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Isoprenylation of Plant Proteins *in Vivo*

ISOPRENYLATED PROTEINS ARE ABUNDANT IN THE MITOCHONDRIA AND NUCLEI OF SPINACH*

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Protein isoprenylation *in vivo* is demonstrated using spinach seedlings labeled with [^{3}H]mevalonate. This report provides evidence for the occurrence of a large number of isoprenylated proteins in plants. Seedlings, without roots, were labeled quantitatively through the cut stem. Mevinolin treatment of the seedlings resulted in increased incorporation of radiolabel into proteins. Approximately 30 labeled bands could be detected after autoradiography of SDS-polyacrylamide gel electrophoresis-separated polypeptides, ranging in molecular mass from 6 to 200 kDa. Methyl iodide hydrolysis resulted in the release of covalently bound farnesol, geranylgeraniol, phytol, and some unidentified isoprenoid compounds from mevalonate-labeled proteins. It was found that all cellular fractions contained some isoprenylated proteins, although most were located in the mitochondria and nuclei. Subfractionation of the nucleus revealed that the majority of isoprenylated proteins in this compartment were components of the nuclear matrix. The results demonstrate that *in vivo* labeling of a complex organism can be performed using a plant system in order to study protein isoprenylation and distribution of modified proteins in different cellular compartments.

Isoprenylation of proteins is a common cellular event, which until recently has not been studied in plants. It has been estimated that as much as 2% of total mammalian cell protein is isoprenylated, which corresponds to 60–80 different proteins/cell (1). Proteins previously shown as being isoprenylated include small GTP-binding proteins (2–4), nuclear lamins (prelamin A and lamin B) (5, 6), and γ-subunits of heterotrimeric G-proteins (7).

Recently, a large amount of interest has been focused on protein isoprenylation since it was established that several members of the Ras superfamily were modified in this manner (2–4). These proteins regulate a variety of functions including control of cell growth and differentiation, cytokinesis, and membrane trafficking (8).

So far, three different prenyl protein transferases have been identified: farnesyl protein transferase (9) and geranylgeranyl protein transferases I (10) and II (11). These enzymes catalyze the transferal of the isoprene moieties from isoprenyl pyrophosphates (farnesyl pyrophosphate, C_{15}, or geranylgeranyl pyrophosphate, C_{20}), produced in various cellular compartments (12, 13), to at least 1 cysteine residue near the carboxyl terminus of the substrate protein by the formation of a thioether linkage. Farnesyl protein transferase and geranylgeranyl protein transferase I recognize a specific sequence of amino acid residues, here referred to as a -C$X_1X_2X_3$-box, where X_1 and X_2 could be any amino acid residues. The last residue of this motif (X_3) appears to direct which isoprene pyrophosphate should be attached. Serine, methionine, glutamine, cysteine, and alanine signal for farnesylation (see Ref. 14) whereas leucine and phenylalanine are targets for geranylgeranylation (10). Geranylgeranyl protein transferase II activity is dependent on an as yet unidentified internal sequence, as well as a carboxyl-terminal motif, designated -CC, -C*X*C or -CC*XX*, which contains 2 cysteines (11). There have been several reports of protein-bound isoprenoids other than farnesol and geranylgeraniol, including dolichyl phosphate (15) and isopentenyl adenine (16), although the nature of the covalent binding in these cases and the enzymes catalyzing these modifications remain to be identified.

Protein isoprenylation is a ubiquitous event, occurring in organisms as diverse as yeast and humans. However, although the chemical mechanism of protein isoprenylation is now beginning to be unraveled, little is known about the extent of this post-translational modification or its specific physiological function. Many studies have been performed in yeast and mammalian cell cultures utilizing radiolabeled precursors of the mevalonate pathway for the tagging of isoprenylated proteins. Studies of the isoprenylation process by these methods in more complex systems or in whole organisms have proved limited. In a recent investigation (17), it was found that pieces of etiolated spinach leaves, incubated in a [^{3}H]mevalonate solution, incorporated the label into protein-bound farnesol, geranylgeraniol, phytol, and longer chain polyprenols, although only one radiolabeled band was identified by SDS-PAGE.[1] This work established that plants possess isoprenylated proteins that may be studied in an appropriate *in vivo* system. Cell cultures of tobacco also show incorporation of [^{14}C]mevalonate into a protein-associated form (18). Additionally, the occurrence of plant protein isoprenylation has been implied by the results of several recent studies. For example, farnesyl- and geranylgeranyl protein transferase activities have been found in cell extracts of *Atriplex nummularia*, and isoprenylation has been performed *in vitro* with ANJ1 (a higher plant homologue of the bacterial molecular chaperone DnaJ) as a substrate (19). Farnesyl protein transferase β-subunit has recently been cloned and se-

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[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high performance liquid chromatography.

quenced from *Pisum sativum* (20). Small GTP-binding proteins of the Ras superfamily have been cloned and sequenced from *Nicotiana plumbaginifolia* (21), *Zea mays* (22), and *Arabidopsis thaliana* (23) and shown to contain carboxyl-terminal motifs corresponding to those found in mammalian isoprenylated proteins. In an *in vitro* system, a small GTP-binding Ras-related protein from *P. sativum* could be geranylgeranylated (24).

The results reported in this article demonstrate that isoprenylation of plant proteins occurs *in vivo*, and a system has been established to study the mechanism of this process. The data provide evidence that in plant cells isoprenylation of proteins occurs extensively, and isoprenylated proteins are located in many cellular compartments, in particular the mitochondria and nuclei.

EXPERIMENTAL PROCEDURES

Materials—Spinach seedlings (*Spinacea oleracea* L.) were grown on vermiculite in a growth room at 22–25 °C, with a 10-h photoperiod, for 2–3 weeks. (*R,S*)-[5-^{3}H]mevalonolactone (specific activity, 11.6 Ci/mmol) was prepared as described by Keller (25). Mevinolin was the generous gift of Dr. A. W. Alberts (Merck). Before use, the mevalonolactone and mevinolin were converted from the metabolically inactive lactone form to the active dihydroxy acid form. This was achieved by evaporation of the solvent in which the compound was stored under a nitrogen stream, addition of 0.05 M NaOH to increase the pH to >11, and incubation at 30 °C for 40 min, followed by evaporation again to dryness. The compounds were then dissolved in the required amount of solvent (water or spinach medium) to the desired concentration. Radiolabeled protein molecular mass standards were obtained from Amersham Corp. All chemicals were obtained from Sigma, unless otherwise indicated, and solvents were of reagent grade.

Labeling of Spinach Proteins in Vivo—For all experiments, roots were excised from the seedlings and the experimental solutions fed in through the cut stem. Unless otherwise indicated, a preincubation treatment with 30 μM mevinolin in spinach growth medium (5 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM NH_4Cl, 156 μM EDTA, 72 μM $FeSO_4$, 46 μM H_3BO_3, 6.2 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.7 μM MoO_3, 0.2 μM NH_4VO_3) was routinely performed for 20 h prior to initiation of labeling. Eppendorf tubes (0.5 ml) were used for the labeling, each containing 20 μl of solution for two plants. Labeling with [^{3}H]mevalonate was carried out at room temperature under laboratory illumination usually for 24 h with 50–100 μCi/seedling (according to the experiment) in spinach medium also containing 30 μM mevinolin. Once the seedlings had taken up the labeling solution, it was replaced by spinach medium with mevinolin for the remainder of the required labeling period.

Labeling of spinach proteins with [^{35}S]methionine (Amersham) was essentially as described above. Seedlings were fed with 1 μCi/plant in spinach medium, and labeling continued for 6 h in the presence or absence of mevinolin. Plants were given an appropriate 20-h pretreatment prior to labeling in spinach medium with or without mevinolin.

Preparation of Radiolabeled Protein Samples for Electrophoresis—After the labeling period, each sample (two plants) was homogenized using an Ultra-Turrax blender in 1 ml of ice cold double distilled water and quickly filtered through one layer of nylon net (20 μm) to remove cell debris and tissue pieces into 10 ml of ice-cold acetone. [^{35}S]Methionine-labeled material was collected by centrifugation for solubilization at this point. Mevalonate-labeled proteins were washed extensively (four times each with acetone and chloroform:methanol (2:1) followed by three extractions with ethanol and three with chloroform:methanol:water (1:1:0.3)). The extraction procedure for cell fractionation experiments was occasionally curtailed due to the limited amount of protein/sample. These samples were extracted at least once with each solvent as described above. The remaining precipitated material was solubilized with 3% lithium dodecyl sulfate, 75 mM Tris-HCl (pH 8.0), 1.5% glycerol, 75 mM dithioerythritol by incubation at 70 °C for 10 min. Protein concentration was determined according to Marder *et al.* (26). Trypsin digests were performed on solubilized material at 37 °C for 1 h at an enzyme concentration of 100 μg·ml^{-1} and a protein concentration of 8 mg·ml^{-1}. The protein profiles of the solubilized samples were resolved on 13% acrylamide gels (27), which were stained with Coomassie Blue R-250, impregnated with AMPLIFY (Amersham, United Kingdom), dried, and exposed to film (Hyperfilm-MP, Amersham) at −80 °C for 3–8 weeks. Separate gels were run for Western blotting, which was performed as described by Towbin *et al.* (28).

Hydrolysis of Bound Lipid—Samples to be analyzed by HPLC were prepared from approximately 2.5 mg of lipid-extracted proteins. Methyl iodide hydrolysis was performed basically according to the method of Casey *et al.* (4); however, prior to the incubations, the tube was flushed with nitrogen to eliminate oxygen from the system. Alkaline hydrolysis was carried out as described earlier (15).

Lipid Analysis—Samples were dissolved in a small volume of ethanol for injection onto HPLC. Analysis of the lipids released from protein samples was performed by reversed phase HPLC using a C-18 column (Hewlett Packard Hypersil ODS 3 μm) and a two-step linear gradient, basically as described previously (13, 29). The first gradient was run for 15 min and consisted of methanol:water (7:3) (solvent A) to methanol:water (9:1) (solvent B). The second gradient was employed for 15 min directly after the first (solvent B to methanol:2-propanol:hexane (2:1:1)). The flow rate throughout the analysis was 1.5 ml·min^{-1}. The radioactivity of the eluate was measured using a radioactivity flow detector (Radiomatic Instruments, Tampa, FL) and UV absorption followed at 210 nm. Unlabeled standards were utilized to identify labeled products of hydrolysis.

Cellular Fractionation of Spinach Seedlings—Cellular fractionation was performed after the methods of Morré *et al.* (30) and Glaser *et al.* (31) from 2.5 g of plant material (mevalonate-labeled seedlings). All procedures were carried out on ice or at 4 °C. Seedlings were homogenized in 300 mM sucrose, 50 mM MOPS (pH 7.8), 5 mM $MgCl_2$, 2 mM EDTA using a mortar and pestle. The homogenate was filtered through one layer of nylon net and centrifuged at 5,000 × *g* for 90 s. The pellet, containing intact chloroplasts and nuclei, was resuspended in 200 mM mannitol, 30 mM MOPS (pH 7.8), 2 mM EDTA (buffer A) and layered onto a cushion of 2 M sucrose in buffer A. Centrifugation was performed using a swing-out rotor for 30 min at 50,000 × *g*. Chloroplasts were removed from the interface, and nuclei were recovered in the pelleted material.

The supernatant from the initial centrifugation (5,000 × *g*, 90 s) was spun again for 3.5 min at 20,000 × *g* to pellet the crude mitochondrial fraction. The remaining supernatant was centrifuged at 146,000 × *g* for 1.5 h to separate the microsomal and cytosolic fractions. The crude mitochondrial fraction was resuspended in 250 mM sucrose, 10 mM MOPS (pH 7.2), 1 mM EDTA and purified on a discontinuous Percoll gradient (31). All samples were resuspended in double distilled water and immediately precipitated by the addition of 10% trichloroacetic acid. Proteins were extracted with organic solvents, as described above, prior to electrophoretic analysis or scintillation counting. Nuclei were further fractionated according to the method described by Wolda and Glomset (6) and after Long *et al.* (32). Basically, soluble nuclear proteins and the nuclear membrane were removed by incubation with 0.2% Triton X-100, DNA- and RNA-bound proteins were released by digestion with DNase and RNase (at 250 and 150 μg·ml^{-1}, respectively), and histones were removed by a 1.6 M NaCl wash.

RESULTS

Incorporation of Mevalonate Pathway Metabolites into Spinach Proteins—The autoradiograph shown in Fig. 1 demonstrates that after metabolic labeling of spinach seedlings with [^{3}H]mevalonate, label remains specifically associated with cellular proteins. This radioactivity results from lipids covalently bound to proteins, since an extensive solvent extraction procedure had been carried out prior to electrophoresis. At least 30 labeled bands of different apparent molecular masses were observed, ranging from greater than 200 kDa to less than 6 kDa. The strongly labeled band at around 3 kDa, indicated in Fig. 1 by an *arrow*, is residual unincorporated mevalonate and probably mevalonate metabolites. The 25–50-kDa region of gels from all experiments could be seen to contain the highest density of radiolabel. A similar labeling pattern was observed in [^{3}H]mevalonate-labeled wheat leaves (data not shown).

Labeling of proteins with [^{3}H]mevalonate was found to be related to both the concentration of radiolabel supplied to the plants (data not shown) and the time of labeling (Fig. 1). Weakly labeled bands could be seen after 5 h of exposure to the radiolabeled substrate (not shown). Labeling of proteins was found to be increased with time up to 21 h and then to decline (Fig. 1). The data shown suggest that a metabolic product(s) of mevalonate is covalently attached to plant proteins during labeling *in vivo*. This experiment was performed using 100 μCi

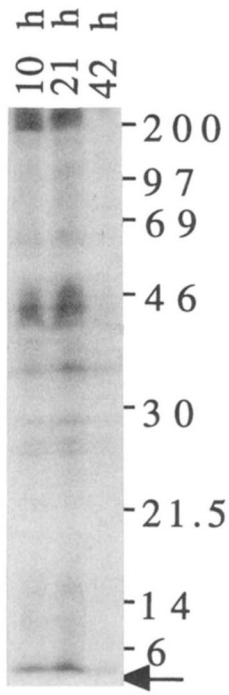

FIG. 1. **Time dependence of [^{3}H]mevalonate labeling of spinach seedling proteins.** An autoradiograph of lipid-extracted protein samples separated by SDS-PAGE from spinach seedlings labeled *in vivo* with 100 μCi of [^{3}H]mevalonate/plant is shown. Labeling was performed for 10, 21, and 42 h, respectively, in the presence of 30 μM mevinolin. Homogenization, extraction, solubilization, SDS-PAGE, and autoradiography were carried out as described under "Experimental Procedures." The gel was loaded with 90 μg of protein/lane. The position of the molecular mass markers is indicated on the *right* of the figure.

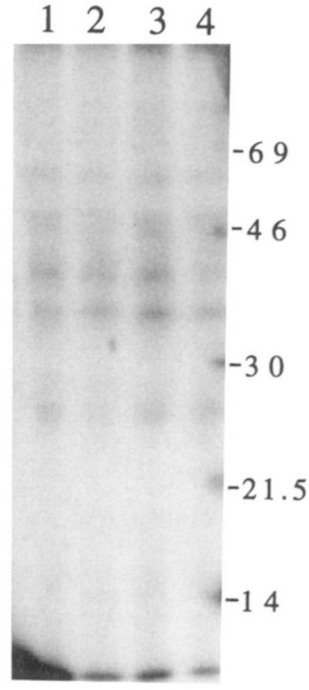

FIG. 2. **Solvent extraction of spinach proteins labeled *in vivo* with [^{3}H]mevalonate.** Seedlings were labeled with 50 μCi/plant in the presence of 30 μM mevinolin for 24 h. At the end of this time, the samples were homogenized. Solvent extractions were performed as described under "Experimental Procedures," and samples were sequentially solubilized for SDS-PAGE and autoradiography. *Lane 1*, acetone-insoluble material; *lane 2*, chloroform:methanol-insoluble material; *lane 3*, ethanol-insoluble material; *lane 4*, chloroform:methanol:water-insoluble material.

of [^{3}H]mevalonate/seedling. The radiolabeled bands detected by autoradiography are, therefore, more intense here than in the following experiments where only 50 μCi/plant was utilized. By feeding spinach seedlings [^{3}H]mevalonate (50 μCi/plant) in this manner it was possible to incorporate 500-1500 dpm·μg^{-1} total protein. The percentage of supplied [^{3}H]mevalonate incorporated into proteins using this spinach system was estimated to be 0.05–0.1%.

Fig. 2 shows that the protein-associated radiolabel cannot be extracted from any individual protein bands during the solvent washing steps. It was observed that the extensive extraction procedure carried out was not necessary to remove noncovalently attached lipids in the plant system used since SDS-PAGE is an excellent additional purification step. However, in order to determine the exposure time required for detection of labeled proteins, extractions were performed to allow an accurate estimation of protein-bound radioactivity by scintillation counting prior to electrophoresis. In addition, omitting solvent extractions results in a large quantity of radiolabel in the low molecular mass region of the gel (Fig. 2, *lane 1*), possibly obscuring any labeled bands in this area. The results in Fig. 2 also show that all labeled bands observed are due to covalently bound, rather than loosely associated, lipid moieties derived from mevalonate.

The radiolabeling is demonstrated to be associated specifically with protein, as all radiolabeled bands are digested upon incubation with trypsin (Fig. 3). The appearance of tryptic fragments still carrying a radiolabeled moiety can be observed.

The extent of labeling was found to be dependent on a preincubation with mevinolin (Fig. 4*A*), a competitive inhibitor of hydroxymethylglutaryl-CoA-reductase (33). Without mevinolin preincubation, labeling of proteins was feasible (275 dpm·μg^{-1} protein). However, a pretreatment with mevinolin was observed to increase the level of incorporated radiolabel, possibly due to a build up of unmodified protein precursors (see Ref. 34) and a decrease in the internal pool of free isoprenyl groups to be attached during this time. It was found that a 24-h pretreatment with the inhibitor resulted in a doubling of the incorporation of [^{3}H]mevalonate as compared with the control (no preincubation). If mevinolin was excluded from the labeling solution and no mevinolin preincubation treatment was given to the plants, an identical pattern of labeled bands could be observed after electrophoresis and autoradiography as that shown in Figs. 1 and 2, although levels of incorporation were reduced further (data not shown).

Interestingly, mevinolin treatment of the seedlings appears to result in a significant increase in total protein synthesis, as can be observed by pretreatment and [^{35}S]methionine labeling of seedlings in the presence or absence of mevinolin (Fig. 4*B*). The pattern of radioactive bands produced by labeling with [^{3}H]mevalonate (Figs. 1 and 2) was found to be very different from that observed after labeling under similar conditions with [^{35}S]methionine (Fig. 4*B*). This illustrates that the radioactivity associated with proteins after labeling with [^{3}H]mevalonate was due to association of a product of mevalonate with specific proteins, rather than degradation of the radiolabel to precursors of amino acid biosynthesis. This degradation pathway is known as the mevalonate shunt (35). We have also observed in a separate experiment that homogenization of unlabeled seedlings (pretreated with mevinolin for 44 h) in the presence of [^{3}H]mevalonate (50 μCi/plant) did not result in labeled proteins being visible by autoradiography of the lipid-extracted proteins. The only band visible on this autoradiograph was that of free mevalonate at the electrophoretic front (not shown).

Characterization of the Covalently Bound Isoprenoids—Methyl iodide hydrolysis of the lipid-extracted radiolabeled

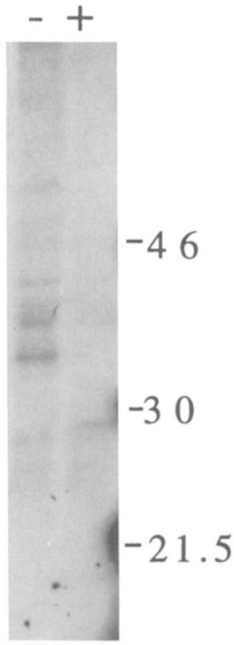

FIG. 3. **Autoradiograph of a trypsin digest of [^{3}H]mevalonate-labeled spinach proteins.** Seedlings were labeled (50 μCi/plant) as described (see "Experimental Procedures"). Samples were homogenized, lipid-extracted, and solubilized for SDS-PAGE. Prior to electrophoresis, one sample (+) was digested with trypsin (100 $\mu g \cdot ml^{-1}$) for 1 h at 37 °C. The undigested control sample (−) was incubated without trypsin under identical conditions. The positions of molecular mass markers are indicated.

proteins was demonstrated to release several tritiated isoprenoids, which were characterized by subsequent HPLC analysis. Spinach proteins were shown to possess covalently bound farnesol, geranylgeraniol, and phytol as well as some unidentified products of mevalonate (Fig. 5*A*). These data demonstrate the attachment of isoprenoids to plant proteins *in vivo* via a thioether linkage. The ratio of geranylgeraniol to farnesol was seen to vary between different experiments (as also observed in our laboratory for animal cell samples), but the geranylgeraniol peak always had a significantly larger area (seen in seven separate hydrolyses). The identity of the compound eluting at the solvent front is presently under investigation and may prove to be polyprenyl phosphates.

Alkaline hydrolysis released a different set of isoprenoids from the labeled proteins (Fig. 5*B*). No farnesol was released under these conditions; however, some geranylgeraniol was liberated as well as long-chain polyprenols. Many products released by both methods of hydrolysis remain to be identified.

Subcellular Distribution of Isoprenylated Proteins—Fractionation of [^{3}H]mevalonate-labeled seedlings revealed that the radiolabel was associated mainly with mitochondrial and nuclear proteins (Table I). All cellular fractions obtained were found to contain a certain amount of radiolabel, although levels in the cytosol and chloroplasts were low, and very few labeled bands could be detected in these samples (Fig. 6). Unique radiolabeled bands can be observed in the various cellular fractions. In chloroplasts, bands of 28 and 24 kDa can be seen. A cytosolic protein of 55 kDa is labeled as well as bands present only in the microsomal fraction of 52, 24, 16, 9, and 5 kDa. Preliminary data suggest that the majority of the label present in chloroplasts is associated with membranous (thylakoid and/or envelope) rather than stromal proteins.

The nuclear and mitochondrial fractions both contained many labeled polypeptides (Fig. 6), and the majority of labeled bands observed in the whole plant protein extract originate from these two fractions. The nuclear fraction shows major

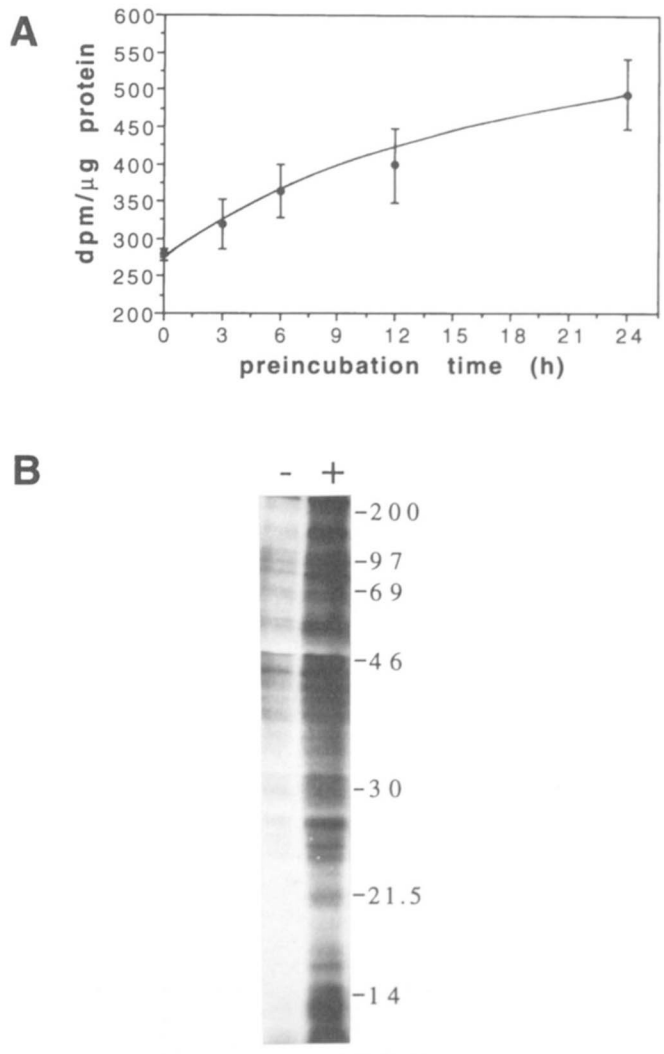

FIG. 4. **Effect of mevinolin on the metabolic labeling of proteins in spinach seedlings.** *A*, graph showing the radiolabeling of spinach proteins *in vivo* with respect to the time of preincubation of the seedlings with 30 μM mevinolin. Seedlings were preincubated for the indicated period with mevinolin and then labeled for 24 h with 50 μCi/plant [^{3}H]mevalonate as described under "Experimental Procedures." Samples were homogenized, lipid-extracted, and solubilized. Labeling level was assessed by scintillation counting of the solubilized material. Results shown are the mean of three replicates ± S.E. *B*, seedlings were preincubated and labeled with [^{35}S]methionine (1 μCi/plant) in the presence or absence of 30 μM mevinolin. The preincubation and labeling times were 20 and 6 h, respectively. Samples were homogenized, acetone-precipitated, solubilized, and separated by SDS-PAGE. The gel was loaded with 90 μg of protein/well, and labeled bands were detected by autoradiography.

labeled bands at 47, 37, 33, and 5 kDa, as well as nine minor labeled components. Strongly labeled bands were detected in the mitochondrial fraction at 47, 33, and 6 kDa, with 10 weaker bands also being observed. There are some similarities in the patterns of bands detected in the nuclear and mitochondrial lanes, which are not due to significant levels of cross-contamination between the two fractions. Western blotting of these fractions has shown that the mitochondrial fraction contained undetectable levels of nuclear contamination, utilizing antibodies raised to proteins of the nuclear pore complex (data not shown). The nuclear fraction contained approximately 15% mitochondrial contamination as assessed by using two antisera raised to soluble mitochondrial proteins (not shown). The absence of significant levels of cross-contaminating membranes in all organelle fractions was seen by electron microscopy (data

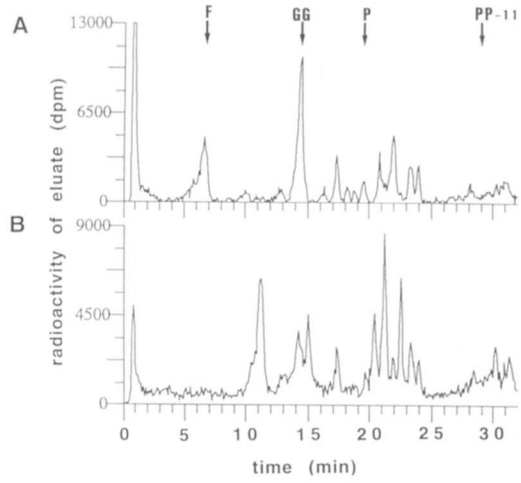

FIG. 5. **HPLC separation of lipids covalently bound to proteins of spinach seedlings.** Spinach seedlings were labeled under standard conditions (see "Experimental Procedures"). After extensive lipid extraction, proteins were subjected to methyl iodide hydrolysis (*A*) or alkaline hydrolysis (*B*). Released isoprenoids were extracted from the hydrolysis mixture and analyzed as described under "Experimental Procedures." The elution times of unlabeled standards are shown: farnesol (*F*), geranylgeraniol (*GG*), phytol (*P*), and polyprenol-11 (*PP-11*).

TABLE I

The distribution of radiolabel among various cellular fractions

Seedlings were labeled with [^{3}H]mevalonate and fractionated as described (see "Experimental Procedures"). After lipid extraction and solubilization, the radiolabel associated with the proteins of each fraction was determined by scintillation counting. Data shown represent the mean values of eight experiments. Standard errors are not given for data, other than in the final column, as specific levels of labeling in the whole plant homogenate differed significantly between experiments (500–1500 dpm·μg^{-1} protein), resulting in numerical differences in labeling of cellular fractions. Therefore, it was considered more appropriate to directly compare data within one experiment to a particular fraction and to calculate errors between experiments from these values (final column).

Cellular fraction	Incorporation of radiolabel	% of total labeling	% of mitochondrial labeling
	dpm·μg^{-1} protein		*dpm·μg^{-1} protein*
Mitochondrion	3170	39.2	100
Nucleus	2350	29.0	74.0 ± 21.1
Cytosol	800	9.9	25.2 ± 4.3
Microsomes	1220	15.1	38.4 ± 15.8
Chloroplast	550	6.8	17.3 ± 5.0

not shown).

To further study the nuclear location of labeled bands observed in the initial fractionation experiments, a nuclear subfractionation protocol was carried out. It can be seen in Fig. 7*A* that sequential treatment of the nuclear pellet with 0.2% Triton X-100, DNase (250 $\mu g \cdot ml^{-1}$) and RNase (150 $\mu g \cdot ml^{-1}$), and 1.6 M NaCl resulted in the release of different subsets of proteins. The autoradiograph of this gel, shown in Fig. 7*B*, establishes that the majority of the labeled polypeptides in the nucleus are located in the Triton/salt-insoluble subfraction (nuclear matrix, N_m), although two labeled bands (at 37 and 62 kDa) were specifically removed by the Triton X-100 treatment (S_1).

DISCUSSION

Isoprenylation of proteins has until now been studied in isolated cells (yeast and tissue culture), systems which take up sufficient labeled precursors and contain available protein acceptors. By using plants fed with radiolabel through the stem, our results show that the isoprenylation process can be studied in a complex organism *in vivo*. The labeling procedure developed for this study proved to be an effective tool for investigation of the mechanisms of the isoprenylation events. The level of isoprenylation found in these experiments with spinach seedlings is comparable with that reported previously (0.5%) in an optimized yeast system (36).

Protein isoprenylation appears to be a frequently occurring event in the plant cell, and it is suggested from the time course of labeling, shown in Fig. 1, that protein-bound products of

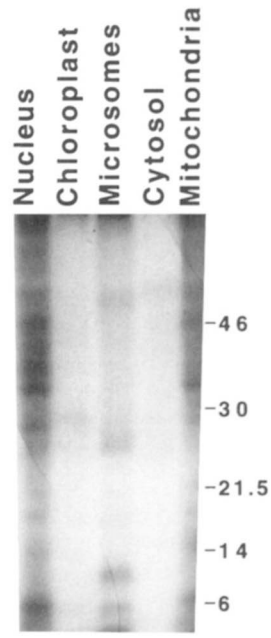

FIG. 6. **Autoradiograph of an SDS-PAGE gel showing proteins of various cellular fractions labeled *in vivo* by [^{3}H]mevalonate.** Seedlings were labeled and fractionated as described under "Experimental Procedures." Nuclear, chloroplastic, microsomal, cytosolic, and mitochondrial fractions were prepared from the plant homogenate. Samples were lipid-extracted and solubilized. The gel was loaded with 90 μg of protein for all fractions except the mitochondrial fraction, for which only 42 μg of protein was loaded, to allow detection of labeled bands in all fractions on the same autoradiograph.

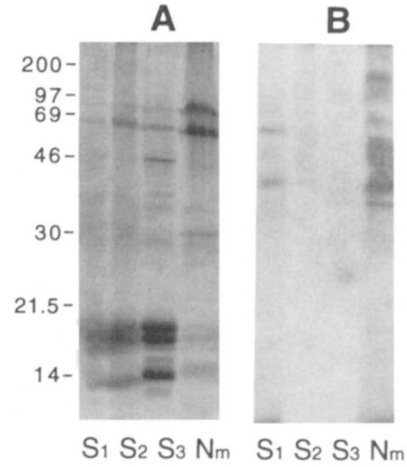

FIG. 7. **Localization of [^{3}H]mevalonate-labeled proteins in nuclear subfractions.** Seedlings were labeled with [^{3}H]mevalonate and fractionated as described under "Experimental Procedures." Nuclei obtained from this procedure were further subfractionated by sequential treatment with 0.2% Triton X-100, RNase (150 $\mu g \cdot ml^{-1}$) and DNase (250 $\mu g \cdot ml^{-1}$), and 1.6 M NaCl. Proteins present in the supernatant after each treatment are shown in *lanes S1*, *S2*, and *S3* respectively. The *lane Nm* shows the proteins remaining in the pellet after the above procedure. Proteins were detected by staining with Coomassie Blue (*A*). An autoradiograph of this gel is shown in *B*. Molecular mass standards for both *A* and *B* are shown on the *left* of the figure.

mevalonate are degraded simultaneously with the proteins to which they are associated, or they are specifically cleaved from them. Previous work (34, 37) indicates that in most cases the former hypothesis is most plausible.

The fact that mevinolin (present during preincubation and labeling) results in stimulation of [^{3}H]mevalonate metabolite incorporation into proteins may be due to effects both on the endogenous pools of precursors for isoprenylation (protein and isoprene) and on protein synthesis itself. Mevinolin has been shown (Fig. 4*B*) to stimulate [^{35}S]methionine labeling of proteins. The reason for this stimulation is, as yet, unknown, and it may represent a genuine induction of protein synthesis. It has previously been demonstrated that certain mevalonate pathway enzymes are up-regulated after mevinolin treatment (38, 39). However, it is possible that the increased incorporation of [^{35}S]methionine into proteins is a consequence of a higher intracellular concentration of the substrate resulting from a mevinolin-induced alteration of cell membrane permeability, since mevinolin may decrease the cellular concentrations of certain membrane components. Thus, it is not yet possible to determine whether the increase observed in [^{3}H]mevalonate incorporation into proteins in the presence of mevinolin (this paper and Ref. 34) is due to stimulation of protein synthesis or effects on isoprene and protein acceptor pools. Neither of these possibilities can be excluded until a method for estimating the intracellular concentration of isoprenes is developed.

It is firmly established by a number of studies that both farnesol and geranylgeraniol can be covalently attached to cysteine residues in proteins by thioether bonds. The results presented here demonstrate that spinach proteins also contain thioether-linked farnesol and geranylgeraniol, as well as additional longer-chain lipids. One of these has been identified as phytol. The occurrence of farnesol and geranylgeraniol modification of proteins is widely accepted and proved using physicochemical methods. However, several longer-chain isoprenes (C_{45}–C_{110}) have been reported as being covalently bound to proteins (15, 17, 40), although the linkage involved was not determined, and the isoprenoid structures have yet to be confirmed. Thus, the posttranslational modification of proteins with isoprenes other than farnesol and geranylgeraniol remains largely unknown but represents an interesting area for future research in all organisms.

It was routinely found that there was a significantly larger amount of geranylgeraniol than farnesol thioether-linked to spinach proteins as has also been shown in other systems, for example Chinese hamster ovary (41) and HeLa (42) cells. In our experiments, methyl iodide hydrolysis always resulted in a ratio of geranylgeraniol:farnesol greater than 2, and longer-chain isoprenes (such as phytol) were also seen, although at a significantly lower abundance. In a previous study (17), the proteins of etiolated spinach seedlings were shown to covalently bind geranylgeraniol:farnesol in a ratio of 0.7. The variation in the levels of these thioether-linked isoprenes is probably a result of the very different developmental state of the seedlings used for the two studies. The methyl iodide hydrolysis results reported for green spinach tissue in this paper are more comparable with those of mammalian cells (41, 43) than etiolated spinach material in that the predominant protein-bound isoprene is geranylgeraniol. We have observed extensive modifications in the pattern of isoprenylated proteins in plants of different physiological condition, as well as during development and aging (this paper and Ref. 17). It appears that isoprenylated proteins may be important regulatory factors in the plant cell, and their amount and type of covalent association with isoprenoids vary depending on cellular requirements. It is also possible that the synthesis of certain isoprenylated proteins is induced by light. We are now analyzing the importance and extent of these alterations in isoprenylation pattern under a variety of conditions.

Upon alkaline hydrolysis, a set of isoprenoid products is released that are not related to those liberated by methyl iodine. The identity of most of these compounds remains to be determined; however, alkaline hydrolysis does appear to release long-chain polyprenols. Thus, it is clear that there are isoprenoid compounds associated with plant proteins via linkages other than thioether. We are presently also investigating whether any of the unidentified compounds detected after alkaline hydrolysis could be isoprenyl cysteines released or isoprenyl peroxides formed during the procedure.

The isoprenylated protein profile found in green spinach tissue shows similarities to those detected in other material. It has been reported previously that many isoprenylated proteins are membrane-associated (7, 18, 44–47), and this is also observed in spinach seedlings. The identity of the labeled bands shown in this paper is not known at the present time. However, it is possible that the band of 68 kDa located in the nuclear matrix represents a plant homologue of nuclear lamins (Fig. 7) (5, 6), and the microsomal band at 24 kDa could be a small GTP-binding protein (Fig. 6) (44, 45, 48, 49). Isoprenylated proteins of 44–69 kDa have previously been reported in the nuclear matrix of murine lymphoma cells (50), and we have demonstrated the occurrence of labeled nuclear matrix proteins in this region. The isoprenylated γ-subunits of heterotrimeric G-proteins have a molecular mass of 5–8 kDa (51–53). As heterotrimeric G-proteins have now been identified in plant microsomes (54, 55), it is most likely that the radiolabeled band at 9 kDa observed in our experiments, unique to the microsomal fraction, corresponds to a plant homologue of mammalian G-protein γ-subunits. The microsomal isoprenylated band of 52 kDa (Fig. 6) may be farnesylated ANJ1 (51 kDa), which was demonstrated to be associated with *Atriplex* microsomal membranes *in vitro* (19). The identity of the isoprenylated proteins in plant mitochondria remains to be established as there are no previous reports of protein isoprenylation in this organelle. However, it is possible that plant mitochondria contain an isoprenylated homologue of DnaJ. A protein (SCJ1) displaying significant homology to this molecular chaperone has recently been identified in yeast mitochondria (56). The deduced amino acid sequence of SCJ1 does not include any recognizable isoprenylation signal sequence, but it is possible that plant proteins contain different motifs directing isoprenylation. Rat liver mitochondria are known to produce large quantities of farnesyl pyrophosphate (57), which may be used for isoprenylation of mitochondrial proteins. However, it cannot, at this point in the investigation, be excluded that some of the bands recovered in the mitochondrial fraction originate from peroxisomes.

The involvement of isoprenylated proteins in a range of cellular functions has previously been shown in animal and yeast systems. Identification of plant homologues to these proteins would be of great interest, and the methodology developed here will be applied to this end in the future. The occurrence of plant-specific isoprenylated proteins has been shown by their occurrence in chloroplasts. Isoprenylated proteins of other cellular compartments may prove to be unique to plants as we have observed many isoprenylated proteins that cannot be accounted for by comparison with labeled proteins identified in other organisms. As the mevalonate pathway is more diverse in plant than in animal cells (35), it may be expected that there are differences in particular aspects of protein isoprenylation in the two systems.

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