have been discovered, and such prenylation reactions are now recognized as important posttranslational modifications that are critical for the biological function of these proteins (Glomset et al., 1990; Clarke, 1992; Schafer and Rine, 1992; Omer and Gibbs, 1994). It is interesting that most of the prenylated proteins have regulatory or structural functions in pathways that control different aspects of cell reproduction such as growth, differentiation, membrane movement and proliferation, and membrane vesicle transport. These proteins include all known members of the Ras superfamily of small GTP-binding proteins such as Ras, Rac, Rho, and Rab, as well as nuclear lamins (Schafer and Rine, 1992; Boguski and McCormick, 1993; Ferro-Novick and Novick, 1993). The requirement of prenylation for the biological function of these proteins in the above cellular processes has been demonstrated directly for Ras and a small group of other prenylated proteins (Hancock et al., 1989; Rossi et al., 1991; Boguski and McCormick, 1993; Li et al., 1993; Alexandrov et al., 1994; Cook and McCormick, 1994; Gibbs et al., 1994; Leevers et al., 1994; Pfeffer, 1994; Seabra et al., 1994; Stokoe et al., 1994; Seabra, 1995). Based on the hydrophobic nature of the C_{15} and C_{20} isoprenoids, it is now generally accepted that the attachment of prenyl groups increases the affinity of the proteins to membranes, where they interact with other proteins (Schafer and Rine, 1992; Brown and Goldstein, 1993). It is tempting when considering the central role of sterol biosynthesis for membrane function and cell growth to speculate that protein prenylation has been recruited as a mechanism by which the cell can adjust its growth activities as a function of FPP and GGPP fluxes through the isoprenoid pathway.

Despite the central and significantly more complex role of the sterol biosynthesis pathway for plant cells, very little

[1] The work reported in this paper was supported by a grant from the U.S. Department of Energy (DOE 85ER13375) to W.G. S.Y. was supported by a postdoctoral fellowship from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD FI-0159–93). A.E.L. was supported by a predoctoral National Institutes of Health training grant (NIH 5-T32–6M07127).

* Corresponding author; e-mail gruissem@nature.berkeley.edu; fax 1–510–642–4995.

Abbreviations: Ab, antibody(ies); CV, column volume(s); FPP, all-*trans*-farnesylpyrophosphate; FTase, farnesyl protein transferase; GG, all-*trans*-geranylgeraniol; GGPP, all-*trans*-geranylgeranylpyrophosphate; GGTase-I, geranylgeranyl protein transferase-I; GST, glutathione *S*-transferase; MAb, monoclonal antibody(ies); Rab GGTase (GGTase-II), Rab geranylgeranyl transferase; REP, Rab escort protein.

is known about protein prenylation or the types of prenylated proteins in plants. Based on studies of yeast and mammalian prenyltransferases, we have attempted to establish the presence of these enzymes in plants. This was done as a first step toward a long-term goal of investigating the possible role of prenyl protein transferases in linking the sterol biosynthesis pathway to cell growth regulation.

Three different protein prenyltransferases have been identified in mammalian cells and yeast that catalyze the prenylation of conserved Cys's in the C terminus of proteins using either C_{15}-FPP or C_{20}-GGPP. FTase is a conserved heterodimeric enzyme in mammalian cells, yeast, and plants (Reiss et al., 1991a, 1991b; Crowell et al., 1993; Gomez et al., 1993; Trueblood et al., 1993; Yang et al., 1993; D. Schmitt, K.L. Callan, S.-H. Kim, W. Gruissem, unpublished results; S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, W. Gruissem, unpublished results) that recognizes a conserved C-terminal *Caa*X motif (C = Cys, *a* = aliphatic amino acid, X = variable) (Reiss et al., 1991, 1991b; Schafer and Rine, 1992). The heterodimeric GGTase type I, or GGTase I, shares the α subunit with FTase but has a distinct β subunit (Seabra et al., 1991; Mayer et al., 1992; Trueblood et al., 1993). The enzyme recognizes the specific C-terminal *Caa*L motif (Finegold et al., 1991; Hancock et al., 1991; Yokoyama et al., 1991) but is promiscuous in yeast mutants in which FTase activity is absent (Trueblood et al., 1993). GGTase I has been identified in mammalian cells, yeast, and plants (Finegold et al., 1991; Yokoyama et al., 1991; Mayer et al., 1992; Morehead et al., 1995; D. Schmitt, K.L. Callan, S.-H. Kim, W. Gruissem, unpublished results). A distinct GGTase-II (Rab GGTase) that modifies the Rab subclass of the superfamily of Ras-like proteins has been characterized in mammalian cells and yeast (Khosravi-Far et al., 1991; Rossi et al., 1991; Seabra et al., 1992a, 1992b; Jiang et al., 1993; Li et al., 1993) but has not yet been identified in plants. This enzyme is a heterotrimeric protein composed of a heterodimeric catalytic component, homologous to FTase and GGTase-I, known as Rab GGTase, and a third protein known as REP, which is believed to present the Rab protein substrate to the catalytic component. The geranylgeranylation of Rab proteins requires a conserved CCXX, XXCC, or XCXC motif, in addition to other less well-defined domains in the protein (Seabra et al., 1992a, 1992b; Beranger et al., 1994a, 1994b; Cremers et al., 1994; Sanford et al., 1995) (Fig. 1).

Although the specificity and biochemical action of all three prenyl transferases has been established in animals and yeast (Glomset et al., 1990; Clarke, 1992; Schafer and Rine, 1992; Omer and Gibbs, 1994), considerably less is known about their regulation during development and relative to the activity of the isoprenoid biosynthesis pathway or about their precise role in the control of cell division and growth. In particular, it is currently not understood if and how Rab GGTase controls the function of Rab proteins in membrane-vesicle transport in response to the regulation of the isoprenoid pathway and the availability of the intermediate GGPP. This problem is further compounded in plants, in which GGPP is a last common intermediate for several isoprenoid end products, including GAs, carotenoids, and Chls. It is therefore important to identify whether Rab GGTase exists in plants. Its identification allow future studies of whether Rab GGTase controls access to the GGPP branchpoint to maintain efficient prenylation of Rab proteins relative to other enzymes that utilize the GGPP intermediate.

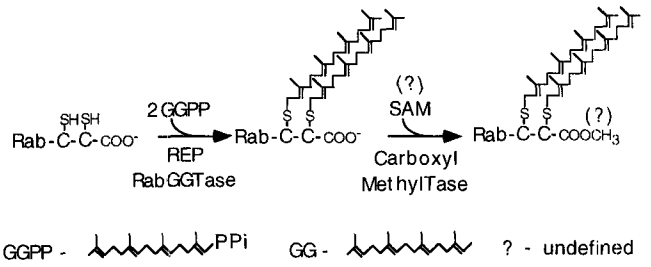

Figure 1. Schematic presentation of the chemical modification occurring on the C terminus of Rab proteins as identified in mammalian and yeast systems. The two Cys residues that are located in either XXCC or XCXC and CCXX (which are not represented for the sake of simplicity) sequence motifs (C, Cys; X, variable) are geranylgeranylated in a reaction that uses GGPP as substrate. The geranylgeranylation requires two protein components: REP, which interacts with the Rab protein and presents it to the catalytic component, and Rab GGTase. In plants there is no direct evidence for the existence of the REP/Rab GGTase enzyme complex, nor is it known whether both Cys residues are modified by GG. Following the geranylgeranylation, carboxyl methylation of the C-terminal carboxyl group occurs in some members of the Rab protein family such as Rab3A. Whether this modification occurs in the other members of the Rab protein family including the plant homologs is still unknown. SAM, *S*-Adenosyl-Met; Carboxyl MethylTase, carboxy methyltransferase.

In this report we establish the presence in plants of Rab GGTase activity that is specific for prenylation of Rab proteins we have isolated from tomato (Loraine et al., 1996). The plant enzyme attaches C_{20}-geranylgeranyl to conserved Cys residues in the C termini of the Rab proteins, which are the only known substrates of the mammalian and yeast enzymes (Khosravi-Far et al., 1991; Kinsella and Maltese, 1991, 1992). Rab proteins are associated with membrane-bound compartments involved in vesicle-mediated transport (reviewed by Ferro-Novick and Novick, 1993; Novick and Brennwald, 1993; Zerial and Stenmark, 1993). All known Rab proteins have C-terminal Cys prenylation motifs (CC, CCXX, and CXC) and are thought to be geranylgeranylated on each Cys residue (Farnsworth et al., 1994). Cys prenylation is required for Rab association with the different intracellular membranes and secretory vesicles. Several Rab proteins have now been cloned from plants (Drew et al., 1993; Nagano et al., 1993; Youssefian et al., 1993; Bednarek et al., 1994; Borg and Poulsen, 1994), but little is known about their subcellular localization and function (Bednarek et al., 1994; Borg and Poulsen, 1994; Park et al., 1994), and their prenylation status has not been addressed. We show that the tomato (*Lycopersicon esculentum*), YPT1 homologs, Rab proteins (LeRab1B and LeRab1C) (Loraine et al., 1996), and the mammalian Rab1A serve as substrates for a Rab GGTase in protein extracts made from various plant tissues. Plant Rab GGTase requires GGPP, the wild-type Rab C-terminal prenylation

motif, and additional upstream sequences for efficient prenylation of the Rab substrates. Together, our results demonstrate that plants, like mammalian cells and yeast, most likely utilize the isoprenoid GGPP for the modification of Rab proteins via a biochemically conserved Rab GGTase. They establish the basis for further molecular and genetic dissection of this enzyme that couples isoprenoid biosynthesis with membrane trafficking critical for cell growth.

MATERIALS AND METHODS

Plant Material

Roots and stems were from hydroponically grown tomato (*Lycopersicon esculentum* cv VFNT Cherry LA1221) plants. Tobacco (*Nicotiana tabacum*) BY-2 suspension cells (Katsuta and Shibaoka, 1992; Nagata et al., 1992) were subcultured every 7 d into fresh medium containing 4.3 g L^{-1} Murashige-Skoog salts, 100 mg L^{-1} inositol, 1 mg L^{-1} thiamine, 0.2 mg L^{-1} 2,4-D, 255 mg L^{-1} $KHPO_4$, and 30 g L^{-1} Suc. Tomato VFNT Cherry suspension cells were subcultured every 7 d and were grown as described by Loraine et al. (1996).

Plant and Yeast Extracts

Stems from 4-week-old tomato plants were cut into 2- to 4-cm sections and immediately frozen in liquid nitrogen. Roots were harvested from hydroponically grown tomato plants and stored frozen until use. Frozen material was ground to a powder with dry ice in a coffee grinder or Waring blender. Ten to 20 g of ground, frozen stem material were allowed to thaw in 10 mL of ice-cold buffer A (50 mM Hepes-KOH, pH 7.9, 250 mM mannitol, 5 mM EDTA, 0.1% [w/v] BSA, 5 mM DTT) containing 0.3 mM diethyldithio-carbamic acid, 0.5 g of PVP, and protease inhibitors (0.5 mg mL^{-1} each antipain, leupeptin, and aprotinin; 1.6 mg mL^{-1} benzamidine; 1 mg mL^{-1} PMSF [Sigma] or 1 mM Pefablock [Boehringer Mannheim]; and 1,10-phenantroline [Sigma]). The mixture was homogenized using a mortar and pestle or a Polytron set at 75% maximum speed in five short bursts lasting 5 to 10 s each. The mixture was allowed to cool on ice for 15 s between bursts. All subsequent steps were performed at 4°C. The homogenate was filtered through six layers of cheesecloth and centrifuged for 10 min at 10,000*g*. The supernatant was collected and recentrifuged for 1 h at 100,000*g*. The 100,000*g* supernatant was either used directly in prenylation reactions or fractionated by an initial 0 to 30% NH_4SO_4 cut, followed by a 30 to 50% NH_4SO_4 cut. Proteins precipitated in the 50% NH_4SO_4 cut were resuspended in buffer B (50 mM Hepes-KOH, pH 7.9, 1 mM EDTA, 2 mM DTT), including the protease inhibitor cocktail. The resuspended protein extract was dialyzed overnight against buffer B, aliquotted, and stored frozen at −80°C until use.

Tobacco BY-2 suspension cells were collected from 3-d-old cultures by filtration. All subsequent steps were done at 4°C. Cells were washed with buffer C (100 mM Tris, pH 7.8, 5 mM EGTA, 5 mM EDTA, 10 mM β-mercaptoethanol, 10% [w/v] glycerol) and then ground in a mortar with acid-washed, silanized sand in buffer C containing 2 mg mL^{-1} leupeptin, antipain, and aprotinin. Lysates were centrifuged for 10 min at 8,000*g*, recentrifuged for 30 min at 30,000*g*, and recentrifuged again for 2 h at 100,000*g*. The 100,000*g* supernatant was collected and concentrated in a Centriprep-10 filtration unit (Amicon, Beverly, MA), dialyzed against buffer D (50 mM Tris, pH 7.8, 10% glycerol, 1 mM DTT), aliquotted, and stored frozen at −80°C until use. Protein extract from tomato VFNT Cherry suspension-cell culture was prepared in the same way except that 0.3 mM diethyldithio-carbamic acid and 0.5 g of PVP were added to the grinding buffer.

Crude extracts from *Saccharomyces cerevisiae* strain *mad2–2* (*bet4 ts*) (mutated in the α-subunit homolog of the yeast Rab GGTase) and its corresponding parent strain were made as described previously (Moores et al., 1991) except that extracts were subjected to an additional centrifugation step at 100,000*g* for 2 h. The supernatant from this step was dialyzed against buffer B, aliquotted, and stored at −80°C until use. Protein concentrations were determined using Protein Assay Reagent (Bio-Rad) and BSA (Sigma) as the standard.

Production of Recombinant Rab Proteins

A DNA sequence for the Glu-Glu epitope sequence MEYMPME (Schaffhausen and Benjamin, 1982; Grussenmeyer et al., 1985; Rubinfeld et al., 1991) was introduced into the cDNAs immediately upstream of the N terminus of the tomato Rab1 protein homologs (LeRab1B, and LeRab1C) using PCR to create epitope-tagged GST::LeRab1B and GST::LeRab1C fusion proteins. GST::LeRab1B and GST::LeRab1C fusion proteins were purified from bacterial protein extracts using glutathione-Sepharose (Pharmacia) according to the manufacturer's instructions. Briefly, 1 L of *Escherichia coli* PR475 cells (lon^-, $ompT^-$) carrying the pGEX2T-GST::LeRab expression vector were grown at 37°C to mid-logarithmic phase. Expression of the GST::LeRab fusion protein was induced by addition of isopropylthio-β-D-galactoside to a final concentration of 0.1 mM for 2 to 4 h at 28°C. Cells were harvested by centrifugation and lysed in a French press at 500 p.s.i. The bacterial lysate was applied to 1.2 mL of self-packed glutathione-Sepharose columns. After the columns were washed extensively with PBS, the fusion protein was eluted with 10 mM GSH in 50 mM Tris-HCl, pH 8.0. Fractions containing the fusion protein were concentrated and dialyzed on ice against buffer B (without protease inhibitors) using a membrane with an M_r cutoff of 10,000 on a collodion dialysis apparatus (Schleicher & Schuell). The same procedure was used to produce GST in *E. coli* cells containing the pGEX2T-GST expression vector without insert.

To produce the recombinant mammalian poly(His)-tagged Rab1A, the plasmid pAR-Rab1A was digested with *Nde*I and *Bam*HI. The DNA fragment containing the Rab1A-coding region was gel purified and subcloned into pET14-b (Novagen, Madison, WI). Protein production was induced in *E. coli* BL 21 DE3 cells carrying the pET14-b-Rab1A DNA construct described above. Following purification and concentration of the Rab1A protein as described

above, an additional purification step was included using 0.8 mL of Ni-nitrilo-tri-acetic acid resin (Quiagen, Chatsworth, CA) (packed at 1.5 mL min^{-1} into an HPLC porous PEEK self-pack column supplied by Perceptive Biosystems, League City, TX) and Biocad Sprint protein liquid chromatography (Perceptive Biosystems). The column was prewashed with 10 CV of lysis buffer (50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 0.5% [v/v] Triton X-100). The protein sample was injected (100 μL) and the column was washed with 10 CV of lysis buffer and then with 50 CV of wash buffer (50 mM phosphate buffer, pH 6.0, 300 mM NaCl, 10% [v/v] glycerol, 25 mM imidazole). Elution was with a 20-CV gradient of 25 mM to 0.5 M imidazole in wash buffer at 1 mL min^{-1}, and 1-mL fractions were collected. The major peak containing the purified Rab1A eluted at 0.25 M imidazole. The samples containing purified Rab1A were concentrated and dialyzed against buffer B using the collodion apparatus as described above.

Prenylation Reactions

Reactions were carried out in 50 μL of 50 mM Hepes-KOH, pH 7.9, 5 mM $MgCl_2$, 0.3 mM Nonidet P-40, 25 mM EDTA, 0.4 nmol of recombinant Rab protein, protease inhibitor cocktail (as described above), 0.5 μM [^{3}H]GGPP or 0.5 μM [^{3}H]FPP (30 Ci $mmol^{-1}$, American Radiolabeled, St. Louis, MO), and 75 μg of plant (or yeast) protein extract at 28°C for 30 min. To avoid potential degradation of the recombinant Rab proteins by proteases present in the plant or yeast protein extracts, the reactions were kept on ice prior to addition of GGPP and incubation at 28°C. Reactions were terminated by addition of SDS-PAGE sample buffer. Equal aliquots from each reaction were subjected to SDS-PAGE, and the resulting gels were fixed for 30 min in 10% (v/v) acetic acid and 50% (v/v) methanol, soaked in Amplify (Amersham), dried onto filter paper, and fluorographed at −80°C for several days. Reactions were quantified by densitometric scanning of bands corresponding to the radioactively labeled recombinant protein substrates (Molecular Dynamics, Sunnyvale, CA).

Competition Reactions with Nonlabeled FPP or GGPP

Dilution series of either FPP or GGPP were prepared in 0.25 M NH_4HCO_3 and 70% (v/v) ethanol. Stock concentrations were adjusted such that addition of 1 μL of nonlabeled FPP or GGPP stock solution to the reaction resulted in final concentrations of 0.25, 0.5, 1, 2, 4, or 20 μM unlabeled competitor. The unlabeled competitor was added to each reaction prior to the radioactively labeled substrate.

Immunoprecipitations

Following the geranylgeranylation reaction, 30-μL reaction aliquots were mixed with 20 μL of anti-Glu-Glu MAb coupled to protein-G-Sepharose, 50 μL of H_2O, and 100 μL of 2× IP buffer (1× IP buffer: 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% [v/v] Triton X-100) and incubated overnight at 4°C with mild agitation. The Glu-Glu protein-G-Sepharose beads were then washed with 1× IP buffer as follows: 3 times for 30 s, 3 times for 5 min, once for 1 h, and once for 30 s. All wash steps were done by delicately shaking the beads. Following each wash step, the beads were collected by centrifugation at 8000*g* for 1 min in a microfuge centrifuge (Eppendorf). After the final wash, the beads were resuspended in 40 μL of H_2O and 10 μL of 5× denaturation buffer (Laemmli, 1970) without β-mercaptoethanol to minimize denaturation of the IgG molecules.

Protein Blots

Polyclonal Ab to GST::LeRab1B were raised in rabbits. Gel electrophoresis and protein blot procedures were carried out according to standard protocols (Ausubel et al., 1995). Following electrotransfer of the proteins to nitrocellulose membranes (Schleicher & Schuell), membranes were decorated with anti-Rab polyclonal Ab (purified on a GST column) by incubation at room temperature for 2 h. Membranes were then washed and incubated with blotting grade goat anti-rabbit horseradish peroxidase-conjugated Ab (Bio-Rad) and developed with ECL (Amersham).

TLC Separation of GGPP, FPP, and Their Derivatives

[^{3}H]GGPP and [^{3}H]FPP and their derivatives were separated on 0.2-mm silica-gel plates (Merck, Darmstadt, Germany) in isopropanol:NH_4SO_4:H_2O solvent (6:3:1 [v/v]). Following chromatography, TLC plates were sprayed with EN^{3}HANCE (DuPont, NEN) and exposed to x-ray film for detection of labeled compounds.

Miscellaneous Techniques

Proteins were separated by electrophoresis on SDS-polyacrylamide gels following established procedures (Laemmli, 1970) and stained with Colloidal Brilliant Blue G (Sigma).

The protein concentration in extracts was determined with the Bio-Rad Protein Assay Kit. The concentration of purified Rab proteins was determined either by using the same kit or by estimating the density of the purified protein band on the stained gel relative to standards.

RESULTS

Purification of Recombinant Rab Protein Substrates and Stability of GGPP

Mutational analysis of mammalian Rab proteins has established that Rab GGTase requires upstream amino acid sequence determinants in addition to the C-terminal CC motifs for efficient prenylation of the protein (Beranger et al., 1994, 1994b; Cremers et al., 1994; Sanford et al., 1995). To establish biochemical evidence for Rab GGTase in plants, it was therefore critical to synthesize a recombinant unmodified Rab substrate that could be used in prenylation assays with [^{3}H]GGPP and plant-protein extract. To accomplish this, we expressed proteins in *E. coli* from tomato cDNAs encoding homologs of the yeast YPT1 and mammalian Rab1 proteins as GST-Rab fusion proteins with a Glu-Glu MAb epitope tag at the N termini of the full-length Rab proteins (GST::LeRab1B and GST::LeRab1C).

The Glu-Glu-tagged proteins can complement a *ypt1* deletion mutant when expressed in yeast, confirming that they are functional YPT1 homologs (Loraine et al., 1996). The canine Rab1A protein was used as a positive control in the prenylation reactions. Figure 2 shows that the purified proteins were approximately 90% pure as judged by the protein profile in stained SDS gels.

GGPP is the last common intermediate in the isoprenoid biosynthetic pathway that is utilized by several other enzymes in the plant cell as a substrate for abundant end products such as Chl and carotenoids, as well as for GA synthesis. Although several of the GGPP-utilizing enzymes are localized to the plastid, they would likely contaminate a protein extract. We therefore determined the stability of GGPP after incubation in different protein extracts by assaying GGPP and derivatives by TLC to control for enzymatic activities that utilize GGPP as a substrate in the absence of the purified Rab proteins. Figure 3 shows, as an example, that GGPP was stable in a fractionated tomato stem protein extract that we used for most of the experiments described in this paper. In contrast, GGPP was very unstable in root protein extracts and was most likely converted into GG by nonspecific phosphatases. Addition of α,β-methylene-ATP prevented the conversion of GGPP to GG in the tomato root protein extract at a concentration of 10 mM but had no effect on the stability of GGPP in the stem extract. Even under these conditions we were not able to detect Rab GGTase in root protein extracts (data not shown), whereas the enzyme activity was readily detectable in the stem extract (see below).

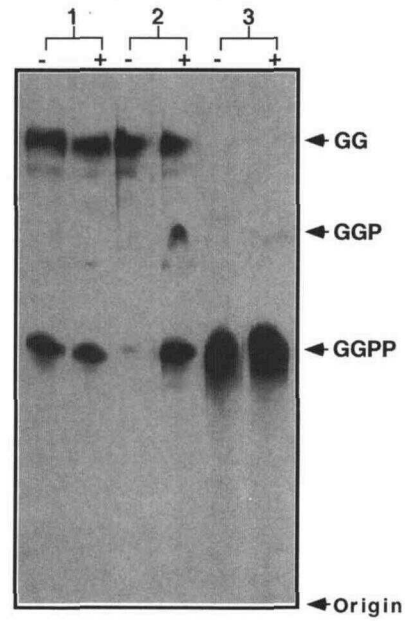

Figure 3. Fluorogram of GGPP and its derivatives GGP and GG as separated by silica-gel TLC. [^{3}H]GGPP was incubated for 30 min at 28°C with geranylgeranylation reaction buffer in the presence (+) or absence (−) of α,β-methylene-ATP (a phosphatase inhibitor). GGPP was incubated either alone (3) or in the presence of 70 μg of protein extract from tomato stem proteins precipitated at the 30 to 50% NH_4SO_4 cut (1) or tomato root proteins precipitated at 80% NH_4SO_4 (2). The locations of GGPP, GGP (all-*trans*-geranylgeranylmonophosphate), GG, and the origin of the separation run are marked with arrows.

Tomato Rab Proteins Are Geranylgeranylated by Yeast and Plant Protein Extracts

Based on the ability of LeRab1B and LeRab1C to complement a *ypt1* mutant (Loraine et al., 1996), we predicted that the recombinant plant proteins could serve as substrates for the yeast Rab GGTase. Figure 4 shows that both GST::LeRab1B and GST::LeRab1C were geranylgeranylated when incubated with [^{3}H]GGPP and a protein extract prepared from a wild-type yeast strain. In contrast, no prenylated protein was detected when LeRab1B or LeRab1C was incubated with a protein extract prepared from the *mad2–2* (*bet4 ts*) yeast strain that has a temperature-sensitive mutation in the BET4 gene, which encodes the homolog of the mammalian Rab GGTase α subunit (Li et al., 1993). Together, these results establish that the recombinant LeRab1B and LeRab1C can serve as protein substrates for yeast Rab GGTase and that the prenylation of the proteins is specific for the Rab GGTase enzyme.

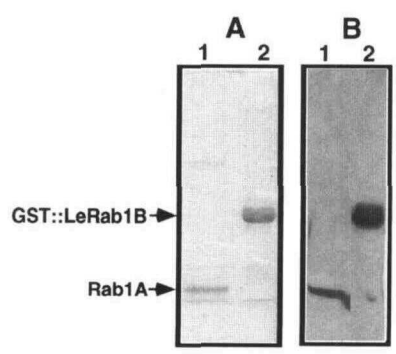

Figure 2. SDS-PAGE of purified Rab proteins used in the prenylation experiments as substrates for geranylgeranylation. Lane 1, Poly(His)-tagged canine Rab1A that was purified over an Ni-nitrilo-tri-acetic acid column. Lane 2, GST::LeRab1B fusion protein purified over a GST-Sepharose column. A, Colloidal Brilliant Blue G-stained gel. B, Western blot of an identical gel decorated with polyclonal Ab raised against GST::LeRab1B. Arrows mark the location of GST::LeRab1B and canine Rab1A.

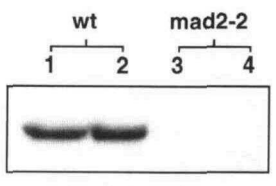

Figure 4. Fluorogram of [^{3}H]GG-labeled GST::LeRab1B and GST::LeRab1C following incubation with yeast protein extract. GST::LeRab1B and LeRab1C were incubated with protein extract prepared from either wild-type (wt) or the mutant *mad2–2* (*bet4 ts*) yeast strains and [^{3}H]GGPP. Following incubation at 28°C for 30 min, the reactions were stopped by addition of SDS-PAGE denaturation buffer and the proteins were fractionated on SDS-PAGE. The gel was then fixed and fluorographed to detect labeled proteins. Lanes 1 and 3, GST::LeRab1B; lanes 2 and 4, GST::LeRab1C.

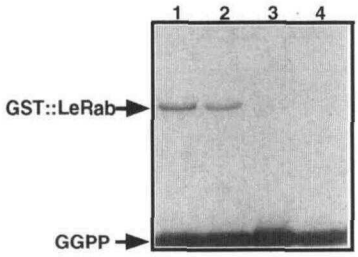

Figure 5. Fluorogram of a gel run following the geranylgeranylation reaction shows that GST and GST::LeRab1CmCC were not labeled following incubation with [^{3}H]GGPP and tomato-stem protein extract. Different proteins used as substrates in the geranylgeranylation reaction were: GST::LeRab1B (lane 1), GST::LeRab1C (lane 2), GST (lane 3), and GST::LeRab1CmCC (lane 4). Arrows mark the locations of the [^{3}H]GG-labeled GST::LeRab proteins and the free, non-protein-bound [^{3}H]GGPP.

To determine whether plant protein extracts contain Rab GGTase activity that can prenylate LeRab, GST::LeRab1B and GST::LeRab1C fusion proteins were incubated with [^{3}H]GGPP and tomato-stem protein extract. Figure 5 shows that this incubation resulted in labeling of GST::LeRab1B and GST::LeRab1C fusion proteins and that the labeled products were resistant to denaturation conditions of SDS-PAGE and could be separated from the free [^{3}H]GGPP. Incubation of purified GST alone did not produce a detectable labeled product, confirming that the LeRab protein was the substrate for geranylgeranylation rather than the GST portion of the fusion protein. Mammalian and yeast Rab GGTase attaches the GG moiety via a thioether linkage to Cys's in CC, CXC, or CCXX (X = variable amino acid) sequence motifs located on the C terminus of the Rab protein. The tomato Rab homologs used in this study contain CCXX-type (LeRab1C) and XCCX-type (LeRab1B) sequence motifs at their C termini. To investigate whether the two Cys residues in these motifs are critical for geranylgeranylation, a mutant form of GST::LeRab1C was used in the prenylation reaction in which the two Cys residues in the C terminus of the protein were changed to Ser and Gly (designated GST::LeRab1CmCC). Figure 5 shows that no labeled protein product was detected following incubation with the plant extract and separation of the protein substrate by SDS-PAGE, suggesting that LeRab1C is likely prenylated at the C-terminal Cys residues.

Although the results shown in Figure 5 strongly suggest that the exogenous LeRab1B and LeRab1C were used as a substrate by a Rab GGTase activity present in the protein extract, they do not exclude the possibility that the observed product could result from labeling of an endogenous substrate that was subject to competition by GST or LeRab1CmCC. Therefore, to provide direct evidence for the labeling of the LeRab proteins, we used an anti-Glu-Glu MAb coupled to protein-G-Sepharose to immunoprecipitate the epitope-tagged GST::LeRab1B and GST::LeRab1C fusion proteins following their incubation with [^{3}H]GGPP in the presence or absence of tomato-stem protein extract. The results in Figure 6 show that the anti-Rab Ab detects both immunoprecipitated GST::LeRab1B and GST::LeRab1C after incubation with tomato protein extract

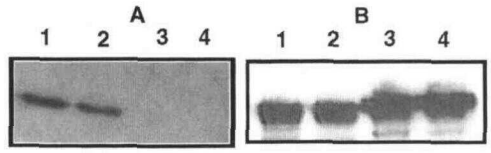

Figure 6. Immunoprecipitated and [^{3}H]GG-labeled GST::LeRab1B and GST::LeRab1C. Glu-Glu peptide-tagged GST::LeRab1B and GST::LeRab1C were incubated at 28°C with [^{3}H]GGPP in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of tomato-stem protein extract. After 30 min the GST::LeRab1 fusion proteins were immunoprecipitated with protein-G-Sepharose-coupled anti-Glu-Glu Mab. Immunoprecipitated proteins were released from the Sepharose beads by denaturation in SDS-PAGE denaturation buffer that lacked β-mercaptoethanol and fractionated on an SDS-PAGE. The gel was either fixed and fluorographed (A) or electrotransferred to a nitrocellulose membrane that was decorated with anti-LeRab1B polyclonal Ab (B). Lanes 1 and 3, GST::LeRab1B; lanes 2 and 4, GST::LeRab1C.

or a control reaction with buffer only, confirming that the anti-Glu-Glu MAb efficiently precipitates the proteins. Most importantly, prenylation of both LeRab1B and LeRab1C was detected only when tomato protein extract was present during the incubation of the GST-fusion proteins with [^{3}H]GGPP. We conclude that plants contain a Rab GGTase-like activity that can correctly prenylate the tomato Rab proteins at conserved C-terminal Cys residues.

The similarity in the detected prenylation level between GST::LeRab1B and GST::LeRab1C, as demonstrated in Figures 4 to 6, allowed us to use either one of the fusion proteins in later experiments.

The Mammalian Rab1A Protein Is Geranylgeranylated by Protein Extracts from Plants

Since both LeRab1B and LeRab1C can be classified as Rab1-like proteins (Loraine et al., 1996), we were interested

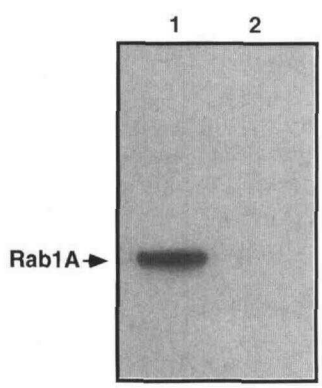

Figure 7. Labeling of canine Rab1A by plant-protein extract. Recombinant canine Rab1A was produced as poly(His)-tagged protein and purified by metal chelate chromatography (see "Materials and Methods"). The purified protein was incubated with [^{3}H]GGPP and tobacco BY-2-cell line protein extract. The reaction was stopped by addition of SDS-PAGE denaturation buffer and proteins were then fractionated on a gel, which was fixed and fluorographed. The location of the [^{3}H]GG-labeled canine Rab1A is marked by an arrow (lane 1). No labeled protein was detected in a control reaction to which no Rab protein was added (lane 2).

to learn whether the Rab GGTase-like activity in the plant protein extract was sufficiently conserved to recognize the mammalian Rab1 protein as a substrate in the geranylgeranylation. The results in Figure 7 show that the canine Rab1A produced in *E. coli* was labeled after incubation with [^{3}H]GGPP and a tobacco BY-2 cell extract. No labeled protein product was detected when the BY-2 cell protein extract was incubated with [^{3}H]GGPP alone. Similar results were obtained with the tomato stem-protein extract (data not shown). The prenylation of the mammalian Rab1A protein in the tobacco and tomato protein extracts, together with the prenylation of LeRab1C in yeast protein extracts, provides compelling evidence for the conservation of the biochemical Rab GGTase-like activity and the critical Rab sequence determinants for the prenylation reaction among mammals, plants, and yeast.

LeRab Proteins Are Substrates for Prenylation by GGPP but Not by FPP

Rab proteins characterized in mammals and yeast are modified exclusively by the isoprene C_{20}-GG (Farnsworth et al., 1990; Khosravi-Far et al., 1991, 1992; Seabra et al., 1992a, 1992b; Brown and Goldstein, 1993; Farnsworth et al., 1994), in contrast to other prenylated proteins (e.g. p21^{Ras}), which are usually modified by the C_{15}-farnesyl FTase (Schafer and Rine, 1992; Omer and Gibbs, 1994) but can be modified by GGTase I in cells that lack active FTase (Trueblood et al., 1993). To establish that LeRab is prenylated exclusively by C_{20}-GG, we attempted prenylation of the protein substrates in the presence of [^{3}H]FPP instead of [^{3}H]GGPP as the prenyl moiety. The GST::LeRab1B fusion protein was incubated with tomato protein extracts prepared from either tomato VFNT suspension-culture cells or tomato stem sections. The results in Figure 8A show that the protein was labeled only in the presence of [^{3}H]GGPP and not in the presence of [^{3}H]FPP, irrespective of the source of the plant protein extract. The difference in the apparent molecular mass of the protein labeled by the two protein extracts was reproducible and could be the result of either postprenylation C-terminal processing of the Rab protein (Schafer and Rine, 1992) or a difference in the geranylgeranylation of one or both Cys residues. Furthermore, addition of a 40-fold excess of unlabeled FPP (20 μM) over [^{3}H]GGPP (0.5 μM) present in the reaction did not compete for the labeling of LeRab1B, whereas addition of 0.5 μM unlabeled GGPP significantly reduced labeling of LeRab1B to less than 40% of the control (Fig. 8B). These results, together with the results in Figure 5, suggest that the prenylation of LeRab1B specifically requires GGPP and a Rab GGTase-like enzyme activity.

The LeRab1CmCC Mutant Is Not Prenylated but Inhibits Prenylation of LeRab1C

The Rab consensus motifs XXCC, CCXX, or XCXC were shown to be absolutely required, but not sufficient, for the attachment of C_{20}-GG. Additional domains in the Rab protein were shown to be critical for recognition and/or efficient prenylation by Rab GGTase (Seabra et al., 1992a, 1992b). It has been proposed that these additional N-terminal domains are required in the interaction among Rab, Rab GGTase, and REP that presents Rab to Rab GGTase (Beranger et al., 1994, 1994b; Sanford et al., 1995). If the plant Rab GGTase-like activity we have identified is similar to the mammalian and yeast enzymes, we would expect that geranylgeranylation of LeRab1C in the tomato protein extract should be inhibited in a quantitative manner by the LeRab1CmCC mutant protein. LeRab1CmCC cannot be prenylated because the CCXX motif has been mutated (Fig. 5), but the protein should still be capable of binding to the enzyme complex. Figure 9 shows that GST::LeRab1CmCC effectively competes for the prenylation of GST::LeRab1C but that GST alone has no effect. Inhibition of GST::LeRab1CmCC labeling was detected with as little as 1.42 nmol of GST::LeRab1CmCC, which is only a 4-fold excess of mutant over wild-type LeRab1C (Fig. 9C), and

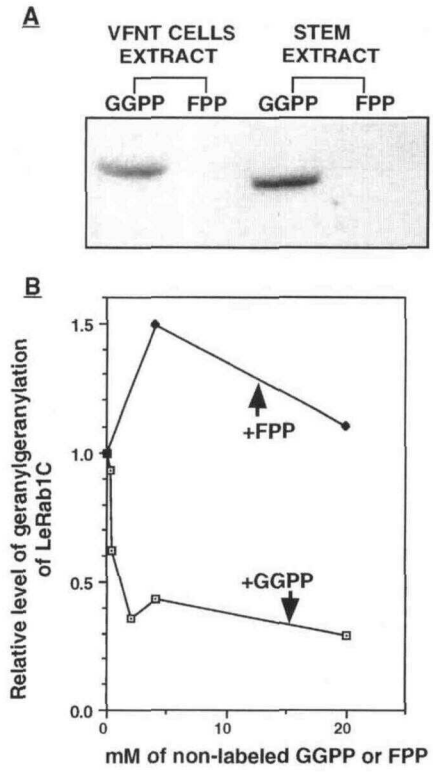

Figure 8. GST::LeRab1B is labeled by [^{3}H]GGPP but not by [^{3}H]FPP. A, Fluorogram of a gel on which GST::LeRab1B protein was fractionated following its incubation with either [^{3}H]GGPP or [^{3}H]FPP in the presence of either tomato stem protein extract or tomato VFNT cherry cell line protein extract. B, Quantitation of a competition experiment in which GST::LeRab1C was incubated with tomato stem protein extract and 0.5 μM [^{3}H]GGPP alone or in the presence of 4 and 20 μM nonlabeled FPP (■) or 0.25, 0.5, 2, 4, and 20 μM nonlabeled GGPP (⊡). Reactions were stopped by the addition of SDS-PAGE denaturation buffer. This was followed by fractionation of the proteins on gels, which were fixed and fluorographed. Quantitation of labeling was carried out by densitometric scanning of labeled bands on the x-ray film. Relative levels of labeling were calculated by subtracting the background level from each of the readings and dividing it by the value of the control reaction, to which no nonlabeled material was added, and which was taken as 1.

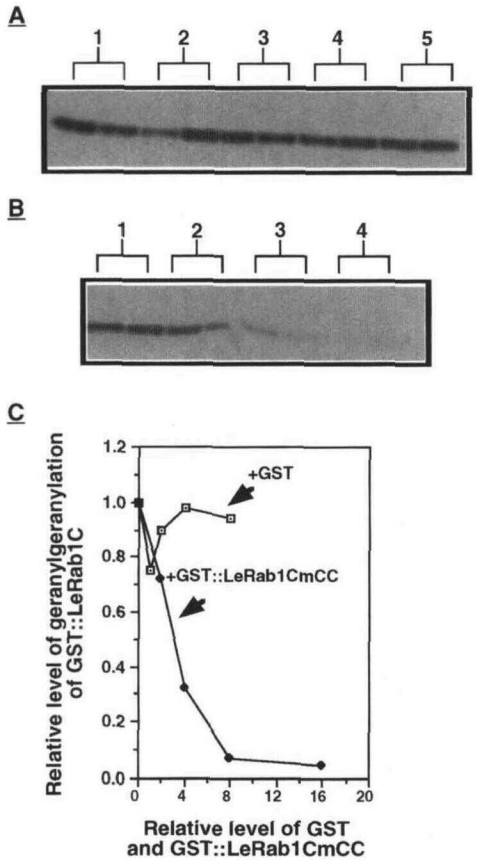

Figure 9. Labeling of GST::LeRab1C by [^{3}H]GGPP is reduced due to the presence of GST::LeRab1CmCC but not due to GST alone. A, Fluorogram of a gel run following duplicate geranylgeranylation reactions in which 0.36 nmol of GST::LeRab1C was incubated with [^{3}H]GGPP and tomato stem protein extract alone (1) or in the presence of GST at the following amounts: 0.36 nmol (2), 0.72 nmol (3), 1.2 nmol (4), and 2.4 nmol (5). B, Similar reactions to those described in A were run but in the presence of GST::LeRab1CmCC at the following concentrations: 0.72 nmol (1), 1.2 nmol (2), 2.4 nmol (3), and 5.7 nmol (4). Each number corresponds to duplicate reactions. C, Quantitation of the results shown in A and B. Levels of GST (⊡) and GST::LeRab1CmCC (■) are presented as relative levels. Quantitation of labeling was carried out by scanning the films with a densitometer. Results are presented as relative levels of labeling calculated by taking the average level of each duplicate and dividing it by the average level of labeling in the absence of any additional protein, which was taken as 1.

almost complete inhibition of LeRab1C labeling was observed with 5.7 nmol (about a 12-fold excess) of LeRab1CmCC in the reaction mixture. These results show that LeRab1CmCC can act as a competitive inhibitor for the prenylation of LeRab1C, most likely through a nonproductive interaction with the Rab GGTase/REP-like enzyme.

DISCUSSION

In this paper we describe the use of unmodified plant and mammalian Rab proteins to establish that plant cells have a Rab GGTase-like activity capable of attaching C_{20}-GG to conserved Cys residues in the C terminus of the substrate proteins. Like the prenylation of Rab proteins by mammalian and yeast Rab GGTase (Seabra et al., 1992a, 1992b; Beranger et al., 1994a, 1994b; Cremers et al., 1994; Sanford et al., 1995), prenylation by the plant enzyme requires additional N-terminal sequences in the Rab protein for efficient geranylgeranylation of the Cys's in the conserved C-terminal prenylation motifs. Together, our results demonstrate that LeRab1B and LeRab1C are likely in vivo prenylation substrates for a Rab GGTase enzyme that is conserved in its biochemical mechanism among mammalian cells, plants, and yeast. Together with previous reports of protein prenylation in plants (Randall et al., 1993; Yang and Watson, 1993; Biermann et al., 1994; Morehead et al., 1995), our biochemical characterization of the plant Rab GGTase-like enzyme described here and similar biochemical and molecular characterization of tomato FTase and GGTase I activities (D. Schmitt, K.L. Callan, S.-H. Kim, W. Gruissem, unpublished results; S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, W. Gruissem, unpublished results) now establishes the presence of a full protein-prenylation enzyme system in plants. These results are significant because they indicate that protein prenylation is an evolutionarily conserved mechanism. We speculate that protein prenylation has been recruited early by the eukaryotic cell to establish a link between the sterol biosynthesis pathway and the biological activity of proteins that are of fundamental importance for cell growth processes. In contrast to mammalian cells, however, just a few target proteins have been identified in plants that are substrates for prenyltransferases. It is now possible to use recombinant or partially purified FTase, GGTase I, and Rab GGTase to search for other plant proteins that are prenylated by these enzymes as an alternative to the application of the heterologous yeast protein extract (Biermann et al., 1994).

The biochemical and molecular characterization of the prenyltransferases in plants also provides an important basis to understand the regulation of these enzymes as a function of the developmental program and in response to environmental, hormonal, and nutritional signals. Although Rab GGTase has been well characterized at the biochemical level in mammalian cells (Seabra et al., 1992a, 1992b; Cremers et al., 1994) and at the genetic level in yeast (Jiang et al., 1993; Li et al., 1993; Fujimura et al., 1994; Jiang and Ferro-Novick, 1994), very little is known about the regulation of the enzyme during cell division and growth, in response to mevalonic acid starvation, or in differentiated cells active in secretory processes. For example, the mammalian REP component of Rab GGTase has been identified as the cause for retinal degradation in choroideremia (Seabra et al., 1993, 1994; Seabra, 1995), but the pathway from the genetic lesion to the pathogenesis of choroideremia is poorly understood. Unlike in mammals and yeast, GGPP in plants is also a precursor for myriad isoprenoid end products, many of which are synthesized in the chloroplast (Bach, 1995), but biochemical or molecular information about the partitioning of the sterol biosynthesis pathway from mevalonic acid to GGPP is scarce. It is generally

unclear how flux through the GGPP branchpoint is regulated or if and how Rab GGTase interfaces with other enzymes at this branchpoint to channel GGPP from the sterol biosynthesis pathway to the prenylation of Rab proteins required for the regulation of membrane trafficking.

To determine the function and regulation of prenyltransferases in plants, we first established their activities in plants as a prerequisite for the molecular cloning. For the biochemical characterization of plant Rab GGTase we cloned homologous Rab proteins as substrates for the enzyme (Loraine et al., 1996). Based on their conserved amino acid sequence, their ability to complement a yeast *ypt1* mutant strain, and their localization to membranes, it has been postulated that the plant-Rab proteins are also modified by the C_{20}-GG isoprene (Drew et al., 1993; Youssefian et al., 1993; Borg and Poulsen, 1994; Park et al., 1994), but no biochemical evidence for such modification in plants has been reported. To detect Rab GGTase-like enzyme-dependent geranylgeranylation in a plant protein extract, we found it necessary to introduce several changes in the assay developed for mammalian Rab GGTase. In addition, the purity and integrity of the Rab protein substrate was critical to detect enzyme activity. Proteolysis of GST::LeRab by thrombin to separate LeRab from the GST protein resulted in a partial degradation of the Rab proteins, which subsequently failed to become modified by GGPP (data not shown). Similarly, we found it necessary to assay the stability of GGPP in each plant protein extract. Protein extracts were prepared from various tissue sources, such as leaves, stems, roots, tomato fruits of different developmental stages, and tissue-culture cells. We found that protein extracts from stem sections of hydroponic tomato plants and tissue-culture cells (in contrast to root tissue, for example) provided both good stability of GGPP and high activity of the Rab GGTase-like activity, although we are unable at present to explain the biochemical basis of this result.

Our observation that tomato and tobacco protein extracts support C-terminal Cys-dependent geranylgeranylation of LeRab1B and LeRab1C, as well as the canine protein Rab1A, is strong evidence for the presence of Rab GGTase in plants. The plant enzyme activity is similar to the geranylgeranylation activity in the yeast protein extract. Furthermore, the apparent lack of LeRab1B and LeRab1C geranylgeranylation in the protein extract prepared from the yeast *mad2–2* mutant strain is additional evidence that the tomato Rab homologs are prenylated by Rab GGTase rather than by GGTase I. Like mammalian and yeast GGTase I, plant GGTase I can efficiently prenylate a peptide substrate with a conserved C*aa*L motif (D. Schmitt, K.L. Callan, S.-H. Kim, W. Gruissem, unpublished results). Partially degraded forms of LeRab failed to become prenylated (data not shown). It is therefore likely that the conserved CC motif in the plant Rab proteins is alone not sufficient for their prenylation, however, thus excluding the possibility that LeRab1B and LeRab1C are prenylated by a GGTase I-like activity. Although we have not analyzed the C-terminal Cys's directly for their modification by C_{20}-GG, the failure of LeRab1CmCC to support geranylgeranylation in vitro and to complement the yeast *ypt1* mutant strain for growth (Loraine et al., 1996) is strong evidence that the two Cys residues serve as the attachment site for the C_{20}-GG moiety. It is interesting that the putative XCCX geranylgeranylation motif of LeRab1B has been described previously only in plants (Nagano et al., 1993) and not in mammals and yeast (the motifs currently identified in mammals and yeast are XXCC, XCXC, and CCXX). This may indicate that the actual location of the Cys residues in the C-terminal region does not greatly influence prenylation by Rab GGTase but could affect other modifications of the C terminus following geranylgeranylation, such as methylation (Farnsworth et al., 1994) or some other modification unique to plants.

The inhibition of in vitro geranylgeranylation of LeRab1C by the LeRab1CmCC mutant protein strongly suggests that the mutant protein can still interact with the enzyme. From this result, we conclude that one or more additional domains in the LeRab protein are also essential for efficient recognition and/or prenylation by the plant Rab GGTase. Our results are consistent with earlier observations that a C-terminal Rab mutant protein competes for efficient geranylgeranylation of the wild-type protein by forming a nonproductive interaction with the REP component of Rab GGTase (Seabra et al., 1992b). It is likely that geranylgeranylation of Rab proteins in plants is also accomplished by a Rab GGTase/REP heterotrimeric enzyme complex by a mechanism as shown in Figure 1. Taken together, our results are consistent with the notion that the Rab geranylgeranylation activity in plants is biochemically similar to the heterotrimeric Rab GGTase-REP enzyme previously identified in mammalian and yeast systems. The biochemical confirmation of the Rab GGTase in plants is an important step to determine the extent to which the biochemical and molecular properties of the plant, animal, and yeast enzymes are conserved and to clarify the regulatory role of the plant enzyme for Rab function during plant growth and development.

ACKNOWLEDGMENTS

The authors wish to thank Drs. C. J. Derr and R. Khosravi-Far (University of North Carolina, Chapel Hill) for providing the pAR-Rab1 construct; Dr. F. McCormick (Onyx Corp., Richmond, CA) for the anti-Glu-Glu MAb; Drs. R. Li (Harvard University, Cambridge, MA) and A.W. Murray (University of California, San Francisco) for the *mad2–2* (*bet4 ts*) yeast strain; S. Jenkins for carefully reviewing the manuscript; and M. Quilter for technical assistance.

Received October 18, 1995; accepted January 23, 1996.

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