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Characterization of Small GTP-Binding Proteins in Plant Cell

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To identify and characterize small GTP-binding proteins in plant cells, GTP-binding studies were performed with electroblotted plant proteins following SDS-polyacrylamide gel electrophoresis using [α-^{32}P]GTP. Three species of small GTP-binding protein (21, 23, and 27 kD) which have a specific GTP-binding property were identified in the membrane and cytosolic fractions of both monocotyledons (*Zea mays*) and dicotyledons (*Glycine max*). Moreover, these three species of small GTP-binding protein were gradually decreased when membranes were treated with hydroxylamine. This result indicates that these small GTP-binding proteins in plant cells are fatty acylated to the membrane lipids. The 27 kDa component was partially purified from hypocotyl membranes of Glycine *max*, following S-300 gel filtration, phenyl-sepharose CL-4B, hydroxyapatite, and Q-sepharose column chromatography. This 27 kD protein was found to have both GTP-binding and GTPase activities.

Keywords: small G-protein, fatty acylation, GTPase

A large numbers of proteins, so called GTPase superfamily which have a common structural motif to turn on by binding GTP and off by hydrolyzing GTP to GDP have been identified in a wide ranges of organisms (Bourne *et al.*, 1991). These proteins are involved in various cellular events including sorting and amplifying transmembrane signals, directing the synthesis and translocation of proteins, guiding vesicular traffic through the cytoplasm, and controling proliferation and differentiation of organisms (Downward, 1990). Among them two classes of regulatory GTP-binding proteins (G protein) have been intensively studied. One class comprises the functionally well characterized heterotrimeric G proteins that transduce receptor-mediated signals across the plasma membrane to specific effector systems such as adenylyl cyclase (Gilman, 1987; Tang and Gilman, 1992) and phospholipase Cβ (Sekar and Hokin, 1986; Michell, 1992). These G proteins are consisting of α, β, and γ subunits, of which α-subunits (39-46 kDa) bind and hydrolyze GTP (Birnbaumer, 1990; Bourne *et al.*, 1991).

The second class of G proteins are the so-called small G proteins, which are monomeric proteins of Mr. 20~28 kDa. More than 60 small G proteins have been characterized by molecular cloning of their genes in a variety of eukaryotes (Downward, 1990; Bourne *et al.*, 1991; Hall, 1993). Based on their sequence homology, these small G proteins are classified as four families, Ras, Rho, Rab, and Arf (Downward, 1990). Proteins in the Ras family have been implicated in the signal transduction that is related to cell proliferation and terminal differentiation (Bollag and McCormick, 1991; Lowy and Willumsen, 1993), while Rho family proteins were known to control the focal adhesion formation and membrane ruffling in animal cells, and the cell polarity in yeast (Ridley and Hall, 1992; Chant and Stowers, 1995). The Rab and Arf family proteins were largely involved in the variety of vesicle transport inside of the cell (Goud and McCaffrey, 1991; Orci *et al.*, 1993).

Several small GTP-binding proteins were cloned and identified in plant cells (Kamada *et al.*, 1992; Ma, 1994; Sano *et al.*, 1994). Most of these small GTP-binding proteins belong to Rab subfamily. Recently Rho family small GTP-binding proteins were also cloned in plants (Yang and Watson, 1993). However, roles of the these small GTP-binding proteins in plant are largely unknown.

In this report we studied biochemical characteristics of the small GTP-binding proteins in plant at protein level.

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MATERIALS AND METHODS

Materials

[α-^{32}P]GTP (specific activity, 3000 Ci/mmol) and [γ-^{32}P]GTP (specific activity, 30 Ci/mmol) were purchased from New England Nuclear, Sephacryl S-300, Phenyl-sepharose CL-4B, and Q-sepharose were obtained from Pharmacia, and Hydroxyapatite was obtained from Bio-Rad.

Membrane Preparation

A membrane fraction from plant tissues was prepared as described previously (Sussman *et al.*, 1980). The supernatant was used as a cytosolic fraction.

Identification of the GTP-binding Proteins in Nitrocellulose Blot

Proteins from the both cytosol and membrane were fractionated in 10 % SDS-polyacrylamide gel, then electrophoretically transferred to the nitrocellulose membrane. This membrane was incubated in the GTP-binding buffer (50 mM Tris-Cl, pH 7.0) including 0.3% Tween 20, 5 mM $MgCl_2$, 1 mM EGTA, and 1 μCi [α-^{32}P]GTP for 2 hrs. at 25 °C, then washed three times with the ice-cold GTP-binding buffer which does not contain GTP. The washed paper was dried and exposed to a X-ray film for 3~5 days.

Purification of the Small GTP-binding Proteins

Purification of the small GTP-binding proteins in plant cells from soybean hypocotyl was followed the schemes developed by Takai *et al.* (1988, 1989).

[α-^{32}P]GTP-binding Assay

GTP-binding assay developed by Takai *et al.* (1988) was used for the identification of small GTP-binding proteins from the column chromatography fractions. Briefly, 10 μl of each column chromatography fraction was incubated with 100 μl of GTP-binding buffer containing 20 mM HEPES (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.8 M NaCl, 6 mM $MgCl_2$, 0.1% Lubrol-PX, and 1 μCi [α-^{32}P]GTP for 1 h at 4°C. The reaction was terminated by addition of 2 ml ice-cold binding buffer containing 5 μM GTP. The samples were filtered nitrocellulose paper and washed three times with same buffer. The filtered papers were air dryied, and radioactivity was measured using liquid scintillation counter.

Measurement of GTPase Activity

GTPase activity in the purified fractions was measured by the method established by Brandt *et al.* (1983) using [γ-^{32}P]GTP.

RESULTS AND DISCUSSION

To identify and characterize the small GTP-binding proteins in plant cells GTP-binding ability was examined with [α-^{32}P]GTP using the electroblotted proteins derived from the both cytosolic and membrane fractions of the corn coleoptiles and soybean hypocotyls after SDS-polyacrylamide gel electrophoresis. Two major small GTP-binding components (27, 23 kD) always appeared in the both cytosolic

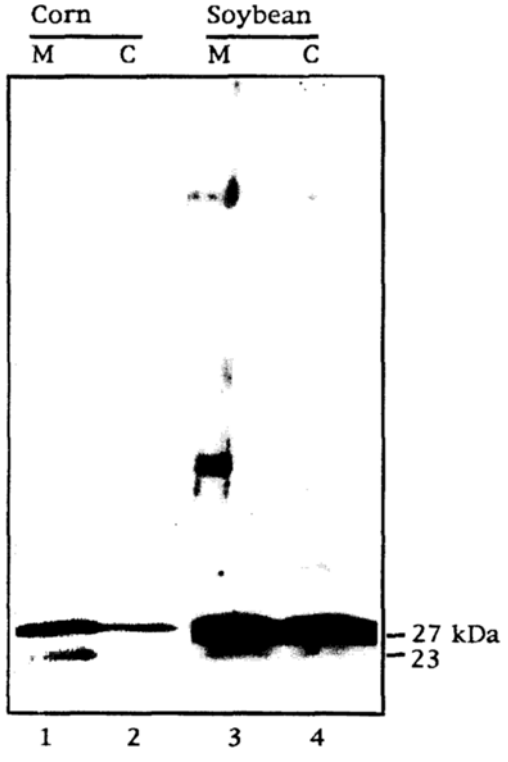

Fig. 1. Autoradiogram of the [α-^{32}P]GTP-binding proteins in plant cells. Proteins were analyzed electrophoretically on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose paper. The nitrocellulose paper was used for GTP-binding study in the presence of [α-^{32}P]GTP as described in Materials and Methods. Lane 1 and 2 contained corn coleptile membrane and cytosolic protein, while lane 3 and 4 contained bean hypocotyl membrane and cytosolic proteins respectively.

(Fig. 1, lanes 2 and 4) and membrane (Fig. 1, lanes 1 and 3) fractions from corn coleoptiles (Fig. 1, lanes 1 and 2) and soybean hypocotyls (Fig. 1, lanes 3 and 4). However, another small GTP-binding protein (21 kD) was identified when the paper was exposed longer period or large amount of proteins were loaded for SDS-PAGE (Fig. 3).

To address whether the three small GTP-binding proteins identified in Fig. 1 specifically bind to GTP, we performed the competitive binding experiments using the GTP-binding buffer which has excessive amount of cold nucleotides. When GTP (5 μM) was added to the GTP-binding buffer, the [α-^{32}P]GTP binding to the 27 and 23 kD components was completely abolished (Fig. 2, compare lane 1 to lane 3). The [α-^{32}P]GTP binding ability to these proteins was inhibited by GDP but other nucleotides such as ADP and AMP did not change this binding ability (data not shown).

In order to compare the chracteristics of GTP-binding proteins in plants with those characterized already, we used membrane proteins of the F9 teratocarcinoma cell for the binding of [α-^{32}P]GTP (Fig. 2, lanes 2 and 4). Two GTP-binding components (25 and 23 kD) were appeared in this cell membrane (lane 4). Either longer exposure of the autoradiogram or large amount of proteins revealed another GTP-binding component (21 kD) like plant tissue (data not shown). The [α-^{32}P]GTP binding ability to these components also abolished by excessive amount of cold GTP (compare lane 2 to lane 4).

These results indicate that the small GTP-binding proteins identified using [α-^{32}P]GTP has a specific binding ability to GTP/GDP. Moreover, identification of these small GTP-binding proteins in both cytosolic and membrane fractions of plant tissue suggests that these proteins also shuttle back and forth from the membrane to cytosol to regulate their activities as observed in animal tissues (Taparowsky *et al.*, 1983; Willumsen *et al.*, 1984; Fukui and Kaziro, 1985). Although a number of small GTP-binding proteins were identified in a variety of plants, we only identified only three species of small GTP-binding proteins using GTP-binding protocol. This limitation of identification of other small-GTP binding proteins is likely in part due to molecular weight between each small GTP-binding protein is relatively similar. Oth-

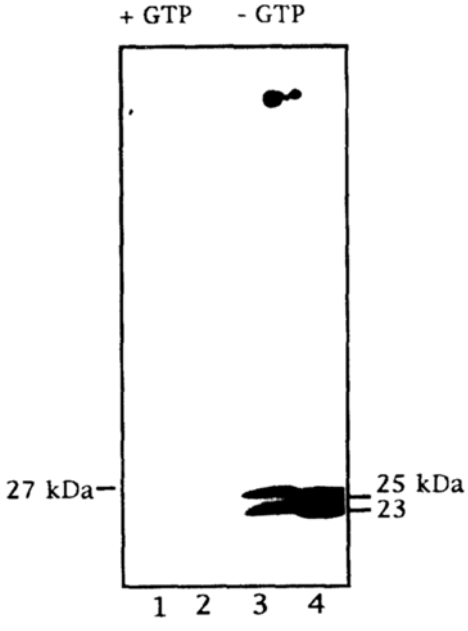

Fig. 2. Effect of GTP on the binding of [α-^{32}P]GTP to membrane proteins. Proteins were separated on a 10% SDS-polyacrylamide gel. Nitrocellulose blots were incubated with [α-^{32}P]GTP either in the presence of 5 μM GTP (lanes 1, 2) or in the absence of GTP (lanes 3, 4). Lanes 1 and 3 containd corn coleptile membrane, while lanes 2 and 4 contained F9 teratocarcinoma cell membrane.

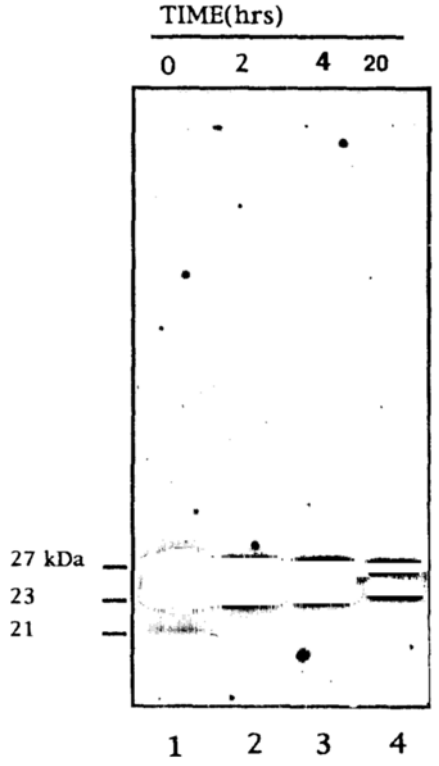

Fig. 3. Effect of hydroxylamine on the small GTP-binding proteins bound to plant cell membranes. Corn coleptile membranes were incubated with 1 M NH_2OH for 0 (lane 1), 2 (lane 2), 4 (lane 3), and 20 hrs (lane 4).

erwise, the method we used is not sensitive enough to identify small amount of this family of protein.

Most of the small GTP-binding proteins in the membranes are known to fatty-acylated to the membrane lipids (Willumsen *et al.*, 1984; Fujiyama and Tamanoi, 1986). To test whether the small GTP-binding proteins in the membrane fraction were fatty-acylated, the membranes were incubated in the 1 M hydroxylamine solution for the different periods, then used for the GTP binding study. The results in Fig. 3 demonstrate that the amount of [α-^{32}P]GTP bound to the all three components decreased in proportion to the increase of incubation time of the membrane in hydroxylamine solution (Fig. 3. compare lane 1 to lanes 2, 3, and 4). To measure the decreased amount of [α-^{32}P]GTP binding to each component after hydroxylamine treatment, the results of the Fig. 3 were analyzed using a densitometer. Almost 60% and 50% of the 27 kD and 23 kD components respectively was released from the membrane which incubated in hydroxylamine solution for 20 hrs.

In addition, the 21 kD was disappeared after 2 hrs incubation of the membrane in hydroxylamine solution. These results indicate the three small GTP-binding proteins in the membrane of plant cells are fatty-acylated to the membrane lipids.

In addition to the GTP binding ability and fatty-acylation to the membrane lipids, the small GTP-binding proteins have a GTPase activity (Downward, 1990; Wittinghofer and Pai, 1991). To examine whether these small GTP-binding proteins have a GTPase activity, the 27 kD component was purified from the membrane fraction of soybean hypocotyl using Sephacryl S-300, Phenylsepharose CL-4B, Hydroxyapatite, and Q-sepharose column chromato-

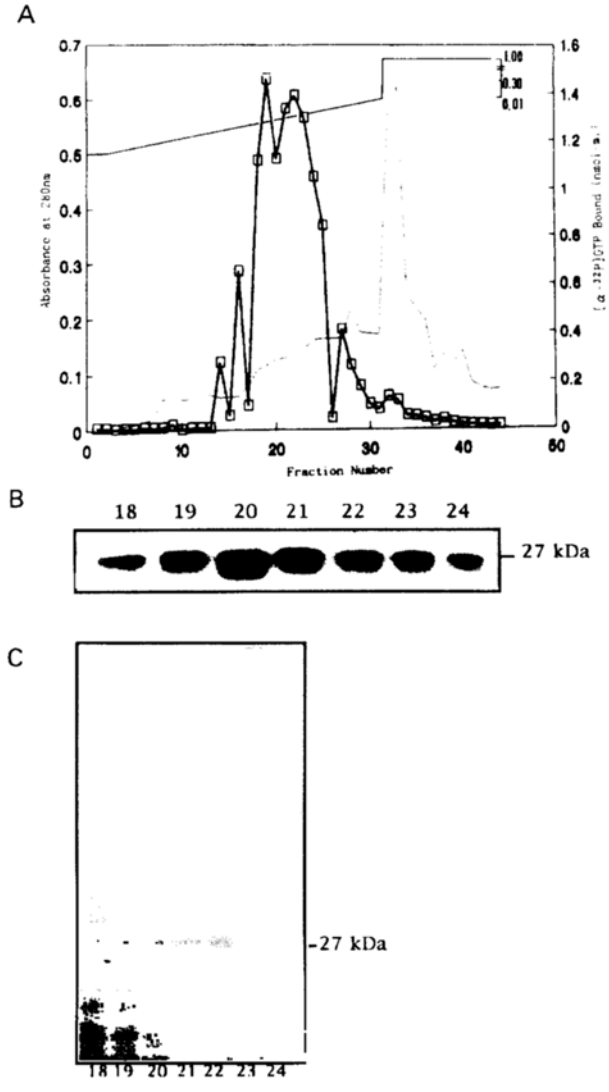

Fig. 4. Results of Q-sepharose column chromatography. A: Protein profile and GTP-binding results. A 20 μl aliquot of each fraction was assayed for [α-^{32}P]GTP-binding activity (rectangles). The amount of protein was determined by absorbance at 280 nm (dotted line). A straight line indicates the concentration of NaCl (M). B: [α- P]GTP-binding results from fractions 18~24. A 20 μl aliquot of each fraction was fractionated on a 10% SDS-polyacrylamide gel, transfered to a nitrocellulose membrane, then GTP-binding was performed as described in Material and Methods using [α-^{32}P]GTP. C: Protein profiles of the fraction 18~24. A 20 μl aliquot of each fraction was analyzed on a 10% SDS-polyacrylamide gel, then stained with siver nitrate.

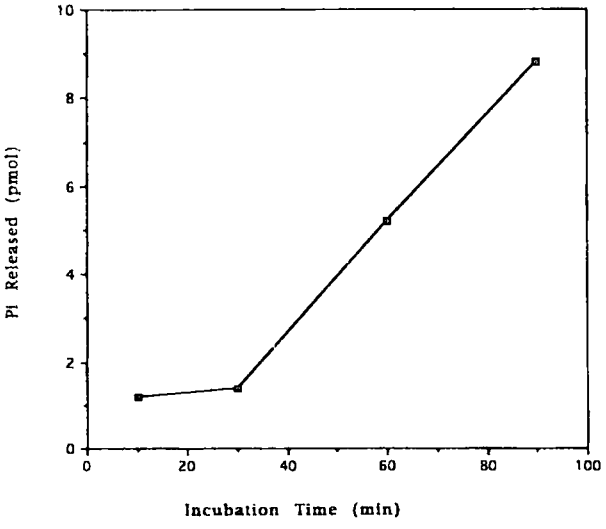

Fig. 5. Time course of the GTPase activity of 27 kDa protein. The GTPase activity of 5 mg of 27 kDa protein was assayed with 1 μM [γ-^{32}P]GTP as described in Materials and Methods for different periods of time at 30°C.

graphies. The results in Fig. 4A demonstrate the protein profile and GTP-binding activity in Q-sepharose fractions. The GTP-binding components were mainly released from the fraction 18 to 26. These fractions were analyzed using SDS-PAGE, then the gel was silver stained (Fig. 4C) to examine purified proteins. The 27 kD appeared as a major component in these fractions and several minor lower molecular weight protein components were also identified. These fractions were also tested the [α-^{32}P]GTP binding ability after electroblotting (Fig. 4B). The only one GTP-binding component (27 kD) which was shown as in Fig. 4B was identified in these fractions. During the purification of 27 kD component other GTP-binding proteins such as 23 and 21 kD was lost due to we chased a major GTP-binding fractions by [α-^{32}P]GTP binding assay in each fraction using nitrocelluose filteration method not in the electroblotted samples.

The GTPase activity in this purified 27 kD was measured using [γ-^{32}P]GTP. The release of the ^{32}P-labeled phosphate was increased time dependently (Fig. 5). This result indicates that the 27 kD component has a GTPase activity.

These results indicate that three GTP-binding components identified in this study truely a member of small GTP-binding proteins. However, the identity of each component and the role for these proteins in the plant cell were remained to be identified.

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