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OCCURRENCE OF PRENYLATED PROTEINS IN PLANT CELLS

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In this paper evidence is presented for the occurrence of prenylated proteins in plants. When spinach leaves were incubated in the presence of [^{3}H]mevalonate non-extractable lipids were found in the protein fraction after extraction with organic solvents. Alkaline hydrolysis liberated phytol, polyprenyl phosphates-11-15 and also, in contrast to animal cells, polyprenols-11-15. Complete removal of farnesol and geranylgeraniol required the cleavage of thioether linkages by iodomethane. The results indicate that several polyisiprenoid lipids in plant cells are covalently bound to proteins. So far a protein fraction dominated by one or more proteins in the 23 kDa region has been identified.

In recent years the presence of prenylated proteins in animal cells has attracted much interest. It has been demonstrated that prenylation is one of the basic requirements for regulation of cell growth (1,2) as well as the activation of *ras* oncogenic proteins (3). The sequence of events involved in this process is now well established and some of the prenyltransferases involved have been characterized (4,5). Originally, it was believed that the main or exclusive function of prenylation was to target proteins regulating growth to the cell membrane or the extracellular space and, consequently, that the number of proteins undergoing such modification was limited. However, recent studies have established that in many animal cells as much as 2% of the total protein, corresponding to 40-60 individual proteins per cell, are prenylated (6). It is therefore likely that prenylation is a common event in various cell functions.

Recently we have detected a highly active mevalonate pathway in homogenates and subcellular fractions of spinach leaves, resulting in high rates of synthesis of various lipid end products (7). It was also found that some of these products could not be recovered by lipid extraction. In order to establish whether, and to what extent polyisoprenoid compounds are covalently bound to proteins in plants, we investigated their occurence in spinach leaves after

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metabolic labeling with [^{3}H]mevalonate. These results demonstrate the existence of prenylated proteins in plants.

Materials and Methods

Materials- Polyprenols, used as standards, were prepared as reported earlier (8). All-*trans*-farnesol and all-*trans*-geranylgeraniol were gifts from Dr T. Takigawa of the Kururay Co., Japan. (R,S)[5-^{3}H] mevalonolactone was prepared as described by Keller (9). All other chemicals were purchased from Sigma Chemical Co.(St Louis, MO).

Plant material- Spinach (*Spinacia oleracea*) was grown in the dark for 12-15 days. The leaves were incubated (0.5 g wet weight per 2 ml) in 0.9% solution of sodium chloride containing 1.5 - 2 mCi (R, S)[5-^{3}H] mevalonolactone (7 Ci/mmol) at room temperature for 24 hours.

Extraction- The leaves were then washed and homogenized using Ultra-Turrax blender. The homogenate was filtered and the solid residue were extracted three times with acetone, three times with chloroform/methanol (2:1) , twice with ethanol and once with chloroform/methanol/water (1:1:0.3). The remaining protein pellet was solubilized in 50 mM Tris-Cl, pH 7.0, containing 2% SDS.

Hydrolytic treatments- Acid and alkaline hydrolysis and iodomethane treatment were performed as described earlier (10,11) Lipids were extracted with cholorform: methanol: water (1:1:0.3) when polar and nonpolar lipids were to be separated on DEAE-Sephadex. In the case of analysis of total lipids butanol was used for extraction. For enzymatic dephosphorylation the method of Wong and Lennarz (12) was employed.

Electrophoresis- SDS/PAGE was performed according to Laemmli (13). The gel was impregnated with AMPLIFY (Amersham, England) and the dried gel was placed against a x-Omat AR Film at -80° C for 4 weeks. Protein was determined by the Lowry procedure (14).

Lipid analysis- The lipids were analyzed by reversed-phase HPLC using a C-18 column (Hewlett Packard Hypersil ODS 3 μm). A gradient of 100% methanol:water (7:3) (solvent A) to 100% methanol:water (9:1) (solvent B) was run for 30 min with a flow rate of 1.5 ml/min. For the resolution of long-chain polyprenols solvent A was methanol:water (9:1) and solvent B was methanol:isopropanol:hexane (2:1:1) and the other conditions were unchanged. The absorption of the eluate was monitored at 210 nm and radioactivity was measured with a radioactivity flow detector (Radiomatic Instruments, Tampa, Fl).

All experiments given in this paper were repeated 6 - 9 times and the values in the tables represents the mean values.

Results

Labeling of spinach lipids- Upon incubation of spinach cotyledon leaves for 24 h in a medium containing [^{3}H]mevalonate, a high rate of incorporation of radioactivity was observed in various lipids including sterols, a-tocopherol, squalene, ubiquinone and plastoquinone as well as phytol (Table 1). Labeling appeared also in farnesol, geranylgeraniol, polyisoprenols and polyisoprenyl phosphates; the latter two compounds were present as several homologues with 11-15 residues. After extensive lipid extraction, a small portion, approximately 1% of the phytol, and a larger fraction of the polyprenols, polyprenyl phosphates, farnesol and geranylgeraniol, 20-30% of each, remained associated with protein.

Analysis of bound lipids- In order to analyze the protein-lipid interactions and to ascertain the covalent nature of the binding, the residue obtained after lipid extraction was subjected to alkaline hydrolysis and re-extraction. This extract was resolved by DEAE-Sephadex chromatography into nonpolar and polar fractions. Upon HPLC of the nonpolar

Table 1. *Lipids non-extractable from spinach homogenate metabolicaly labeled with [^{3}H]mevalonate*

Lipid	Total	Non-extractable	
	(dpm/mg protein)		(% of total)
Sterols and a-tocopherol	750,000	nd	
Squalene	207,000	nd	
Phytol	124,000	1,670	1.3
Ubiquinone and plastoquinone	54,000	nd	
Polyprenols	5,370	1,510	28
Polyprenyl-P	640	210	33
Farnesol	424	81	19
Geranylgeraniol	308	98	32

Total lipids were recovered by lipid extraction after alkaline hydrolysis. Non-extractable lipids denote the lipid fraction recovered after alkaline hydrolysis of the protein fraction previously extracted as described in Materials and Methods. The radioactivity in the lipid fractions obtained by HPLC was determined using a radioactivity flow detector. nd = not detectable.

fraction, peaks containing farnesol + geranylgeraniol, phytol and polyprenols were revealed (Figure 1A). In addition there were some minor peaks, which were not identified. The insert in Fig. 1A illustrates the pattern obtained with a different HPLC system, more appropiate for the

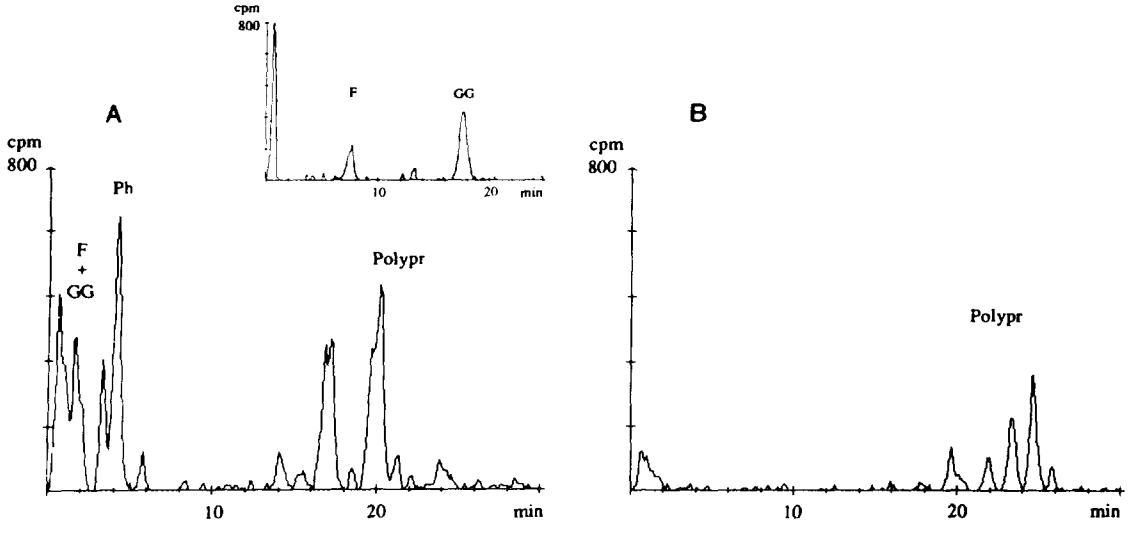

Fig 1. *HPLC separation of the non-extractable lipids in immature spinach leaves.*
After extensive lipid extraction, the proteins were subjected to alkaline hydrolysis and the lipids released separated on DEAE Sephadex. All isoprenoids shown were identified by using standards. A: non-polar lipids, B: polar lipids. The insert shown in A illustrates the HPLC separation used for the identification of short-chain polyprenols. F = farnesol, GG = geranylgeraniol, Ph = phytol, Polypr=polyprenols

resolution of shorter polyprenols. The compounds eluted at 7 and 17 minutes were identified as farnesol and geranylgeraniol, respectively. The polar portion of the lipid extract after alkaline hydrolysis of the protein fraction contained polyprenyl phosphates (Figure 1B).

Amount of bound lipid- Quantitation of the products released from the lipid-extracted proteins by various treatments revealed that acid hydrolysis liberated essentially one product,i.e., phytol (Table 2). Alkaline hydrolysis released a larger amount of phytol, all the polyprenols and polyprenyl phosphates and a portion of the farnesol and geranylgeraniol. Iodomethane treatment was required for complete liberation of the two latter compounds. These results show that the various lipid compounds are covalently bound to proteins and that in the case of farnesol and geranylgeraniol, a thioether linkage is involved.

SDS-PAGE of proteins- In order to identify the protein(s) carrying radioactive lipids, SDS-PAGE and subsequent autoradiography of the lipid extracted proteins were performed. The main band associated with labeled lipid had an apparent molecular weight of appoximately 23 kDa (Figure 2). This band was broad and it is possible that it contains several closely migrating prenylated proteins. It cannot be excluded that also additional bands, containing less radioactivity, may have been revealed by using a longer exposure time during autoradiography.

Discussion

In recent years it has been demonstrated that a number of proteins in animal cells can be modified co- or post-translationally by covalent attachment of lipids, including myristic and palmitic acid, phosphatidylinositol, dolichyl phosphate, farnesol and geranylgeraniol (15,16). Both the types of lipids known to be involed and the number of proteins containing covalent linkages are increasing continuously, and there is growing evidence that these modifications play an important role in the regulation of the control of cell growth and signal transduction (17).

Table 2. *Chemical treatments to release lipids from the protein fraction*

Treatment	Lipid removed (dpm/mg protein)				
	Farnesol	Geranyl-geraniol	Phytol	Poly-prenols	Poly-prenyl-P
Acid hydrolysis	0	0	660	41	5
Alkaline hydrolysis	81	98	1580	212	31
Iodomethane	302	205	21	0	0

After sequential lipid extraction the proteins were subjected to various hydrolytic treatments and the products identified by HPLC.

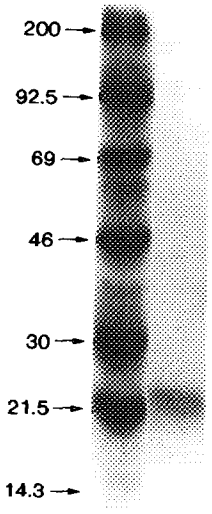

Fig 2. *SDS-gel electrophoretic pattern of the proteins associated with non-extractable [^{3}H]labeled lipids.*
The radioactivity was detected by autoradiography. The standards employed are marked with arrows.

Plant tissues have not yet been investigated with respect to modification of proteins by prenylation. In the present study both farnesol and geranylgeraniol were found to be linked to protein in spinach leaves and, similar to animal cells, the binding involved is a thioether linkage. Likewise, one-third of the labeled polyprenyl phosphates in spinach is associated with proteins. The type of interaction and the influence of this association on glycoprotein synthesis remain to be determined. However, it appears that in plants more mevalonate-derived lipids are covalently bound to proteins compared to animal cells. It appears from our data that the prenylated proteins contain not only farnesol and geranylgeraniol but also longer polyisoprenes (11-15 isoprene units). It is conceivable that similar, longer-isoprene containing proteins will be found in the future also in animal tissues. Since only a small fraction (1.3%) of the phytol was not extractable, the identification of possible phytol-binding protein(s) will require further investigation.

In the case of plants, the only previously known example of the involvement of lipid in posttranslational protein modification is the palmitoylation of the chloroplast D1 protein of photosystem II (18). The occurance of prenylation of plant proteins has not yet been demonstrated. Interestingly, the prenylated protein fraction described in the present study has an approximate molecular weight of 23 kDa similar to that found in animal cells (17). Whether this fraction consists of one or several proteins and whether there are prenylated proteins in other molecular weight ranges, not revealed by the labeling technique used here, remain to be investigated.

Taken together, the present data demonstrate the occurance of prenylated proteins in plant cells and their modification during maturation, indicating that processes responsible for plant cell growth may be regulated by proteins that are activated by prenylation.

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References

1. Farnsworth, C.C., Wolda, S.L., Gelb, M.H., and Glomset, J.A. (1989) J. Biol. Chem.264, 20422-20429.
2. Maltese, W.A. and Sheridan, K.M. (1990) J. Biol. Chem.265, 17883-17890.
3. Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989) Cell 57, 1167-1177.
4. Reiss, Y., Goldstein, J.L., Seabra, M.C., Casey, P.J., and Brown, M.S. (1990) Cell 62, 81-88.
5. Yokoyama, K., Goodwin, G.W., Ghomashchi, F., Glomset, J.A., and Gelb, M.H. (1991) Proc. Natl. Acad. Sci. U. S. A.88, 5302-5306.
6. Epstein, W.W., Lever, D., Leining, L.M., Bruenger, E., and Rilling, H.C. (1991) Proc. Natl. Acad. Sci. U. S. A.88, 9668-9670.
7. Swiezewska, E., Dallner, G., Andersson, B., and Ernster, L. (1993) J. Biol. Chem. 268, 1494-1499.
8. Chojnacki, T., Jankowski, W., Mankowski, T., and Sasak, W. (1975) Anal. Biochem.69, 114-119.
9. Keller, R.K. (1986) J. Biol. Chem.261, 12053-12059.
10. Thelin, A., Löw, P., Chojnacki, T., and Dallner, G. (1991) Eur. J. Biochem.195, 755-761.
11 Maltese, W.A. and Erdman, R.A. (1989) J. Biol. Chem.264, 18168-18172.
12. Wong, T.K., Decker, G.L., and Lennarz, W.J. (1982) J. Biol. Chem.257, 6614-6618.
13. Laemmli, U.K. (1970) Nature227, 680-685.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem.193, 265-275.
15. Sinensky, M. and Lutz, R.J. (1992) Bioessays14, 25-31.
16. Chow, M., Der, C.J. and Buss, J.E. (1992) Curr. Opin. Cell Biol. 4, 629-636.
17. Maltese, W.A. (1990) FASEB. J.4, 3319-3328.
18. Mattoo, A.K. and Edelman, M. (1987) Proc. Natl. Acad. Sci. U. S. A.84, 1497-1501.