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Characterization of a Calcium- and Lipid-Dependent Protein Kinase Associated with the Plasma Membrane of Oat†

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ABSTRACT: A protein kinase that is activated by calcium and lipid has been partially purified from the plasma membrane of oat roots. This protein kinase cross-reacts with four monoclonal antibodies directed against a soluble calcium-dependent protein kinase from soybean described previously [Putnam-Evans, C. L., Harmon, A. C., & Cormier, M. J. (1990) *Biochemistry 29*, 2488–2495; Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991) *Science 252*, 951–954], indicating that the oat enzyme is a member of this calcium-dependent protein kinase family. Immunoblots demonstrate that the membrane-derived protein kinase is slightly larger than that observed in the cytosolic fraction of oat. Limited digestion of the membrane-derived kinase with trypsin generates a smaller water-soluble kinase that is still activated by calcium but is no longer activated by lipid. When posthomogenization proteolysis is minimized, the bulk of the immunoreactive kinase material is localized in the membrane. These results suggest that a calcium-dependent protein kinase observed in the supernatant fraction of oat extracts may originate in situ from a calcium- and lipid-dependent protein kinase which is associated with the oat plasma membrane. They further indicate that, in contrast to animal cells, the predominant calcium- and lipid-dependent protein kinase associated with the plasma membrane of plant cells has biochemical properties and amino acid sequence unlike protein kinase C.

In higher plants, as in other eukaryotes, calcium plays an important role as a second messenger, and has been implicated in cell elongation and division, protoplasmic streaming, and hormone action [Reviewed in Marmé and Dieter (1983), Hepler and Wayne (1985), and Carofoli (1987)]. In contrast, the role of cAMP in plants is questionable (Spieteri et al., 1989). Despite the recognition that calcium plays a second-messenger role in plants, little is known concerning the molecular mechanism by which changes in cytoplasmic calcium act.

This past year, the predominant calcium-dependent protein kinase in the cytosolic fraction of soybean cells was purified to homogeneity (Putnam-Evans et al., 1990), and a cDNA clone encoding the kinase was also isolated (Harper et al., 1991). The kinase is different from any previously identified plant or animal kinase in that it requires calcium but not calmodulin or phosphatidylserine for activity (Harmon et al., 1987; Putnam-Evans et al., 1990). The protein has an amino-terminal kinase catalytic domain fused to a carboxy-terminal domain which shows greatest homology to calmodulin and contains four calcium-binding sites (Harper et al., 1991). As such, this kinase is capable of binding calcium directly and represents the prototype for a new family of calcium-regulated protein kinases.

The studies reported in this paper were prompted by the observation that the predominant kinase activity associated with the plasma membrane of higher plants is also calcium-dependent (Schaller & Sussman, 1988; Klucis & Polya, 1988; Klimczak & Hind, 1990). Here we demonstrate that a protein kinase found in both the soluble and the plasma membrane fraction of oat roots is immunologically related to the cytosolic calcium-dependent protein kinase from soybean (Putnam-Evans et al., 1990). Our results indicate that in oat this plasma membrane bound kinase is dependent upon both calcium and lipid and suggest that limited proteolysis of the membrane-

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associated form of the kinase generates a purely soluble form that is no longer dependent upon lipid. They further indicate that the predominant calcium- and lipid-dependent protein kinase associated with the plasma membrane of higher plants is a novel protein kinase, unlike protein kinase C.

Materials and Methods

Plasma Membrane Isolation. Oat root microsomes were isolated as described in Surowy and Sussman (1986), and plasma membranes were purified by partitioning in an aqueous dextran–poly(ethylene glycol) two-phase system (Larsson et al., 1987; Sandstrom et al., 1987). The final plasma membrane pellet was resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT),[1] 1 mM EDTA, and 10% (v/v) glycerol at a protein concentration of 5–10 mg/mL and stored at −80 °C. Typically, 25 mg of plasma membrane protein was obtained from 500 g of roots.

Partial Purification of Protein Kinase Activity. All steps were carried out at 4 °C. Plasma membranes (100 mg) were brought to 2 mg of protein/mL in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 4 mM EDTA, 0.5 M KCl, and 10% (v/v) glycerol and centrifuged at 100000*g* for 45 min. The pellet (called salt-washed plasma membranes) was resuspended at 2 mg of protein/mL in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol and treated with 0.9% (v/v) Triton X-100. After centrifugation at 100000*g* for 45 min, the pellet was resuspended at 2 mg of protein/mL, treated again with Triton X-100 under identical conditions, and centrifuged at 100000*g* for 45 min. The supernatants from both Triton X-100 extractions were combined and applied to a 11 cm × 1.5 cm (internal diameter) column of Q-Sepharose (Sigma) equilibrated with chromatography buffer [10 mM Tris-HCl (pH 7.5), 1 mM DTT, 10% (v/v) glycerol, and 0.9% (v/v) Triton X-100]. The flow rate was 0.6 mL/min. After application, the flow rate was increased to 1.2 mL/min, and the column was washed with 20 mL of chromatography buffer, followed by 13 mL of chromatography buffer containing 0.1 M NaCl. Kinase activity was eluted from the column with a 180-mL linear concentration gradient of 0.1–0.4 M NaCl in chromatography buffer. Seven-milliliter fractions were collected. Fractions containing activity were pooled and concentrated 7-fold using a 70-mL stirred cell (Nucleopore) equipped with a YM-10 membrane (Amicon). The concentrate (3 mL) was mixed with 1 mg of blue dextran and 1 mg of cytochrome *c* as visual molecular weight markers and applied to a gel filtration column (165 cm × 1.5 cm) of Sephacryl S-300 (Sigma). Kinase activity was eluted with chromatography buffer containing 0.1 M NaCl at a flow rate of 0.33 mL/min, and 5-mL fractions were collected. Two peaks of kinase activity were observed, and fractions were pooled and stored at −80 °C. Cytosolic protein kinase activity was also partially purified following the same scheme.

Protein Kinase Assay. Protein kinase activity was assayed in a total volume of 50 μL containing 50 mM Mes/Tris (pH 6.2), 6 mM $MgCl_2$, and 50 μg of histone type IIIS (lysine-rich fraction; catalogue number H-5505 from Sigma) as exogenous substrate. The reaction was initiated by the addition of 20 μM ATP containing 10 μCi of [γ-^{32}P]ATP, except for Table I where 100 μM ATP was used. Samples were incubated for 20 min at room temperature (22 °C). Each reaction was stopped by applying the entire assay mixture to a 2 × 2 cm^2 of Whatman 3MM paper, which was immediately immersed in cold 10% (w/v) trichloroacetic acid containing 10 mM disodium pyrophosphate (10 mL per paper square) and washed for 15 min. The squares were then washed 3 times with 10% (w/v) trichloroacetic acid containing 10 mM disodium pyrophosphate at 22 °C, once with the same solution at 60 °C, then with 95% (v/v) ethanol at 60 °C, and then with ether at 22 °C. All washes were for at least 15 min. Paper squares were allowed to dry, and radioactivity was quantitifed by measurement in a liquid scintillation counter.

The above assay mixture contained 73 μM calcium, as determined by atomic absorption spectrophotometry, with most of the calcium being contributed from the commercial preparation of histone substrate. For the experiment in Figure 6, histone substrate was extensively dialyzed and assays performed in the presence of 0.2 mM EGTA. Exogenous calcium was added in the form of $CaCl_2$ and the concentration of free calcium determined from the association constant for Ca·EGTA as described previously (Schaller & Sussman, 1988).

In order to examine lipid-dependent kinase activity, lipids were dissolved in chloroform, dried under nitrogen, and then resuspended by sonication into buffer for assays. Partially purified phosphatidylcholine from soybean [containing 40% (w/w) phosphatidylcholine; catalogue number P-3644 from Sigma] was used as a source of plant lipid.

Protein Determination. Protein concentrations were measured by the method of Lowry et al. (1951). When high concentrations of detergent were present, the method of Bensadoun and Weinstein (1976) was used. Bovine serum albumin (catalogue number A9647 from Sigma) was used as standard.

Phase Partitioning with Triton X-114. Triton X-114 was precondensed as described in Bordier (1981). A 1% (v/v) solution of Triton X-114 in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 10% (v/v) glycerol, and 0.1 M NaCl was prepared from a 15% (v/v) stock solution of Triton X-114 in 10 mM Tris-HCl (pH 7.4) and 0.15 M NaCl. For partitioning, 1 part kinase (50 μL) was mixed with 9 parts of the 1% (v/v) Triton X-114 solution (450 μL) in a 1.5-mL microfuge tube and placed on ice for 30 min. The sample was incubated for 10 min in a 37 °C water bath and then centrifuged for 30 s in a microfuge. Two phases were observed: an upper aqueous phase and a lower detergent phase. The phases were separated, and each was brought back to the original volume (500 μL) with 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 10% (v/v) glycerol, and 0.1 M NaCl and to the original concentration of Triton X-114 (1% v/v). Kinase assays were performed by diluting 5 μL of each phase into a 50-μL kinase assay mixture containing 0.5 mg/mL soybean lipid.

SDS–PAGE and Immunoblotting. Protein samples were concentrated by precipitation with trichloroacetic acid in the presence of deoxycholate, as described in Bensadoun and Weinstein (1976). Pellets were dissolved in 20 μL of electrophoresis buffer and proteins separated by SDS–PAGE according to Laemmli (1970). The stacking gel was 5% (w/v) acrylamide, and the running gel was either 8% or 10% (w/v) acrylamide, as indicated. Immunoblotting was performed according to Surowy and Sussman (1986) with a mixture of four monoclonal antibodies. These antibodies were raised against a calcium-dependent protein kinase purified from the cytosol of suspension-cultured soybean cells (Putnam-Evans et al., 1989). Immunodecorated protein bands were visualized using a second antibody (goat anti-mouse) conjugated to alkaline phosphatase. When greater sensitivity was required (Figures 3, 4, and 7), a second antibody (goat anti-mouse) was used, followed by a third antibody (rabbit anti-goat) conju-

[1] Abbreviations: DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

Table I: Partial Purification of Kinase Activity from the Oat Plasma Membrane

fraction	protein (mg)	total act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	+lipid (0.5 mg/mL) total act. (nmol/min)	+lipid (0.5 mg/mL) sp act. (nmol min^{-1} mg^{-1})	lipid stimulation (x-fold)
plasma membrane	71.1	30.4	0.43	68.4	0.97	2.3
KCl-washed plasma membrane	66.6	28.7	0.43	58.0	0.87	2.0
Triton X-100 extract	23.4	67.7	2.9	145.6	6.23	2.2
Q-Sepharose	3.0	15.1	5.0	74.6	24.9	4.9
Sephacryl S-300						
peak 1	0.45	5.5	12.2	36.7	81.5	6.7
peak 2	0.13	7.9	60.8	10.5	80.8	1.3

gated to alkaline phosphatase.

Digestion with Trypsin. Diphenylcarbamyl chloride treated trypsin (10% by weight of protein) was added to the partially purified kinase and the mixture incubated in a water bath at 22 °C for the indicated times. Digestion was terminated by adding soybean trypsin inhibitor at a 10-fold by weight excess over the trypsin.

Materials. Oat seeds (*Avena sativa*, variety Stout) were obtained from Olds Seed Co. (Madison, WI). SDS–PAGE reagents were purchased from Bio-Rad Laboratories (Richmond, CA). ATP was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). [γ-^{32}P]ATP (catalogue number NEG-002Z) was purchased from New England Nuclear (Boston, MA). A 100 mM stock solution of $CaCl_2$ was purchased from Orion Research Inc. (Cambridge, MA). Goat immunoglobulin (IgG) directed against mouse IgG and rabbit IgG directed against goat IgG (conjugated to alkaline phosphatase) were purchased from Kirkegaard & Perry Laboratories (Gaitherburg, MD). Goat IgG directed against mouse IgG and conjugated to alkaline phosphatase was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals, protein standards, and chromatography supports were purchased from Sigma.

Results

Partial Purification of Protein Kinase Activity from the Oat Plasma Membrane. Previous investigators (Klucis & Polya, 1988; Ladror & Zielinski, 1989) studying protein kinase activity in the microsomal fraction of higher plants have found that the majority of activity is associated with the plasma membrane. We isolated plasma membrane from oat root microsomes, followed by treatment with 0.5 M KCl and 4 mM EDTA to remove loosely associated protein. After the addition of 0.9% (v/v) Triton X-100 to membranes, 90% of the total activity was present in the soluble fraction, with 10% remaining in the pellet (Table I).

Plasma membrane protein solubilized by Triton X-100 was separated by a salt gradient on a column of Q-Sepharose. A single major peak of kinase activity eluted at 0.18 M NaCl. Peak fractions were pooled, concentrated, and size-fractionated on a column of Sephacryl S-300 in the presence of Triton X-100. Two peaks of activity were observed, referred to as peak 1 and peak 2 (Figure 1). On the basis of calibration with known globular proteins, in the absence of Triton X-100, peak 1 kinase eluted at M_r = 210 000–250 000, while peak 2 kinase eluted at M_r = 60 000.

The inclusion of Triton X-100 during purification resulted in sharper peaks of kinase activity and a greater recovery of activity at each step of purification. Further analysis indicated that some of the kinase activity loss observed following chromatography (Table I) could be attributed to a lipid requirement. When soybean lipid (0.5 mg/mL) was included in the kinase assay, an approximately 2-fold lipid stimulation of kinase activity was observed using plasma membrane or the

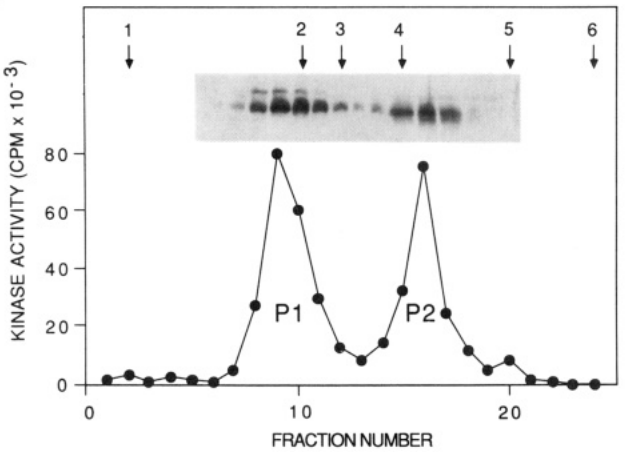

FIGURE 1: Size-fractionation of plasma membrane protein kinase activity on a column of Sephacryl S-300. Peak fractions of kinase activity off the Q-Sepharose column were pooled, concentrated, and applied to a column of Sephacryl S-300. Fractions off the Sephacryl S-300 column were assayed for kinase activity. Two peaks of activity were identified: peak 1 (P1) and peak 2 (P2). In addition, 0.15 mL of each fraction was separated by SDS–PAGE on an 8% (w/v) polyacrylamide gel, electroblotted to nitrocellulose, and immunostained with antibodies specific to the cytosolic protein kinase from soybean (inset). Triton X-100 (0.9% v/v) was present throughout purification. This Sephacryl S-300 column was calibrated without Triton X-100 present in the chromatography buffer. Numbered arrows indicate the elution positions of (1) blue dextran (M_r = 2 000 000), (2) β-amylase (M_r = 200 000), (3) alcohol dehydrogenase (M_r = 150 000), (4) bovine serum albumin (M_r = 66 000), (5) carbonic anhydrase (M_r = 29 000), and (6) cytochrome *c* (M_r = 12 400).

initial Triton X-100 extract as the source of enzyme. Lipid stimulation increased to 4.9-fold following anion-exchange chromatography. After size-fractionation, the lipid stimulation was only observed with the higher molecular weight peak (i.e., 6.7–fold stimulation with peak 1 versus 1.3-fold stimulation with peak 2).

The activity of both peak 1 kinase and peak 2 kinase was dependent on calcium in the absence or presence of lipid. The inclusion of 0.2 mM EGTA, a calcium chelator, in the kinase assay reduced the activity of both peak 1 and peak 2 kinases to less than 10% of the original activity. Activity was restored by the addition of exogenous $CaCl_2$.

Plasma Membrane Associated Kinase Is Immunologically Related to a Cytosolic Protein Kinase. A calcium-dependent protein kinase has been purified to homogeneity from the cytosolic fraction of suspension-cultured soybean cells (Harmon et al., 1987; Putnam-Evans et al., 1990). A mixture of four monoclonal antibodies directed against this enzyme was used to examine the kinase activities partially purified from the oat plasma membrane. Western blot analysis of fractions from the size-fractionation column indicated that both peak 1 and peak 2 kinases contained polypeptides immunologically related to the cytosolic protein kinase (Figure 1, inset). Peak 1 contained two immunostained bands: a major band at M_r = 61 000 and a minor band at M_r = 79 000. Peak 2 contained

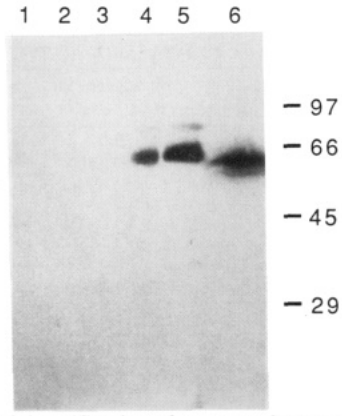

FIGURE 2: Partial purification of oat root plasma membrane kinase. Kinase activity was partially purified as described under Materials and Methods. Fractions containing kinase activity were separated on SDS–PAGE on a 10% (w/v) polyacrylamide gel, transferred to nitrocellulose, and immunostained with antibodies specific to the cytosolic protein kinase from soybean. Lanes 1 through 6 each represent 2.5 μg of protein from plasma membranes (lane 1), salt-washed plasma membranes (lane 2), Triton X-100 solubilized protein (lane 3), the kinase peak off the Q-Sepharose column (lane 4), and peak 1 (lane 5) and peak 2 (lane 6) off the Sephacryl S-300 column. Migration positions of SDS–PAGE molecular mass markers are indicated in kilodaltons.

a single band at M_r = 58 000. This difference in molecular weight (M_r = 61 000 compared to M_r = 58 000) was confirmed by mixing and coelectrophoresis on SDS–PAGE. The intensity of the immunostain correlated well with kinase activity in each fraction eluted from the size-fractionation column. In addition, following each step of kinase purification, coincident with the magnitude of increase in kinase specific activity, there was a corresponding increase in the intensity of the immunostained bands per microgram of protein (Figure 2). These observations indicate that immunostained material copurifies with kinase catalytic activity.

For comparative purposes, the cytosolic protein kinase of oat was partially purified in the presence of Triton X-100 following the same scheme employed to isolate protein kinase activity of the oat plasma membrane. The major peak of kinase activity eluted with a salt gradient similarly to the plasma membrane kinase activity when analyzed by anion exchange on Q-Sepharose. However, when cytosolic kinase activity was analyzed by size-fractionation on Sephacryl S-300, only a single peak of kinase activity was observed. This peak of activity eluted coincident with peak 2 kinase of the plasma membrane and contained an immunodecorated polypeptide of M_r = 58 000.

When tested individually, each of the four monoclonal antibodies was found to react with the same polypeptides of peak 1, peak 2, and the cytosolic kinase (Figure 3), suggesting a close relationship between these three kinases.

Phase Partitioning of Kinase Activity with Triton X-114. Both peak 2 kinase and the cytosolic kinase have an immunodecorated polypeptide of M_r = 58 000 on SDS–PAGE which, within the experimental error of this technique, is consistent with the molecular weight of 60 000 as determined by size-fractionation on Sephacryl S-300. Peak 1 kinase has a major immunodecorated polypeptide of M_r = 61 000 and a minor immunodecorated polypeptide of M_r = 79 000, but an apparent molecular weight of 210 000–250 000 as determined by size-fractionation on Sephacryl S-300. This discrepancy in molecular weight between PAGE in the presence of sodium dodecyl sulfate and column chromatography in a neutral detergent could result from a multimeric kinase. Alternatively, peak 1 kinase could be associated with a detergent micelle since size-fractionation took place in the presence of Triton X-100. Triton X-100 micelles have a calculated molecular weight of approximately 90 000, although micelles containing additional lipids may be considerably larger (Helenius & Simons, 1975).

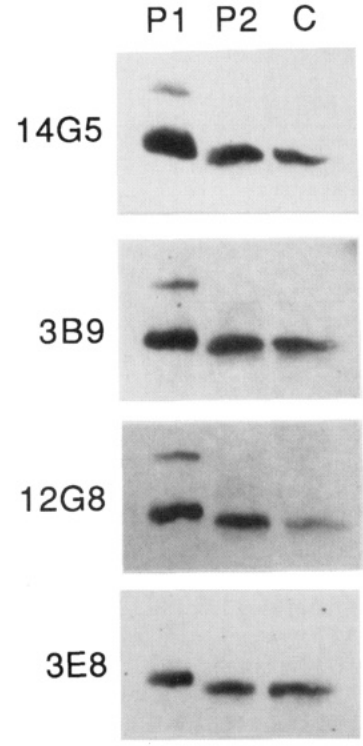

FIGURE 3: Western blot analysis of plasma membrane and cytosolic protein kinase activity with individual monoclonal antibodies. Peak 1 kinase (P1), peak 2 kinase (P2), and the cytosolic protein kinase (C) of oat were each separated by SDS–PAGE on a 10% (w/v) polyacrylamide gel, electroblotted to nitrocellulose, and immunostained with monoclonal antibodies specific to the cytosolic protein kinase from soybean. Four individual monoclonal antibodies, designated 14G5, 3B9, 12G8, and 3E8, were used for analysis. Although not readily visible in the photograph, the monoclonal antibody designated 3E8 does immunodecorate the M_r = 79 000 polypeptide.

We tested the hypothesis that peak 1 kinase represented a micellar-associated kinase while peak 2 kinase and the cytosolic kinase represented free soluble kinase by comparing the ability of each kinase to associate with Triton X-114 during a temperature-induced phase partition. Triton X-114 is a neutral detergent that forms a homogeneous solution at 4 °C but, at temperatures above 20 °C, separates into two phases: an upper phase depleted in detergent (the aqueous phase) and a lower phase enriched in detergent (the detergent phase). Proteins with hydrophobic sequences, such as integral membrane proteins, tend to associate with the detergent phase (Bordier, 1981; Pryde & Phillips, 1986). Using this system, we found that 90% of peak 1 kinase activity associated with the detergent phase. In contrast, 100% of the peak 2 kinase and the cytosolic kinase activities were found in the aqueous phase. This experiment indicated that peak 1 kinase has an affinity for a hydrophobic environment, unlike peak 2 kinase and the cytosolic kinase, and is capable of associating with detergent micelles.

Membrane Association of Kinase in Oat Extracts. The native size of the immunodecorated kinase polypeptides was examined in crude extracts of oat root. Oat roots were rapidly homogenized, and the protein was immediately precipitated with 7% (w/v) trichloroacetic acid. Two immunostained bands were observed in a Western blot of total oat root protein, a band at M_r = 61 000 and a band at M_r = 79 000, the same

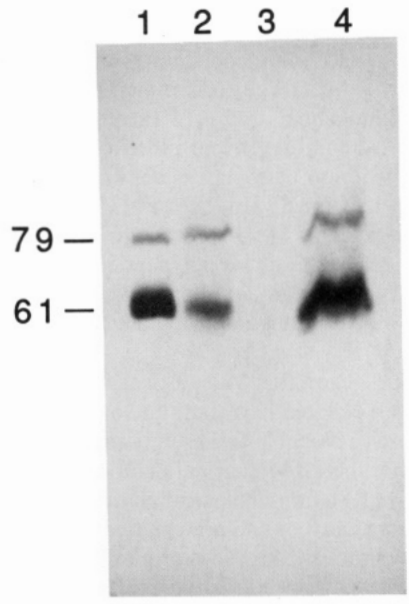

FIGURE 4: Western blot analysis of soluble and particulate fractions of an oat homogenate after rapid extraction and 7% (w/v) trichloroacetic acid treatment. Oat roots were harvested, homogenized in 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA, and strained through cheesecloth. Protein from the whole extract was either precipitated immediately with 7% (w/v) trichloroacetic acid or divided into soluble and particulate fractions by centrifugation in a microfuge for 15 min followed by precipitation with 7% trichloroacetic acid. Protein was separated by SDS–PAGE on a 10% (w/v) polyacrylamide gel, electroblotted to nitrocellulose, and immunostained with antibodies specific to the calcium-dependent protein kinase from soybean. Lanes 2, 3, and 4 represent 80 μg of the whole oat extract, the soluble fraction, and the particulate fraction, respectively. For comparative purposes, a 0.6-μg sample of partially purified peak 1 kinase was also included (lane 1). Positions of the M_r = 79 000 and the M_r = 61 000 immunostained polypeptides are indicated.

bands found in peak 1 kinase (Figure 4). The oat root homogenate was also rapidly separated into a soluble and a particulate fraction by centrifugation, followed by protein precipitation with 7% (w/v) trichloroacetic acid. A Western blot indicated that virtually all of the immunoreactive material was associated with the particulate fraction. These data indicate that the native molecular weight of the kinase is at least 61 000 and further suggest that, in situ, the kinase is associated with the membrane fraction of oat. Peak 2 kinase and the cytosolic kinase with M_r = 58 000 are probably degradation products of the M_r = 61 000 peak 1 kinase.

Lipid Stimulation of Kinase Activity. As mentioned previously, peak 1 kinase activity was found to be partially dependent upon added lipid. The lipid stimulation of peak 1 kinase was examined in more detail. Soybean lipid was found to stimulate kinase activity up to 20-fold when used at a concentration of 2 mg/mL in the kinase assay (Figure 5). The lipid preparation used in this experiment contained 40% (w/w) phosphatidylcholine. However, pure phosphatidylcholine had little effect upon kinase activity, indicating that some other component of the lipid mixture was involved in stimulation. Phosphatidylserine, which fulfills the lipid requirement of protein kinase C in animal cells, also had little effect upon the activity of peak 1 kinase. When the diacylglycerol diolein was included with phosphatidylserine in the kinase assay, there was no increase in stimulation. Several purified lipids, when tested individually, did have an effect upon the activity of peak 1 kinase. Phosphatidylinositol, platelet activating factor, and lysophosphatidylcholine were all able to stimulate kinase activity, but in no case was a stimulation greater than 5-fold observed (Figure 5).

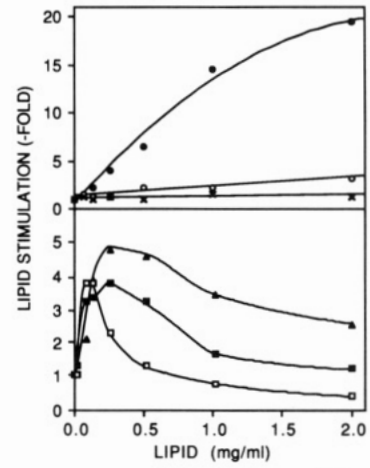

FIGURE 5: Lipid stimulation of peak 1 kinase activity. Peak 1 kinase activity was assayed in the presence of 73 μM Ca^{2+} using histone as a substrate. Lipids were included in the assay at the concentrations indicated. Crude soybean lipid (closed circles); phosphatidylserine (open circles); phosphatidylcholine (crosses); phosphatidylinositol (closed triangles); platelet activating factor (closed squares); lysophosphatidylcholine (open squares). Stimulation is relative to kinase activity obtained in the absence of lipid.

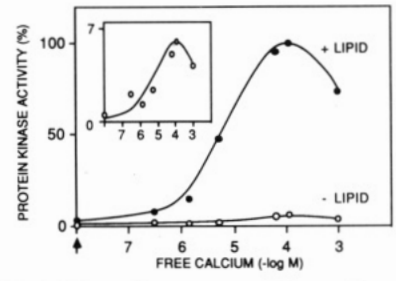

FIGURE 6: Effect of free calcium concentration on the activity of peak 1 kinase in the presence and absence of lipid. The activity of peak 1 kinase was determined in an assay mixture containing 0.2 mM EGTA and varying amounts of $CaCl_2$ to give the free calcium concentrations shown. The arrow indicates the presence of EGTA without the addition of $CaCl_2$. Kinase activity was assayed in the presence (closed circles) and absence (open circles) of 2 mg/mL soybean lipid. Activity is expressed as the percent of the maximum observed. The inset is an expansion of the minus lipid curve; the units for the abscissa and ordinate are identical to the figure, but the ordinate axis is expanded to show only 0–7%.

The activity of peak 1 kinase was dependent on calcium, both in the presence and in the absence of added lipid. However, the maximum activity observed in the presence of lipid was 20-fold higher than the maximum activity observed in the absence of lipid (Figure 6).

Limited Digestion of Peak 1 Kinase with Trypsin. Previous experiments indicated that peak 2 kinase and the cytosolic kinase might be degradation products of peak 1 kinase. The different properties of these enzymes could be explained by the loss of a region from the amino or carboxy terminus of the peak 1 kinase polypeptide. This hypothesis was tested by limited digestion with trypsin. Upon treatment with trypsin, peak 1 kinase gradually lost the ability to associate with Triton micelles, as determined by phase-partitioning with Triton X-114 (Figure 7A). Initially, greater than 80% of the kinase activity was found in the detergent phase, but after 5-min digestion, virtually all of the kinase activity was found in the aqueous phase. A Western blot indicated that, concurrent with the loss of detergent association, the polypeptides of M_r = 61 000 and M_r = 79 000 were degraded, resulting in the appearance of a new polypeptide of M_r = 55 000 (Figure 7B, total digest). This result indicated that a region with a molecular mass of 6000 daltons or less at a terminus of the M_r = 61 000

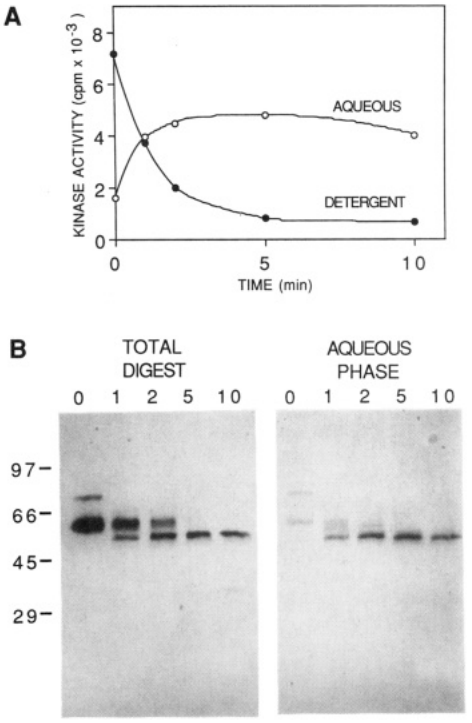

FIGURE 7: Limited tryptic digestion of peak 1 kinase removes a region necessary for detergent association. Peak 1 kinase was digested with 10% (w/w) trypsin for 0, 1, 2, 5, and 10 min at room temperature. In (A), the ability to associate with Triton micelles was tested by phase-partitioning in Triton X-114. Kinase activity was determined with 5 μL from detergent and aqueous phases, diluted 10-fold into assay mixture. In (B), a Western blot analysis was performed on the same tryptic digest of peak 1 kinase. The tryptic digest was analyzed directly by submitting 50 μL of the total digest from each time point to SDS–PAGE on a 10% polyacrylamide gel, electroblotting to nitrocellulose, and immunostaining with antibodies specific to the cytosolic protein kinase from soybean. In addition, a portion of the tryptic digest (50 μL) was partitioned in Triton X-114, and the entire aqueous phase was submitted to SDS–PAGE, electroblotted, and immunostained. For the Western blots, the times of tryptic digestion are indicated in minutes, and the migration positions of molecular mass markers are indicated in kilodaltons.

polypeptide was necessary for micellar association of the kinase. A Western blot of the aqueous phase confirmed this interpretation (Figure 7B, aqueous phase). Initially, very little of the peak 1 polypeptide was present in the aqueous phase, but after tryptic digestion, the degraded product of M_r = 55 000 rapidly appeared. Following tryptic digestion, kinase activity is still dependent on calcium; there is a greater than 10-fold stimulation by added calcium, resembling the degree of calcium dependence observed with the native enzyme. Longer tryptic digestion resulted in a loss of kinase activity and the appearance of an immunodecorated polypeptide at M_r = 36 000 (results not shown).

The kinase activity was also examined for lipid dependence following limited proteolytic digestion. Initially, peak 1 kinase was stimulated 19.8-fold by 2 mg/mL lipid, but upon treatment with trypsin, the stimulation dropped to 7-fold after 1 min, 2.7-fold after 5 min, and 1.7-fold after 10 min. The kinase still exhibited calcium dependence following 10-min digestion with trypsin.

These data indicate that proteolytic digestion of the peak 1 kinase converts it into a lipid-independent form unable to associate with detergent micelles, but which retains its dependence on calcium. These properties of the membranous kinase following limited tryptic digestion are similar to those of the protein kinase derived from the "cytosolic" fraction of oats.

Discussion

A calcium- and lipid-dependent protein kinase has been partially purified from the plasma membrane fraction of oat roots. Immunological studies with monoclonal antibodies specific to a calcium-dependent protein kinase isolated from the soluble fraction of soybean identify two cross-reactive polypeptides associated with the plasma membrane kinase: a major polypeptide of M_r = 61 000 and a minor polypeptide of M_r = 79 000. These two polypeptides could be separate protein kinases with similar properties, or the M_r = 61 000 polypeptide may be a degradative product of the M_r = 79 000 polypeptide.

Our results suggest that a soluble calcium-dependent kinase (M_r = 58 000) isolated from both the oat plasma membrane and the cytosol is probably the result of proteolytic degradation of the membrane-associated calcium- and lipid-dependent protein kinase. An immunological analysis of protein rapidly extracted from oat root and precipitated with trichloroacetic acid indicates that the kinase is originally present as the M_r = 61 000 and M_r = 79 000 polypeptides. Both of these polypeptides are associated almost exclusively with the membrane fraction of the oat extract. In addition, limited proteolysis with trypsin yields a calcium-dependent kinase that is lipid independent and is unable to associate with Triton micelles. These results indicate that the kinase is normally present in oat as a calcium- and lipid-dependent protein kinase that is capable of associating with the plasma membrane. The soluble form probably arises following homogenization of the root tissue, during which proteases are released from the large central vacuole observed in plant cells. Alternatively, proteolysis might also be occurring in situ, and could possibly perform a biological function (Allen, 1987; Hansen et al., 1988).

A cDNA clone encoding the calcium-dependent protein kinase from soybean has been sequenced (Harper et al., 1991). This clone codes for a protein which contains an amino-terminal kinase catalytic domain most similar to those of the calcium- and calmodulin-dependent protein kinases, attached to a carboxy-terminal calmodulin-like region with four perfect calcium-binding hands. The predicted sequence of this kinase can explain its ability to directly bind and be activated by calcium.

The evidence presented here suggests that the soluble form of the calcium-dependent protein kinase observed in crude extracts of oat is derived from a membrane-bound form of the enzyme. Southern blots of soybean and *Arabidopsis thaliana* genomic DNA probed with a restriction fragment of the cDNA encoding the soybean calcium-dependent protein kinase suggest that multiple genes encoding calcium-dependent protein kinases are present in higher plant species (Harper et al., 1991). These molecular cloning data indicate that there exists a family of calcium-dependent protein kinases with close sequence identity. Some members of this family may be soluble whereas others may be membrane-associated. There is no biochemical evidence to indicate that the soybean calcium-dependent protein kinase described by Putnam-Evans et al. (1990) is membrane-bound. Therefore, this soybean isoform may represent a purely soluble member of the family.

Limited digestion of the oat kinase with trypsin indicates that a region located near a terminus of the M_r = 61 000 polypeptide is involved in lipid-stimulation and micellar association. An examination of the terminal regions of the protein predicted from one cloned gene for a soybean calcium-dependent protein kinase does not reveal a domain containing hydrophobic amino acids that could mediate lipid association. It may be that disparate amino acids are involved

in the association or that removal of the terminus destabilizes a tertiary structure necessary for the association. Alternatively, future examination of the sequence for other isoforms may reveal a clear hydrophobic domain.

In the presence of calcium, the calcium- and lipid-dependent protein kinase is stimulated up to 20-fold by soybean lipid. This stimulation is larger than any previous report in the plant literature (Harmon, 1989) and indicates a true dependence on lipid for maximal activity. Although structurally different, the calcium- and lipid-dependent protein kinase partially purified from the plasma membrane of oat root shares some features with animal protein kinase C, the most readily apparent being a strong dependence upon both calcium and lipid for maximal activity. Protein kinase C is also capable of associating with microsomes, and appears to depend upon membrane association for proper regulation of activity (Nishizuka, 1986). There are also some clear biochemical differences between the plant calcium- and lipid-dependent protein kinase and animal protein kinase C. First, all known forms of protein kinase C are stimulated by phosphatidylserine (Nishizuka, 1988), but the plant kinase, though stimulated by lipid, shows little stimulation by phosphatidylserine. Second, protein kinase C can be removed from the membrane by treatment with the calcium chelators EDTA and EGTA (Philips et al., 1989); we find that the oat calcium- and lipid-dependent protein kinase remains associated with the plasma membrane even after treatment with 4 mM EDTA.

We would like to emphasize that this kinase we have described is a lipid-dependent protein kinase. This does not necessarily mean that the kinase is *regulated* by lipids in vivo. The calcium- and lipid-dependent protein kinase was isolated from the plasma membrane, and so it may be that the lipid plays a purely structural role. Further studies are necessary to determine whether or not lipid is capable of acting in a regulatory fashion. However, the data presented here do indicate that the predominant calcium- and lipid-dependent protein kinase associated with the plant plasma membrane is not protein kinase C, as found in animal cells, but instead belongs to a novel class of calcium-dependent but calmodulin- and phosphatidylserine-independent protein kinases.

Association of the calcium- and lipid-dependent protein kinase with the oat plasma membrane is suggestive that the kinase might be involved in signal transduction events that occur at the membrane. We have previously identified the oat plasma membrane H^+-ATPase as a phosphoprotein (Schaller & Sussman, 1988), and preliminary results indicate that the calcium- and lipid-dependent protein kinase partially purified from the oat plasma membrane is capable of phosphorylating the H^+-ATPase in vitro (results not shown). Studies by Kolarov et al. (1988) indicate that kinase-mediated phosphorylation of the yeast plasma membrane H^+-ATPase stimulates activity. Whether phosphorylation modulates activity of the higher plant H^+-ATPase in situ is as yet unknown but is an attractive hypothesis to explain rapid changes observed in electrophysiological measurements of plant cells after treatment with growth-regulating hormones and light. Future work will be aimed at more clearly characterizing the in situ substrates for this protein kinase and its role in the signal transduction pathway.

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