Volume 238, number 1, 87–89 FEB 06337 September 1988

Phosphatidylinositol kinase activity of a plasma membrane-associated calcium-activated protein kinase from pea

D.P. Blowers and A.J. Trewavas

Department of Botany, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, Scotland

Received 8 August 1988

A purified protein kinase preparation from pea is shown to contain phosphatidylinositol kinase activity. The preparation, produced by membrane solubilisation and ATP-agarose affinity chromatography, contains proteins of 18 kDa (pp18, a calcium activated autophosphorylating protein-serine kinase), 67 kDa and 48 kDa and exhibits the phosphorylation of an endogenous lipid when supplied with [γ-^{32}P]ATP. This product is tentatively identified as lysophosphatidylinositol phosphate. When supplied with [γ-^{32}P]ATP and exogenous phosphatidylinositol, the production of phosphatidylinositol phosphate is observed.

Lipid kinase; Phosphatidylinositol phosphorylation; Phosphatidylinositol phosphate; (Pisum sativum)

1. INTRODUCTION

The agonist-induced rapid turnover of plasma membrane-located phosphatidylinositol, with the production of the second messengers inositol trisphosphate and diacylglycerol, is now well characterised in animal systems [1]. The cycle is dependent upon a phospholipase C, numerous kinases and phosphatases, as well as two transferases [2].

In plants, only selected elements of the system has been identified. Boss and Massel [3] first identified the presence of polyphosphoinositides (PIP and PIP_2) in plant membranes and Melin et al. [4] demonstrated the presence of a polyphosphoinositide specific phospholipase C. Additionally, the presence of PI kinase and PIP kinase activities in plant plasma membranes has been demonstrated [5,6]. However, none of the above enzymes have been purified from plants.

In this report we show that a purified calcium- and calmodulin-regulated, plasma membrane-located, protein kinase (pp18) preparation exhibits PI kinase activity.

Correspondence address: D.P. Blowers, Department of Botany, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, Scotland

Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate

2. MATERIALS AND METHODS

[γ-^{32}P]ATP (spec. act. 185 TBq·mmol^{-1}) was obtained from Amersham (England). Other chemicals were obtained from Sigma (England) or British Drug Houses (England).

Unexpanded dark grown pea leaves were grown, harvested and a microsomal membrane preparation isolated as previously described [7]. Details of the purification of the 18 kDa protein kinase are to be published elsewhere [8]. In brief, membrane proteins were solubilised by acetone precipitation and resuspension in an aqueous buffer. ATP-agarose affinity chromatography of the solubilised membrane proteins produced the final purified fraction in an isolation buffer containing 6 mM Mes (2-[morpholino]ethanesulfonic acid), 6 mM Tris-Cl^-, 20.2 mM Na_2EDTA, pH 7.2. On a specific activity basis, purification is over 700-fold.

Protein phosphorylation was performed by labelling of the purified protein kinase fraction in an isolation buffer. Reactions were initiated by the addition of 100 nM [γ-^{32}P]ATP and terminated by the addition of sample buffer [8] prior to heating at 100°C for 10 min and separation by SDS-PAGE.

Lipid phosphorylation was performed by addition of 10 μl of

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

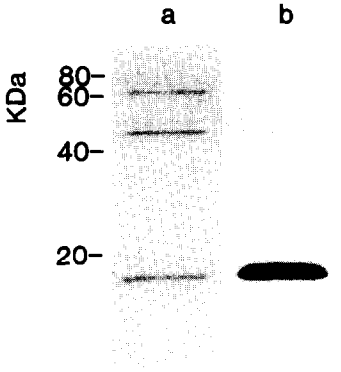

Fig.1. SDS-PAGE analysis of the purified kinase fraction. Lane a, Coomassie brilliant blue R staining. Lane b, autoradiograph of lane a which had been labelled with [γ-^{32}P]ATP prior to separation.

purified protein kinase and 10 μl of the above isolation buffer (with only 0.2 mM Na_2EDTA) with or without 5 $\mu g \cdot \mu l^{-1}$ PI (sonicated). Reactions were initiated by the addition of 100 nM [γ-^{32}P]ATP. At the appropriate times the reactions were terminated by the addition of 130 μl of 1 M HCl and phosphoinositides extracted according to Sugimoto et al. [9].

The phosphorylated lipids were separated by ascending chromatography on silica gel 60 (250 μm) plates (Merck) which had been lightly sprayed with 1% (w/v) aqueous potassium oxalate and activated by heating for 1 h at 110°C. First dimension: Chloroform/methanol/4 M NH_4OH, 9:7:2 (v/v), for 2 h. Second dimension: Propan-1-ol/4 M NH_4OH, 13:7 (v/v), for 3 h. Lipids were detected by spraying with 1% (w/v) I_2 in methanol or by autoradiography.

3. RESULTS

The plasma membrane-located [10] autophosphorylating protein kinase (pp18) [11] was purified by selective solubilisation and ATP-agarose affinity chromatography. Fig.1 is representative of the degree of purity obtained by this procedure now used routinely for pp18 purification. On a specific activity basis, a purification of 714-fold is estimated [8]. Two major proteins, in addition to the 18 kDa pp18, are present (67 and 48 kDa) and it is not known whether these are essential for pp18 activity and/or possess other activities, although they remain associated with pp18 during size exclusion chromatography. Fig.1 also illustrates the

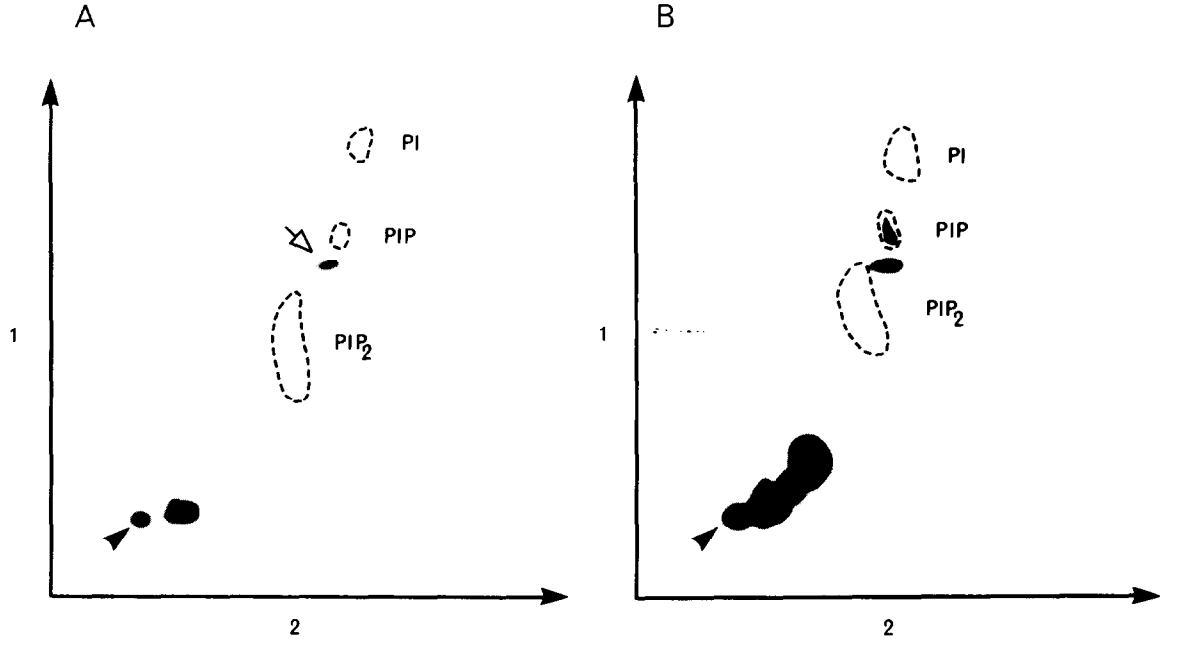

Fig.2. Autoradiographs of two-dimensional thin-layer chromatography separations of endogenous (A) and exogenous (B) lipid phosphorylation by the kinase preparation. The protein kinase preparation was labelled using [γ-^{32}P]ATP for 5 min (A) or 30 min (B) with the addition of exogenous phosphatidylinositol for plate B. Lipids were then extracted. First and second dimensional directions are indicated and points of application are shown by arrowheads. Endogenous phosphorylated lipid product is shown by an open arrow on plate A. Positions of I_2 staining standards are outlined.

autophosphorylation of pp18 obtained when incubated with [γ-^{32}P]ATP.

Labelling of the purified protein kinase preparation using [γ-^{32}P]ATP and extraction of phosphoinositides reveals the presence of an endogenous lipid phosphorylation (marked with an open arrow on fig.2A). This compound migrates between the outlined PIP and PIP_2 markers and possibly represents the phosphorylation of lysoPI to form lysoPIP [12]. A lysophosphatidylinositol kinase has been identified in human platelet microsomes [13], and a similar unidentified inositol lipid was observed by Boss and Massel [3]. Upon the addition of exogenous PI to the phosphorylation mixture the production of PIP is observed (fig.2B). The purified protein kinase preparation thus contains PI kinase activity. A slight enhancement in the level of the putative lysoPIP is also observed, possibly due to lysoPI contamination of the exogenous PI and not the longer incubation time used for exogenous PI phosphorylation. The major spots migrating close to the origin are, as yet, unidentified. However, they may represent inositol phosphates produced by breakdown of the phosphorylated lipids [3].

The addition of 1,2-dioctanoyl-glycerol or PIP failed to produce either phosphatidic acid or PIP_2, respectively (not shown). Initial observations suggest a relative insensitivity of the pp18 endogenous lipid (shown in fig.2A) to digestion by phospholipase C, phospholipase A_2 and alkaline phosphatase when compared to exogenously added lipids (not shown). Insensitivity to enzymic attack would suggest that the lipid is protected by a protein moiety [12].

4. DISCUSSION

To our knowledge this is the first report of the purification of a PI kinase from plants and is thus a considerable advance in dissecting the possible PI turnover-mediated signal transduction in plants. To state that this activity is solely associated with pp18 would be premature, the activities of the 67 kDa and 48 kDa proteins, or combinations thereof, must be investigated. However, the association of lipid kinase activity with a protein kinase is not unusual. Numerous tyrosine kinases are known to phosphorylate PI, PIP and diacylglycerol [14]. Additionally, phosphorylase kinase will phosphorylate PI, producing PIP, and the cAMP-dependent protein kinase will phosphorylate PIP to produce PIP_2 [9]. PI kinase activity is also thought to be associated with the sarcoplasmic reticulum Ca^{2+}-ATPase [12].

A direct demonstration that pp18 is involved in the turnover of phosphatidylinositol is required, as its plasma membrane location favours its involvement. The purification of the other enzymes for completion of the cycle is also necessary.

Acknowledgements: We acknowledge the support of the Agricultural and Food Research Council Cell Signalling and Recognition Program. Our thanks to Dr R. Irvine for his comments and kindly supplying the PIP and PIP_2 standards.

REFERENCES

[1] Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 159–193.
[2] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[3] Boss, W.F. and Massel, M.O. (1985) Biochem. Biophys. Res. Commun. 132, 1018–1023.
[4] Melin, P.-M., Sommarin, M., Sandelius, A.S. and Jergil, B. (1987) FEBS Lett. 223, 87–91.
[5] Sandelius, A.S. and Sommarin, M. (1986) FEBS Lett. 201, 282–286.
[6] Sommarin, M. and Sandelius, A.S. (1988) Biochim. Biophys. Acta 958, 268–278.
[7] Hetherington, A.M. and Trewavas, A.J. (1984) Planta 161, 409–417.
[8] Blowers, D.P. and Trewavas, A.J. (1988) Eur. J. Biochem., submitted.
[9] Sugimoto, Y., Whitman, M., Cantley, L.C. and Erikson, R.L. (1984) Proc. Natl. Acad. Sci. USA 81, 2117–2121.
[10] Blowers, D.P., Hetherington, A.M. and Trewavas, A.J. (1985) Planta 166, 208–215.
[11] Blowers, D.P. and Trewavas, A.J. (1987) Biochem. Biophys. Res. Commun. 143, 691–696.
[12] Varsanyi, M., Tolle, H.-G., Heilmeyer, L.M.G., Dawson, R.M.C. and Irvine, R.F. (1983) EMBO J. 2, 1543–1548.
[13] Thomas, L.M. and Holub, B.J. (1987) Lipids 22, 144–147.
[14] Hunter, T. and Cooper, J.A. (1985) Annu. Rev. Biochem. 54, 897–930.