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Ras protein of the slime mold *Physarum polycephalum* is farnesylated *in vitro*°

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***Physarum* Ppras1 protein was efficiently prenylated by prenyltransferases of spinach. Surprisingly in spite of the C-terminal sequence (CLLL) specific for geranylgeranylation the protein was preferentially farnesylated. Consequences of this observation are discussed.**

Posttranslational modification of proteins *via* covalent linkage of isoprenoid groups is a well-known cellular event occurring in a wide range of eukaryotes [1, 2] including plants [3]. It is required for membrane targeting, protein–protein interactions, and biological activity of key regulatory proteins, such as Ras small GTPases. There are three enzymes catalyzing this process: the best known, farnesyl protein transferase (FTPase), and geranylgeranyl protein transferase I and II (GGPTase I and GGPTase II). Farnesyl protein trans-

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Abbreviations: FPTase, farnesyl protein transferase; GGPTase I, geranylgeranyl protein transferase I; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPTG, isopropyl β-D-thiogalactopyranoside.

ferase (FPTase) catalyses the modification of cysteine with a farnesyl group transferred from FPP while the two latter enzymes transfer a geranylgeranyl group from geranylgeranyl diphosphate (GGPP) to cysteine located at (or close to) the C-terminus of the protein. The requirement for a specific amino-acid sequence of the substrate for all these enzymes is different. Substrates modified by either FPTase or GGPTase I end with a -CaaX box at the C-terminus while GGPTase II substrates with a -aCaC or -aaCC sequence where C stands for cysteine, a – denotes an aliphatic amino acid and X - specific amino acid. Cross-specificity of FPTase and GGPTase I has been showed [4] although proteins with X = Ser, Met, Ala or Gln are processed mainly by FPTase while Leu at this position directs the modification mostly by GGPTase I. Such a capacity to accomodate alternate substrates may be of biological significance providing the prenylated products for the cells mutated in the essential genes coding for FPTase or GGPTase I subunits [5]. In all cases studied so far the enzymes were activated by divalent cations (Zn^{2+} and Mg^{2+}). FPTase has been purified and cloned from yeast and mammalian cells [6, 7]. Recently both α and β subunits of this heterodimeric enzyme have been cloned also from plant cells, i.e. from garden pea [8, 9] and *Arabidopsis* [10]. Mammalian FTPase is a subject of numerous of studies because farnesylation of Ras proteins promotes their membrane association thus enabling the function of a signal transduction pathway. The biological role of FPTase has also been studied in plant cells. It has been suggested that FTPase is required for the plant cell cycle by modulating the transition from G_1 to the S phase and progression through the S phase [8]. There are also indications for a key role for FTPase in abscisic acid (ABA) signaling or ABA-mediated growth regulation [10]. A potential function in the regulation of nutrient transport or allocation was also postulated [11]. According to our previous studies plant FPTase recognizes nonapeptide as an acceptor [12] but small molecules like dithiothreitol (DTT) were also found to serve as a substrate [13]. Upon an *in vitro* assay spinach FPTase transfered diverse isoprenoid groups from the corresponding diphosphates to the nonapeptide acceptor but as expected farnesyl and geranylgeranyl groups were the best substrates [12].

In contrast to FPTase the other two prenyltransferases have not been studied in plants. The occurrence of GGPTase I activity was postulated in the cell extract of *Atriplex nummularia* [14]. Recent data show that not only the C-terminal tetrapeptide but also the upstream internal sequence of the protein are important for the specificity of transferase [15]. Thus it seemed interesting to use a recombinant Ras protein for the characterization of prenyl transferases of spinach. The results of these experiments show that the Ras protein is recognized as a substrate by both FPTase and GGPTase I.

MATERIALS AND METHODS

Plant material. Spinach (*Spinacia oleracea* L) seedlings were grown in darkness for 12 days at 21°C. Whole cotyledons were homogenized in 0.25 M saccharose in 50 mM Tris/HCl (pH 7.5) with an Ultra-Turrax homogenizer three times for 5 s. Subfractionation was performed as described previously [12].

Ras protein. Ppras1 protein, Swiss-prot: P34729, (CLLL terminal sequence) was obtained by overexpression of Ppras1 cDNA [16] in the bacterial system QIAexpress (Qiagen). The expression vector pQE40 introducing a His_6 oligopeptide at the N-terminus of the Ppras1 protein, inducible with IPTG in *Escherichia coli* strain XL1-Blue MRF', was used for the production of the hybrida protein. Cells were suspended in a buffer (300 mM NaCl, 0.1 mM PMSF, 50 mM Na_2HPO_4, pH 7.8), and homogenized by freezing (-80°C) and thawing;

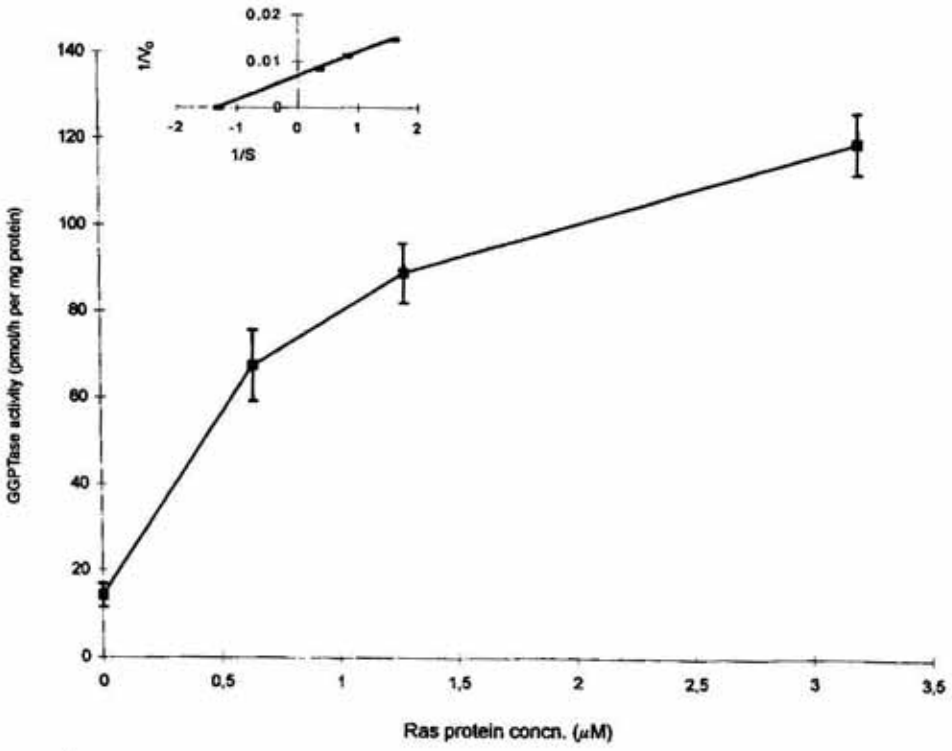

Figure 1. The influence of Ras concentration on GGPTase I activity.

Data are the mean of three experiments. The inset illustrates the Lineweaver-Burk plot.

the lysate was centrifuged (12000 × *g*, 15 min) and loaded onto a Ni-NTA-agarose column. Ppras1 was eluted with a buffer: 300 mM NaCl, 10% glycerol, 0.1 mM PSMF, 50 mM Na_2HPO_4, pH 4.3.

GGPTase I assay. The assay was performed as described previously [12], with some modifications. Recombinant Ras protein instead of the nonapeptide was used as an acceptor. Enzymatic reaction after 60 min was stopped by adding 10% HCl in ethanol. After overnight precipitation at 4°C the mixture was transferred to a Whatmann G filter and then washed carefully first with 2 × 5 ml of 10% HCl in ethanol and then with pure ethanol. Filters were transferred to scintillation vials, dried at room temperature and scintillation counted using Bray cocktail [17]. Protein content was determined by the Bradford method using bovine serum albumin as a standard [18].

Reagents and chemicals. All-*trans* [^{3}H]FPP and [^{3}H]GGPP were obtained from Collection of Polyprenols. All other chemicals were of analytical grade and were used without further purification.

RESULTS AND DISCUSSION

In a typical experiment a crude cytosolic fraction from spinach leaves was used as a source of the enzyme. Formation of isoprenylated Ras protein was determined by incubating the acceptor with tritium labeled isoprenoid diphosphate. Isoprenylated protein was efficiently formed in our *in vitro* system. The amount of the product was linear within 60 min of incubation. Substrate curve for Ras is shown in Fig.1 indicating a low K_m value (approximately 0.74 μM). Both Mg^{2+} and Zn^{2+} were required for the activity of prenyl transferases, optimal concentrations (20 mM and 40 μM, respectively) were used in the assay. When short chain isoprenoid diphosphates, FPP and GGPP, known to modify proteins covalently were compared in our assay as donors of isoprenoid groups both

were found to act as substrates. Surprisingly, farnesylated protein was formed 3 times more effectively than the geranylgeranylated one (Table 1).

Table 1. Influence of isoprenoid diphosphate on GGPTase activity.

Incubations were performed as in Materials and Methods

Type of isoprenoid donor	GGPTase activity (pmol/h per mg protein)
FPP	135.3 ± 13.1
GGPP	47.1 ± 5.7

Proteins with the -CaaL motif are known to be substrates for GGPTase I, and thus are geranylgeranylated. Therefore the above striking results are difficult to explain at the moment. One possible reason is a complex experimental system, as the crude cytosol used as the enzyme source contained all three prenyl transferases. However, cross-specificity of both enzymes has been documented [4] and the activity ratio with the two substrates (Table 1) is too high to be explained by the use of a crude extract. Also one could speculate about the differences in the relative levels of FPTase and GGPTase I activities in spinach cells. The rate of Ras farnesylation highly exceeding that of geranylgeranylation suggests that in our experiments FPTase activity could be much higher than that of GGPTase I.

According to [14] the ANJ1 protein with the C-terminal sequence -CAQQ was found to be farnesylated and to some extent geranylgeranylated. Substitution of leucine for the terminal glutamine in the -CAQQ sequence abolished any geranylgeranylation of this protein and also lowered the level of farnesylation of the mutant protein. Besides, it is worth noting that structural elements upstream of the carboxyl-terminal -CaaX motif have been shown to play a role in the recognition of the substrate protein by mammalian and insect FPTases [15]. Basing on the literature data and our results it might be concluded that the specificity of isoprenylation (i.e. farnesylation *versus* geranylgeranylation) is not strictly determined by the last amino acid in the -CaaX box. This should be further explored with pure enzymes. However, up to now no plant prenyltransferases has been purified to homogeneity. A description of the properties of prenyl transferases seems very important for the characterization of regulation of plant cell metabolism.

REFERENCES

1. Clarke, S. (1992) *Annu. Rev. Biochem.* **61**, 355-386.
2. Casey, P.J. & Seabra, M.C. (1996) *J. Biol. Chem.* **271**, 5289-5292.
3. Swiezewska, E., Thelin, A., Dallner, G., Andersson, B. & Ernster, L. (1993) *Biochem. Biophys. Res. Commun.* **192**, 161-166.
4. James, G.L., Goldstein, J.L. & Brown, M.S. (1995) *J. Biol. Chem.* **270**, 6221-6226.
5. Trueblood, C.E., Ohya, Y. & Rine, J. (1993) *Mol. Cell Biol.* **13**, 4260-4275.
6. Zhang, F.L. & Casey, P.J. (1996) *Annu. Rev. Biochem.* **65**, 241-269.
7. Caldwell, G.A., Naider, F. & Becker, J.M.. (1985) *Microbiol. Rev.* **59**, 406-422.
8. Qian, D., Zhou, D., Ju, R., Cramer, C.L. & Yang, Z. (1996) *Plant Cell* **8**, 2381-2394.
9. Yang, Z., Cramer, C.L. & Watson, J.C. (1993) *Plant Physiol.* **101**, 667-674.
10. Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. & McCourt, P. (1996) *Science* 273, 1239-1241.
11. Zhou, D., Qian, D., Cramer, C.L. & Yang, Z. (1997) *Plant J.* **12**, 921-930.
12. Skoczylas, E. & Swiezewska, E. (1996) *Biochimie* **78**, 139-143.

13. Parmryd, I., Shipton, C., Swiezewska, E., Andersson, B. & Dallner, G. (1996) *Eur. J. Biochem.* **234**, 723–731.

14. Zhu, J.-K., Bressan, R.A. & Hasegava, P.M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8557–8561.

15. Kalman, V.K., Erdman, R.A., Maltese, W.A. & Robishaw, J.D. (1995) *J. Biol. Chem.* **270**, 14835–14841.

16. Kozlowski, P., Fronk, J. & Toczko, K. (1993) *Biochim. Biophys. Acta* **1173**, 357–359.

17. Bray G.A. (1960) *Anal. Biochem.* **1**, 279–285.

18. Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–254.