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Co-purification of Plasma Membrane ATPase and Phosphatidylinositol Kinase from Pea Plasma Membranes

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The plasma membrane ATPase was partially purified by a linear glycerol density gradient centrifugation of the detergent-solubilized plasma membrane proteins and subsequent separation by a size-exclusion column chromatography. A purified ATPase preparation is shown to contain a 97.6kDa protein that was cross-reacted with an antibody raised against mung bean H^+-ATPase. The preparation also exhibited the phosphorylation of exogenous phosphatidylinositol (PI) when supplied with [γ-^{32}P]ATP. These results indicate that one form of plasma membrane ATPase is co-purified with PI kinase.

Key words : Lipid kinase, *Mycosphaerella pinodes*, plasma membrane ATPase, pea (*Pisum sativum* L.), suppressor

Introduction

In plant cells as well as mammalian cells, PI metabolism has been shown to be implicated in transduction of diverse extracellular signals such as plant hormones[26], light[10,11], hypoosmotic stress[5] and fungal elicitors[7,9,18,21,22], and the components of the pathway are ubiquitously present. This pathway involves sequential phosphorylation of phosphatidylinositols by lipid kinases and subsequent hydrolysis of PIP_2 by inositol phospholipid-specific PLC, resulting in production of two functional second messengers such as IP_3 and DAG[2]. In our previous studies[18,21,23], we showed that significant increases in levels of IP_3 and DAG were induced within a few minutes after the start of elicitor-treatment of pea epicotyls, then transcripts markedly accumulated for PAL and CHS, key enzymes in the biosynthetic pathway to the pea phytoalexin pisatin. However, the concomitant presence of suppressors from *Mycosphaerella pinodes*, a fungal pathogen of pea, specifically inhibited or delayed the elicitor-induced defenses, by inhibiting ATPase and PI metabolism[6,15,16,18,19,20,21,22,23,24,25]. Moreover, inhibitors of plasma membrane ATPase were shown to block the elicitor-induced activation of PI metabolism as well as the pisatin accumulation in pea epicotyls[20,22,24,25]. These results suggest that plasma membrane ATPase may interact with signal transduction pathway,

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Abbreviations

BSA, bovine serum albumin; CHS, chalcone synthase; DAG, diacylglycerol; DTT, dithiothreitol; EDTA, ethylenediamine-N,N,N′,N′,-tetraacetic acid; IP_3, inositol 1,4,5-trisphosphate; MES, 2-morpholinoethanesulfonic acid, monohydrate; PAGE, polyacrylamide gel electrophoresis; PAL, phenylalanine ammonia-lyase; PI, phosphatidylinositol; PI kinase, phosphatidylinositol kinase; PI metabolism, polyphosphoinositide metabolism; PIP, phosphatidylinositol 4-monophosphate; PIP kinase, phosphatidylinositol 4-monophosphate kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulfate; TBS, 50mM Tris/HCl, pH7.4, 500mM NaCl; Tris, Tris[hydroxymethyl]aminomethane

in particular PI metabolism. In this report, to further confirm the interaction between the ATPase and PI metabolism, we attempt to purify and to characterize membrane subfractions from the detergent-solubilized plasma membranes of pea.

Materials and Methods

Chemicals — PI, PIP and PIP_2 were purchased from Sigma. [γ-^{32}P]ATP (6,000 Ci/mmol) was obtained from Amersham. Other chemicals were from Wako Pure Chemical Inc.

Plant material — Seeds of *Pisum sativum* L. cv. Midoriusui were sown on moistened vermiculite in a plastic container and grown in darkness at 22°C. Etiolated epicotyls from 9-day-old seedlings were used to prepare plasma membrane fraction as described previously[22].

Preparation of suppressor from *Mycosphaerella pinodes* — A suppressor preparation was isolated from germination fluid of *Mycosphaerella pinodes* (Berk. et Blox) Vestergren, strain OMP-1 (IFO-30342, ATCC-42741) as previously described[14,25].

Solubilization of plasma membrane proteins and the subsequent fractionation by a linear glycerol density gradient centrifugation — Plasma membrane fraction was suspended in 5 mM Tris/HCl (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.25 M sucrose and 0.5% (v/v) Triton X-100 at a protein concentration of 2 mg/ml. The mixture was sonicated for 30 sec 5 times at 4°C in a bath-type sonicator (Branson 3200, Yamato, Tokyo, Japan) and incubated on ice for an additional 30 min. The solubilized membrane proteins were recovered by ultracentrifugation at 106,000 x*g* for 30 min. Under these conditions, over 70% of proteins were recovered into the supernatant fraction[1]. The detergent-solubilized membrane proteins were layered on 32 ml of a 23-43% linear glycerol density gradient that contained 5 mM Tris/HCl (pH 7.6), 1 mM DTT, 1 mM PMSF, 1 μg/ml antipain, 1 μg/ml luepeptin, 0.05% (w/v) Zwittergent 3-14 and were then centrifuged at 106,000 x*g* for 21 h at 4°C. After the centrifugation, the gradient was divided into 32 equal-volume fractions: fractions (1 ml) were collected from the top part of the gradient. Protein concentration was determined with BSA as the standard, by the dye-binding method with a kit from Bio-Rad.

Size-exclusion column chromatography — Active ATPase fractions after linear glycerol density gradient centrifugation were pooled and applied on a HiLoad Superdex 200 pg column (16 mm i.d. × 600 mm; Pharmacia) that had been pre-equilibrated with 10 mM Tris/HCl (pH 7.6) containing 20% (v/v) glycerol, 200 mM KCl, 1 mM DTT, 1 mM PMSF, 1 μg/ml antipain, 1 μg/ml luepeptin and 0.05% (w/v) Zwittergent 3-14. The sample was eluted with the same buffer at a flow rate of 0.55 ml/min.

ATPase assay — ATPase assay was carried out in a reaction mixture containing 30 mM Tris/MES (pH 6.5), 3 mM ATP and 3 mM $MgSO_4$, and the released Pi was determined as described previously[25].

Lipid kinase assay — Ten micrograms of PI or PIP were taken to dryness under N_2 stream and resuspended in a Tris-buffer containing Triton X-100 by sonication in a bath-type sonicator. Lipid kinase activity was measured in a reaction mixture containing 30 mM Tris/MES (pH 6.5), 15 mM $MgSO_4$, 10 μM ATP and 10 μCi of [γ-^{32}P]ATP, in the presence of 10 μg of exogenous lipid (PI or PIP). The reaction was initiated by adding enzyme fraction and incubated for 30 min at 25°C. After adding perchloric acid to stop the reaction, the lipids were extracted and separated on a silica gel plate (LK5D, Whatman) as described previously[19,22]. The phosphorylated lipids were visualized with a Bio-imaging analyzer (Bas 2000 system; Fujix, Tokyo, Japan).

Electrophoresis and immunoblot analysis — SDS-PAGE was performed according to Laemmli[8]. Electrophoresed proteins were trans-

ferred to a PVDF membrane (Bio-Rad) at 140 mA for 1 h with a semidry electroblot apparatus in a buffer containing 20 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS. The protein blot was blocked for 1 h at room temperature with 3% (w/v) gelatin-TBS and incubated for 2 h with an anti-H^+-ATPase (generously gifted by Prof. Dr. Asahi, Fukui Prefectural University, Japan). After extensive washing, the blot was stained with a goat anti-rabbit IgG conjugated with horseradish peroxidase and its substrate 5-bromo-4-chloro 3-naphtol.

Results and Discussion

When the solubilized membrane proteins were separated by a 23-43% linear glycerol density gradient centrifugation, vanadate-sensitive ATPase was distributed in the gradient with one distinct peak at the upper part (Fig. 1A). Moreover, different distributions of proteins and ATPase activity in the continuous gradient showed the successful solubilization of plasma membrane ATPase. The ATPase in each fraction required Mg^{2+} for full activities (not shown) and was vanadate- and suppressor-sensitive (Fig. 1A), just like the membrane-bound form of the enzyme[1,25]. On the other hand, when assayed for the presence of lipid kinase activity, the enzyme fractions with apparent ATPase activities were shown to phosphorylate exogenous PI and PIP to form PIP and PIP_2, respectively (Fig. 1B). Furthermore, the peak of lipid kinases coincided almost exactly with that of ATPase (Fig. 1b), indicating that one form of plasma membrane ATPase was co-sedimented with lipid kinase activities.

Our previous studies[21,22] showed that adding elicitor to isolated plasma membrane fraction of pea resulted in a rapid phosphorylation of PI and PIP, in parallel with an increase in PLC activity. However, the concomitant presence of ATPase inhibitors including a fungal suppressor remarkably decreased the phosphorylation[21,22]. To define the interaction between ATPase and lipid

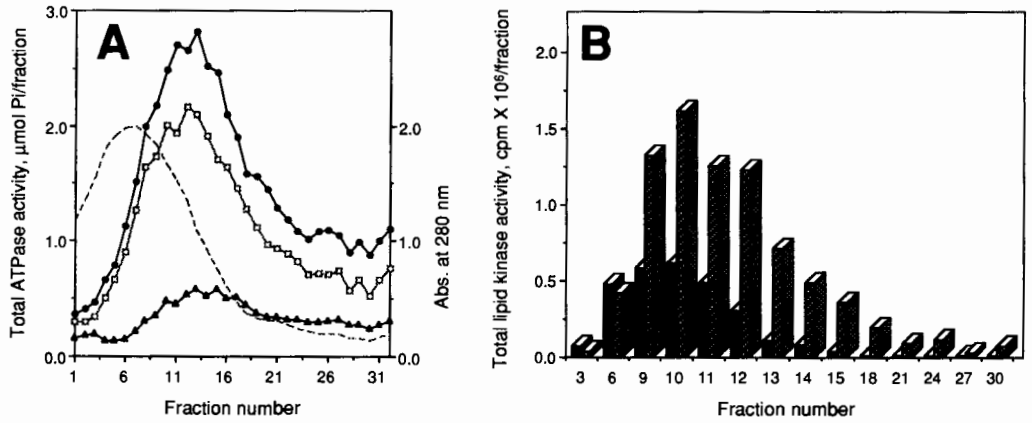

Fig. 1 Fractionation of the detergent-solubilized plasma membrane proteins by linear glycerol density gradient centrifugation.

A, Distributions of proteins and ATPase activity in the continuous gradient after the centrifugation. Fractions (1 ml) were collected from the top part of the gradient and aliquots (2.5 μl) of each fraction were assayed for ATPase as described in the text. The activity was expressed as a total activity (μmol Pi/h/each fraction) in the absence (●) or the presence of fungal suppressor (60 μg/ml, BSA equiv.; □) or vanadate (1 mM; ▲). Solid line shows a distribution of proteins monitored at 280 nm. **B**, Distributions of lipid kinase activity. Lipid kinase activities were measured, using enzyme fractions (No. 3, 6, 9, 10, 11, 12, 13, 14, 15, 18, 21, 24, 27 and 30), in the reaction mixture containing 30 mM Tris/MES (pH 6.5), 15 mM $MgSO_4$, 10 μM ATP, 10 μCi of [γ-^{32}P]ATP and 10 μg of exogenous lipid (PI or PIP). The radioactivities of [^{32}P]PIP (*left column*) and -PIP_2 (*right column*) were quantified with a Bio-imaging analyzer (Bas 2000 system, Fujix, Tokyo, Japan) after their separation on a silica gel plate. Data was expressed as the total activity in each fraction.

kinase(s), we also examined the effect of the suppressor on lipid kinase activities, using a most apparent fraction of ATPase activity (fraction No. 10). When the assay was carried out in the presence of the suppressor, the incorporation of radioactivity into PIP and PIP_2 were markedly reduced (Fig. 2). This result, consistent with our previous finding[21,22] observed in plasma membrane fraction, further suggests that ATPase may play a role in regulating lipid kinase(s). Chen and Boss[3] reported recently that plasma membrane ATPase of carrot cells was regulated by PI and PIP_2. In pea plasma membrane, exogenous addition of inositolphospholipids, i.e. PIP and PIP_2 increased ATPase activity[16], whereas adding neomycin, a capture of PIP_2, to plasma membranes decreased its activity (our unpublished data). Based on these findings, there seems to exist a functional and spatial interaction "cross-talk" between ATPase and PI metabolism in pea plasma membranes.

To further define the association between ATPase and lipid kinase, active fractions of ATPase obtained by the glycerol density gradient centrifugation were collected and then subjected to a size-exclusion column chromatography. The chromatography resulted in separation of five apparent peaks (designated F1 through F5) (Fig. 3A). When each fraction was assayed for ATPase, activity was abundantly recovered by F3. The presence of ATPase was also confirmed by an immunoblot analysis with an antibody raised against mung bean H^+-ATPase (Fig. 3C). In addition, the fraction (F3) was shown to exhibit the phosphorylation of exogenous PI when supplied with [γ-^{32}P]ATP (Fig. 3D). These results indicate that one form of plasma membrane ATPase is co-purified with PI kinase. In

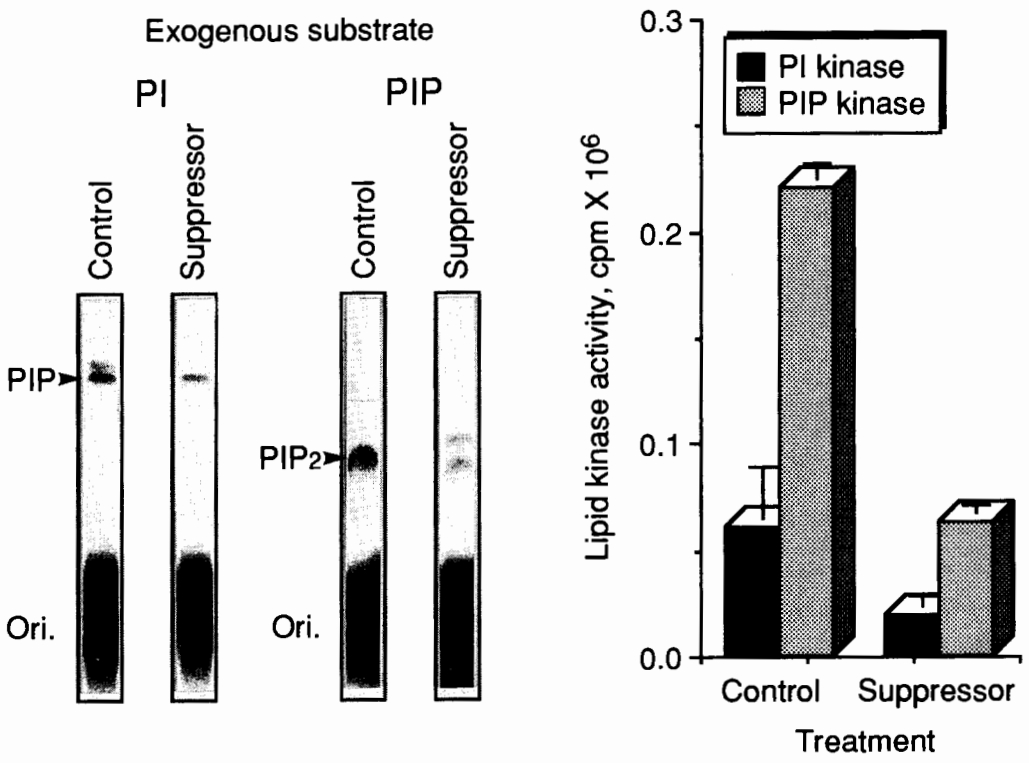

Fig. 2 Effect of fungal suppressor on lipid kinase activity in a partially purified ATPase fraction.
Assay was carried out using a No. 10 fraction after a linear glycerol density gradient centrifugation, in the absence or the presence of the suppressor (100 μg/ml). The left figure shows autoradiograms of [^{32}P]PIP and -PIP_2 after separation on a silica gel plate. The radioactivity of [^{32}P]PIP (*left column*) and -PIP_2 (*right column*) were quantified with a Bio-imaging analyzer (Bas 2000 system, Fujix, Tokyo, Japan). Mean values with S.D. from duplicate experiments are shown in the rightfigure.

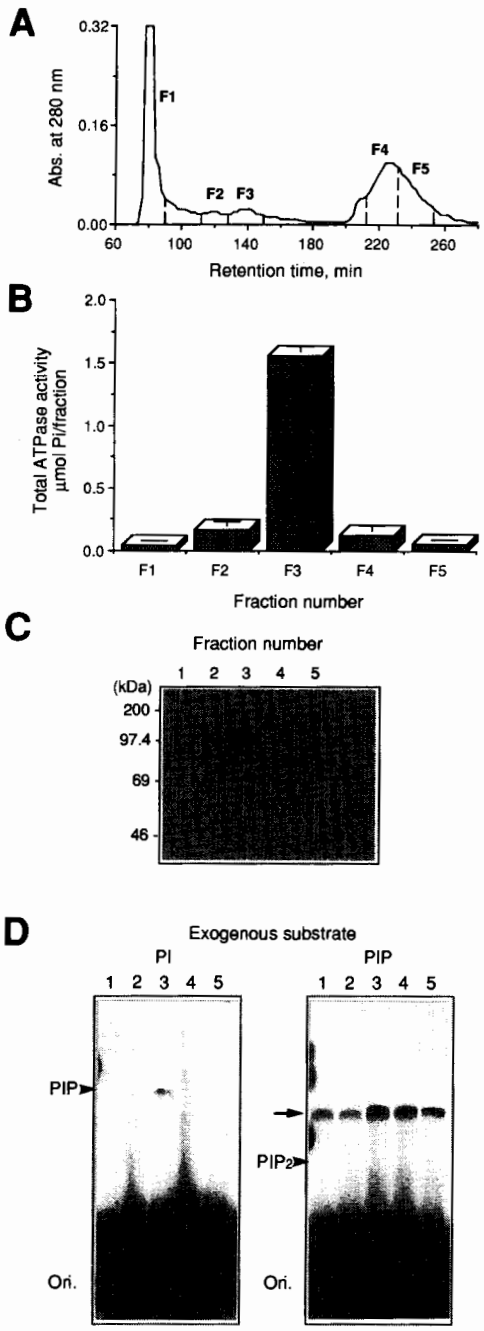

Fig. 3 Size-exclusion chromatography of a partially purified ATPase fraction.
Active ATPase fractions after a linear glycerol density gradient centrifugation were applied on a HiLoad Superdex 200 pg column. **A**, Elution profile monitored at 280 nm. Five apparent peaks (designated F1 through F5) were collected. **B**, Distributions of ATPase activity. An aliquot (5 μl) in each fraction was assayed for ATPase and the activity was expressed as the total activity in each fraction. **C**, Immunoblot analysis with anti-H^+-ATPase. One half micrograms of proteins were separated by 10% polyacrylamide gel and blotted onto a PVDF membrane. The blot was incubated with anti-H^+-ATPase (diluted 1/200) and then visualized by goat anti-rabbit IgG conjugated with horseradish peroxidase. **D**, Distributions of lipid kinase activity. Lipid kinase activity was measured in the presence of exogenous PI or PIP. The reaction was initiated by adding enzyme fraction and incubated for 30 min at 25°C. After adding perchloric acid to stop the reaction, the phosphorylated lipids were extracted, separated on a silica gel plate and were visualized with a Bio-imaging analyzer as described above. Each number at the top indicates the fraction number designated in Fig. 3A. Note that F3 fraction phosphorylated PI to form PIP when supplied with [γ-^{32}P]ATP. However, the addition of PIP failed to produce PIP_2, although an unidentified compound that migrates between PIP and PIP_2 was observed (*arrow*).

the present study, however, the addition of PIP failed to produce PIP_2, although an unidentified compound that migrates between PIP and PIP_2 was observed (Fig. 3D). This may be ascribed to the absence of PIP kinase or its inactivation, probably due to lack of certain regulatory factor(s) during purification steps.

Plasma membrane ATPase in higher plants is thought to be a master enzyme that is involved in a wide variety of cellular events, such as ion and nutrient uptake, the growth and development of cells and regulation of intracellular pH[13]. In mammalian cells, Szamel and Resch[17] reported that ouabain, an inhibitor of Na^+/K^+-ATPase, blocked the mitogenesis in lymphocyte induced by concanavalin A. They[12] further showed that Na^+/K^+-ATPase segregated in subfractions from detergent-solubilized lymphocyte membranes also contained acyl-CoA: lysophosphatidylcoline aclytransferase responsible for signal transduction. In radish plasma membranes, Cocucci and Marre[4] showed that one form of plasma membrane ATPase was co-purified with a putative fusicoccin receptor. Taken together, there is no doubt concerning association of plasma membrane ATPase with other membrane proteins, including receptors and signal transduction proteins. Thus, elucidation of functional and spatial interaction between ATPase and lipid kinase(s) will provide new insights on the physiological roles of ATPase in signal transduction. Further investigations are underway to dissect the complicated signal transduction pathway leading to the induction of defense responses.

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エンドウ原形質膜における ATP アーゼと ホスファチジルイノシトールリン脂質リン酸化酵素の共精製

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エンドウの上胚軸組織から分離した原形質膜画分における ATP アーゼとホスファチジルイノシトールリン脂質リン酸化酵素との相互作用を解析する目的で，双方の原形質膜画分からの可溶化とそれらの部分精製を試みた．原形質膜の Triton X-100可溶化画分をグリセロール連続密度勾配遠心分画に供し，得られた活性画分をさらに分子ふるいカラムクロマトグラフィーによって分離した．この結果，ATP アーゼとホスファチジルイノシトールリン脂質リン酸化酵素は共精製され，非変性条件下では双方の活性を分けることができなかった．