Characterization of an *Arabidopsis* Calmodulin-like Domain Protein Kinase Purified from *Escherichia coli* Using an Affinity Sandwich Technique†

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ABSTRACT: A full-length cDNA encoding a calcium-dependent protein kinase with a calmodulin-like domain from *Arabidopsis thaliana* (AK-1 for *Arabidopsis* kinase-1) has been expressed as a fusion protein (called AK-1-6H) in *Escherichia coli* and purified to near homogeneity with high specific activity (typically 2000 nmol min^{-1} mg^{-1}) using an "affinity sandwich" technique. AK-1-6H protein phosphorylation activity using histone as substrate was stimulated up to 50-fold by the addition of calcium alone or up to 5-fold by the addition of specific phospholipids alone; together calcium and these lipids acted synergistically to give up to 100-fold stimulation. We earlier reported that, of a wide array of lipids tested, only phosphatidylinositol and lysophosphatidylcholine stimulated histone phosphorylation by AK-1 [Harper, J. F., Binder, B. M., & Sussman M. R. (1993) *Biochemistry 32,* 3282–3290]. The properties of lipid stimulation were further explored by testing the effects of lipids on autophosphorylation and on other catalytic properties of the kinase. Although phosphatidylinositol stimulated autophosphorylation up to 11-fold, lysophosphatidylcholine was inactive. Basic peptides such as polylysine (average M_r ~37 100) were potent, mixed-type inhibitors of AK-1-6H with an IC_{50} of 2 nM. In the presence of phosphatidylinositol, the inhibition was reduced and the IC_{50} for polylysine was increased to 341 nM. As with autophosphorylation, lysophosphatidylcholine was inactive in alleviating the basic peptide inhibition, which suggests that this lipid's stimulatory effects using exogenous substrate are distinct from those of phosphatidylinositol. These results are consistent with a model in which phosphoinositides directly interact with the kinase protein and alleviate a catalytic block caused by basic charges.

In higher plants, calcium acts as a second messenger regulating a variety of cellular processes including photomorphogenesis, thigmomorphogenesis, and interactions with pathogens (Hedrich & Schroeder, 1989; Schroeder & Hedrich, 1989; Schroeder & Thuleau, 1991; Trewavas & Gilroy, 1991; Roberts & Harmon, 1992). Other plant second messengers may include inositol trisphosphate (Schumaker & Sze, 1987; Gilroy *et al.*, 1990; Blatt *et al.*, 1990), lysophosphatidylcholine (Palmgren & Sommarin, 1989; Scherer *et al.*, 1989), and the phosphoinositides (Morré *et al.*, 1984; Drøbak & Ferguson, 1985; Memon & Boss, 1990; Kamada & Muto, 1991), but the intracellular receptors or molecular mechanisms of action have not yet been conclusively identified. One mechanism by which these second messengers act in plants is via calcium-dependent protein kinases (Harmon *et al.*, 1987; Roberts, 1989; Battey, 1990; Polya *et al.*, 1990; Putnam-Evans *et al.*, 1990; Weaver *et al.*, 1991) or calcium- and lipid-stimulated kinases (Schäfer *et al.*, 1985; Elliott & Skinner, 1986; Martiny-Baron & Scherer, 1989; Schaller *et al.*, 1992).

In a search for plant protein receptors for calcium, a new type of protein kinase was purified which was stimulated by calcium without the addition of calmodulin (Harmon *et al.*, 1987; Putnam-Evans *et al.*, 1990). This new type of calcium-dependent protein kinase has been cloned and sequenced and found to contain a kinase catalytic domain fused to a domain that contains four EF hands and closely resembles calmodulin (Harper *et al.*, 1991). This kinase is called a calmodulin-like domain protein kinase or CDPK[1] (Roberts, 1993). In plants, CDPKs have been proposed to be associated with the cytoskeleton (Putnam-Evans *et al.*, 1989) or plasma membrane (Schaller *et al.*, 1992) and to phosphorylate the plasma membrane H^+-ATPase (Schaller & Sussman, 1988) and nodulin 26 from the nitrogen-fixing nodules of soybean (Weaver & Roberts, 1992). Until recently, CDPK had only been identified in higher plants, but a CDPK has now been cloned from the malarial parasite *Plasmodium* (Zhao *et al.*, 1993), and biochemical studies suggest its presence in *Paramecium* as well (Gunderson & Nelson, 1987; Son *et al.*, 1993). In addition, an immunologically similar protein has been detected in HeLa cells (Harmon *et al.*, 1989) and rat brain (Son *et al.*, 1993), indicating that these kinases are present in a variety of species across kingdoms. We have previously reported (Harper *et al.*, 1993) the isolation and sequencing of a cDNA clone from *Arabidopsis thaliana* that encodes a CDPK we call AK-1 for *Arabidopsis* kinase-1. AK-1 appears to belong to a gene family containing at least five members (E. Hrabak, personal communication). The predicted amino acid sequence of this *Arabidopsis* CDPK is very similar to the kinases cloned and sequenced from soybean

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[1] Abbreviations: AK-1, *Arabidopsis* kinase 1; AK-1-6H, *Arabidopsis* kinase 1 fusion protein attached to a glutathione S-transferase molecule at the N-terminus and a 6× histidine moiety at the C-terminus; CDPK, calmodulin-like domain protein kinase with a kinase catalytic domain fused to a calmodulin-like domain; CHAPS, 3-[(3-cholamidopropyl)-diethylammonio]-1-propanesulfonate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IC_{50}, concentration of a compound resulting in 50% inhibition of an enzyme; PMSF, phenylmethanesulfonyl fluoride; SB-16, 3-(*N*-hexadecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate.

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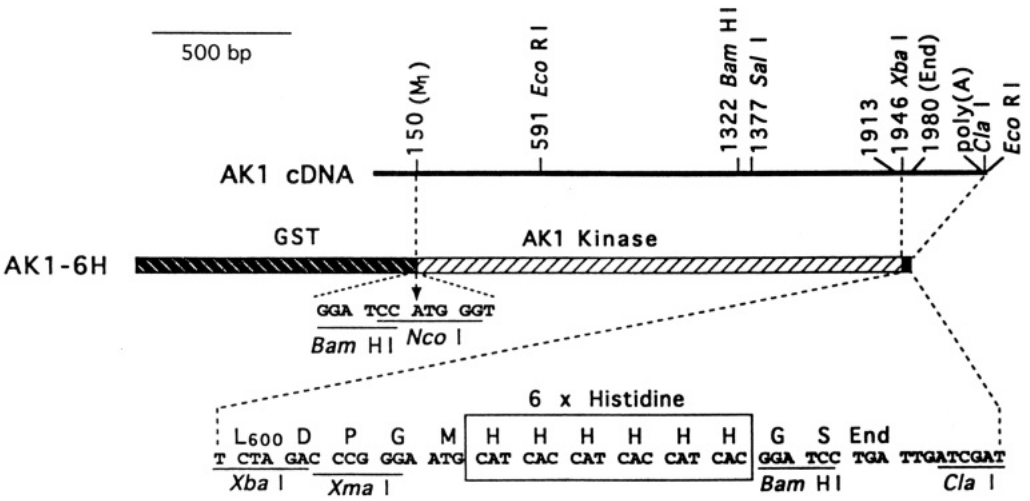

FIGURE 1: Diagram of AK-1-6H fusion protein construct. The AK-1 full-length cDNA (AK-1 cDNA) is diagrammed with important enzyme sites and features indicated. The numbers above AK-1 correspond to base pair positions in the cDNA sequence (GenBank accession number L14771). The structure of the AK-1-6H fusion protein construct (AK-1-6H) is diagrammed below. A glutathione S-transferase (GST) coding sequence from pGEX-2T is fused to the AK-1 N-terminus at amino acid M_1 (base pair position 150). The *Nco*I site in the GST/AK-1 linker is not unique. A 6× histidine motif is added to the C-terminus at amino acid L_{600} (base pair position 1946). Stop codons are indicated as End.

(Harper *et al.*, 1991) and carrot (Suen & Choi, 1991). The predicted amino acid sequence suggests that AK-1, like the other clones from plants and the clone from *Plasmodium* (Zhao *et al.*, 1993), is a single peptide with a kinase domain linked via a junction domain to a calmodulin-like domain with four highly conserved EF hands (Harper *et al.*, 1993).

In our initial study, we expressed AK-1 as an N-terminally truncated protein fused to glutathione S-transferase [called KGF-1 in Harper *et al.* (1993)]. Although this truncated fusion protein was stimulated by calcium alone, we were surprised to find that it was also activated synergistically by specific lipids (Harper *et al.*, 1993) and was biochemically similar to a membrane-associated, calcium- and lipid-dependent protein kinase partially purified from oat roots (Schaller *et al.*, 1992). In particular, lysophosphatidylcholine and phosphatidylinositol stimulate both kinases while phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine do not (Schaller *et al.*, 1992; Harper *et al.*, 1993).

In this study we expressed AK-1 that now contains the N-terminus in *Escherichia coli* as a fusion protein attached to a glutathione S-transferase molecule at its N-terminus and a six-histidine moiety at its C terminus (called AK-1-6H for AK-1 6× histidine). AK-1-6H was purified to homogeneity by a technique we call "affinity sandwich" purification. This fusion protein is being used as a model for investigating the mechanisms of regulation by lipid and calcium. Stimulatory lipids (the phosphoinositides, lysophosphatidylcholine, and lysophosphatidylinositol) activated AK-1-6H by increasing the V_{max} of the enzyme with little effect on its apparent K_m for ATP or the protein substrate. In contrast, calcium altered both the V_{max} and the apparent K_m for ATP and protein substrate, suggesting that these two regulators act via different mechanisms. Phosphatidylinositol, but not lysophosphatidylcholine, interacts directly with AK-1-6H by stimulating autophosphorylation. AK-1-6H was also strongly inhibited at nanomolar concentrations by poly(L-lysine) and poly(L-arginine) in the absence of added lipid. Addition of lipid increased the IC_{50} of poly(L-lysine).

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP was obtained from New England Nuclear (Wilmington, DE). Phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate were obtained from Sigma (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN) and were also kindly supplied by Dr. Richard Anderson (University of Wisconsin). Lipids from all three sources gave similar results. Syntide 2 (amino acid sequence PLARTLSVAGLPKK) was obtained from Dr. Alice Harmon (University of Florida). Glutathione–Sepharose beads were from Pharmacia (Piscataway, NJ) and nickel–nitrilotriacetic acid (NTA) resin was from QIAgen (Chatsworth, CA). All other chemicals were from Sigma.

Construction of AK-1 "Affinity Sandwich" Fusion Protein. A fusion protein was constructed in which AK-1 was sandwiched between a glutathione S-transferase domain on its N-terminus and a 6× histidine motif on its C-terminus.

Important features of the resulting AK-1-6H construct are outlined in Figure 1. AK-1-6H is based on clone Ak1-7 (also called Com7) which represents the entire coding sequence of AK-1 fused in frame to the 3′ (C-terminal) end of the glutathione S-transferase coding sequence in the vector pGEX-2T. Ak1-7 was made in two steps. First, a fusion construct designated pKF1-149 was made which contains the coding sequence for the first 149 amino acids of AK-1 cloned as a *Bam*HI/*Eco*RI fragment from pKME3 (Harper *et al.*, 1993) into pGEX-2T. pKME3 was previously isolated from a PCR amplification of genomic DNA and sequenced to verify the absence of PCR errors. Ak1-7 was completed by cloning the remainder of the kinase coding sequence from pAK-NX-25 (Harper *et al.*, 1993) into pKF1-149 as a 1.6-kbp *Eco*RI fragment.

The affinity sandwich construct AK-1-6H was derived from Ak1-7 by inserting the coding sequence for six histidines at the C-terminal end. A synthetic oligomer encoding a 6× histidine motif was synthesized and inserted as an *Xba*I/*Cla*I fragment between the *Xba*I site at position 1946 of the AK-1 cDNA and a *Cla*I site present in the linker region following the poly(A) sequence. The 6× histidine insertion truncates the C-terminal end of AK-1 at residue Leu_{600}, which is locatedin the divergent C-terminal domain 12 residues distal to the end of the conserved calmodulin-like domain.

"Affinity Sandwich" Purification of Fusion Protein. E. coli transformed with recombinant plasmids were grown under antibiotic selection in LB medium containing 1 M sorbitol

Table 1: Purification of AK-1-6H

fraction	protein (mg)	total activity[a] (nmol min^{-1})			specific activity (nmol min^{-1} mg^{-1})		
		control	calcium	calcium + lipid	control	calcium	calcium + lipid
crude homogenate	31.98	124.7	678	1989	3.9	21.2	62.2
crude supernatant	31.86	133.8	1663	1150	4.2	52.2	36.1
Q-Sepharose	12.0	115.5	2131	3022	9.6	177.6	251.9
nickel column	1.11	61.1	536	407	55.0	483	367.0
glutathione–sepharose	0.533	3.6	140	232	6.8	264	435.2

[a] AK-1-6H activity was measured using syntide 2 as protein substrate.

and 2.5 mM betaine at 37 °C (Blackwell & Horgan, 1991). Overnight cultures of *E. coli* were diluted 10-fold and grown an additional 2 h at 37 °C before addition of 1.0 mM IPTG, and then they were grown an additional 1 h at 25 °C. Cells were pelleted and resuspended in 4 mL of breakage buffer containing 150 mM NaCl, 2 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 50 mM Tris, pH 7.3. Cells were lysed by sonication and centrifuged at 10000*g* for 15 min.

Since this kinase fusion protein contains a glutathione S-transferase molecule fused to its N-terminus and a 6× histidine moiety fused to its C-terminus, we used two specific affinity columns to select for full-length fusion protein. We call this method "affinity sandwich" purification.

Before using affinity sandwich purification, we applied the crude supernatant to a 4-mL Q-Sepharose column preequilibrated in buffer containing 20 mM Tris, 2 mM dithiothreitol, 2 mM $CaCl_2$, and 0.1 mM PMSF, pH 7.5. The column was then washed with 10 mL each of this Tris buffer with increasing amounts of NaCl and a decrease in the pH. The buffers used contained (A) 50 mM NaCl, pH 7.5; (B) 100 mM NaCl, pH 7.5; (C) 200 mM NaCl, pH 7.5; (D) 200 mM NaCl, pH 7.0; (E) 200 mM NaCl, pH 6.6; (F) 500 mM NaCl, pH 6.6; (G) 1.5 M NaCl, pH 6.6. Most kinase activity eluted with fraction F.

Fraction F from the Q-Sepharose column was diluted 3-fold with 50 mM Tris, pH 8.0, and applied to a 2-mL nickel–nitrilotriacetic acid resin (nickel) column. This nickel column had been preequilibrated with 50 mM Tris, pH 8.0. The column was washed with 50 mM Tris, 0.1 mM PMSF, pH 8.0, and 10 mM imidazole to remove contaminating proteins. Fusion protein was eluted by competition with imidazole with 6 mL of 50 mM Tris buffer and 0.1 mM PMSF, pH 6.0, containing 350 mM imidazole. This fraction was diluted 5-fold with 50 mM Tris, pH 7.3, and applied to a 2-mL glutathione–Sepharose column. The column was washed with 6 volumes of buffer containing 150 mM NaCl, 50 mM Tris, 2 mM dithiothreitol, and 0.1 mM PMSF, pH 7.3. Fusion protein was then eluted by competition with free glutathione with 3 volumes of elution buffer containing 50 mM Tris, 5 mM glutathione, and 0.1 mM PMSF, pH 8.0. All purification steps were carried out at 4 °C.

Recovery of kinase activity was monitored throughout purification as described below using either histone IIIS or syntide 2 as substrate and protein content was analyzed using the method of Bradford (1976). Recovery of protein and specific activity of kinase activity at each step is outlined in Table 1. Typically this method of purification with three column chromatography steps gave approximately a 7–10-fold purification of kinase activity with a specific activity of between 400 and 600 nmol min^{-1} (mg of protein)$^{-1}$ using syntide 2 as substrate. Proteins from each step of the purification were electrophoresed in a 10% polyacrylamide gel according to the methods of Laemmli (1970), and a typical gel is shown in Figure 2. Typically, a 500-mL preparation

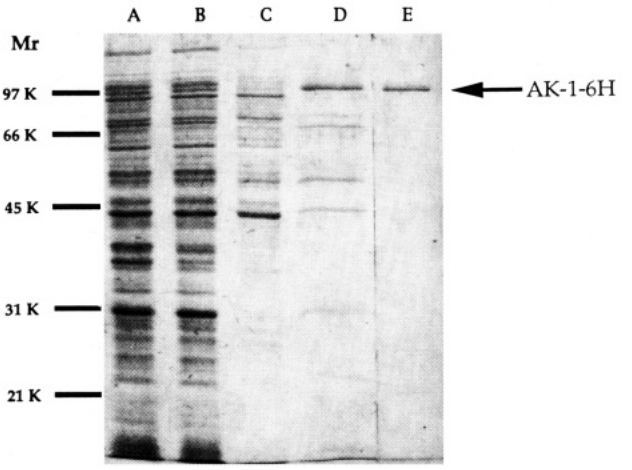

FIGURE 2: Purification of AK-1-6H. Proteins from various steps of the purification were separated by electrophoresis on a sodium dodecyl sulfate–10% (w/v) polyacrylamide gel and visualized with Coomassie Brilliant Blue. AK-1-6H was not expressed to a high enough amount to visualize until it was affinity purified. One microgram of protein was loaded in each lane. Lane A, total protein from sonicated *E. coli*; lane B, soluble proteins applied to the Q-Sepharose column; lane C, peak fraction eluted from the Q-Sepharose column with maximum kinase specific activity; lane D, proteins eluted from the nickel-NTA column; lane E, final purification on the glutathione–Sepharose column. Lines on the left side indicate molecular weight markers and the arrow marks the position of AK-1-6H.

yielded 0.5 mg of purified, active AK-1-6H. Using these methods, no measurable kinase activity is recovered in the elution buffer from *E. coli* that have been transformed with the pGEX-2T parent vector.

Protein Phosphorylation Activity. Protein kinase activity of the AK-1-6H fusion protein was assayed in 20 mM Tris, pH 7.3, 6 mM $MgCl_2$, 50 μM ATP, and 100–300 ng of AK-1-6H. Previous work has shown that this kinase assay buffer in the absence of added $CaCl_2$ or EGTA contains 83 μM calcium (Schaller *et al.*, 1992). Free calcium levels were buffered to either 2.5 nM or 100 μM [free calcium concentrations from calculations in Caldwell (1970)]. To obtain 2.5 nM, no $CaCl_2$ was added and 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) was added to give 2.5 nM free calcium. To obtain 100 μM calcium, 1 mM $CaCl_2$ was added and buffered with 0.97 mM EGTA. Crude lipid mixtures and purified lipids were suspended in buffer by probe sonication. Thin-layer chromatography indicated that no detectable breakdown of purified lipids occurred by this procedure (data not shown). Lipids were stored under argon.

We found that the best exogenous substrates were syntide 2, myelin basic protein, and histone IIIS. Of these, syntide 2 was the best substrate. Other substrates tested included protamine chloride, cytochrome *c*, myosin light chains, and actin. The apparent K_ms for syntide 2 and histone IIIS in the presence of calcium were approximately 0.048 mg/mL (or 32.1 μM) and 0.11 mg/mL, respectively. Kinetic parameters for AK-1-6H (apparent K_m and V_{max}) were determined with

plots. Unless otherwise noted, 0.5 mg/mL histone or 0.2 mg/mL syntide (133 μM) were used in our assays. Each sample contained 10 μCi of [γ-^{32}P]ATP. Assays were initiated by the addition of ATP and allowed to incubate for 20 min at room temperature. For most experiments this varied between 19 and 23 °C; for kinetic measurements involving ATP and protein substrate, the ambient temperature was 20 °C. Two methods were used to terminate the assay.

The first is a modification of the method described in Schaller *et al.* (1992). Briefly, the assay mixture was spotted onto a 2.5-cm^2 piece of Whatman 3MM paper and the paper was immersed in 10 mL/square cold 10% (w/v) trichloroacetic acid and 10 mM pyrophosphate to quench the reaction. Unincorporated radioactivity was removed with four 15-min washes with the trichloroacetic acid plus pyrophosphate solution, followed by one wash with 95% ethanol and one wash with ether. The paper squares were then allowed to dry.

In the second method, the assay mixture was spotted onto cellulose phosphate paper (P81) circles from Whatman. This paper has been shown to bind basic proteins even after phosphorylation (Casnellie, 1991). The paper circles were then immersed in 10 mL/circle 75 mM phosphoric acid followed by four 5-min washes with phosphoric acid. Both methods gave similar results; however, the second method was quicker and had less background and most figures in this paper use this method. In control experiments, we found that neither crude lipid nor poly(L-lysine) altered the ability of substrate protein to bind to the P81 paper (data not shown).

Radioactivity incorporated into substrate protein was counted by measuring Cerenkov radiation with a scintillation counter.

Autophosphorylation was measured under the conditions described above except that no exogenous substrate was added. The reactions were stopped by addition of Laemmli stop buffer and the proteins were electrophoresed on a 10% polyacrylamide gel according to the methods of Laemmli (1970) and stained with Coomassie Brilliant Blue. The protein kinase band was excised and solubilized in 300 μL of 85% (v/v) phosphoric acid and 300 μL of 30% (v/v) H_2O_2 at 60 °C overnight, and radioactivity was measured using liquid scintillation counting.

RESULTS

(I) Affinity Sandwich Purification of AK-1-6H. We have previously reported the biochemical activity and regulation of a partially purified AK-1 fusion protein [called KGF-1 in Harper *et al.* (1993)]. In our original description of AK-1, we used methods modified from Smith and Johnson (1988) for the expression and partial purification of an N-terminally truncated AK-1 fusion protein fused to glutathione S-transferase alone (Harper *et al.*, 1993). When similar methods were used to express and purify a full-length clone of AK-1, we were unable to obtain pure, full-length protein (based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis). In this study we have overcome this difficulty by fusing six histidines to the C-terminus of the fusion protein, allowing a second affinity column to be used to select for full-length fusion protein. This two-step purification selected for full-length fusion protein over truncated products because only the full-length fusion protein contained both the C-terminus with the 6× His (selected for by the nickel column) and the N-terminus with the glutathione S-transferase (selected for with the glutathione column). The proteins contained in each step of the purification are shown in Figure 2. The final step in the purification yielded one major protein band with a M_r of approximately 100 000, which is close to the predicted M_r of 99 000 for the kinase fusion protein. We find that this band represents >95% of the protein recovered when Coomassie-stained bands are individually excised and solubilized and the absorbance at 610 nm is analyzed. This final fraction typically had a kinase activity with a specific activity of 400–600 nmol min^{-1} (mg of protein)$^{-1}$ using syntide 2 as substrate (Table 1). Some preparations yielded even higher specific activities of 2800 nmol min^{-1} (mg of protein)$^{-1}$. This is a much higher specific activity than we have previously reported (Harper *et al.*, 1993) and is probably due to the higher purity of the kinase preparation used in the current study.

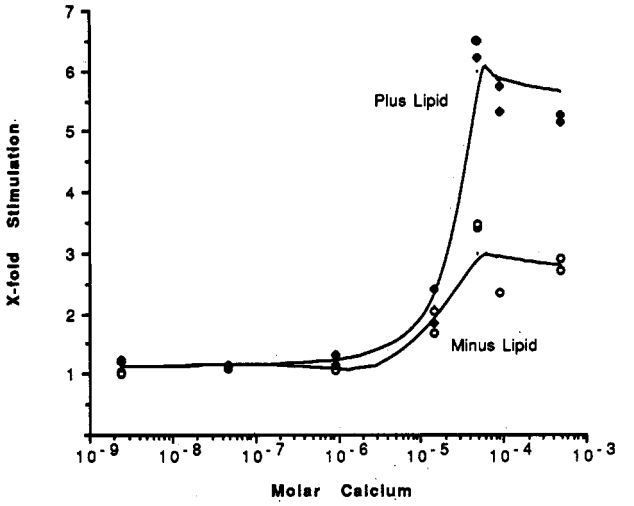

FIGURE 3: Calcium dependence of AK-1-6H activity. The stimulation of AK-1-6H by calcium was measured at various concentrations of $CaCl_2$ in the absence (○) or presence (●) of 0.5 mg/mL of crude lipid. Maximum activity was seen with approximately 100 μM $CaCl_2$. To obtain free calcium levels of 0.1, 2, 10, 30, 100, 200, 400, and 1000 μM, 1 mM $CaCl_2$ was added and calcium buffered with the following millimolar concentrations of EGTA, respectively: 1.77, 1.11, 1.07, 1.045, 0.97, 0.873, 0.673, and 0. To obtain a free calcium level of 2.5 nM, no extra calcium was added and 1 mM EGTA was added. The assay buffer with no added calcium or EGTA has been measured to contain 83 μM calcium (Schaller *et al.*, 1992). Calculations for this calcium were from Caldwell (1970). AK-1-6H activity was monitored using 0.5 mg/mL histone IIIS as a phosphorylation substrate. All data in this figure are normalized to phosphorylation levels in the absence of added lipid and at 2.5 nM calcium.

(II) Calcium and Lipid Activate AK-1-6H Synergistically. In our initial studies on partially purified, truncated AK-1, we measured up to 6-fold stimulation of AK-1 by calcium (Harper *et al.*, 1993). In the current study with a more purified AK-1-6H, we found up to 50-fold stimulation by calcium (Table 1). It is likely that truncated products of the AK-1 fusion protein are active and are calcium-independent; with a purer preparation of full-length AK-1-6H we can measure the true extent of activation by calcium. The activity of the AK-1-6H fusion protein was typically stimulated 2–5-fold by the addition of crude lipid from soybean using histone as protein substrate. However, phosphorylation of the 14 amino acid long peptide syntide 2 was stimulated no more than 2-fold by lipids. The stimulation by crude lipid was not due to a shift in the calcium sensitivity of AK-1-6H (Figure 3). Calcium gave half-maximal activation at approximately 33 μM in the presence or absence of added crude lipid. Crude lipid stimulated AK-1-6H in a concentration-dependent manner with half-maximal stimulation at 54 ± 17 μg/mL in the absence of calcium and 48 ± 18 μg/mL in the presence of calcium. Figure 4 shows one such comparison.

We compared how calcium and lipid independently alter the enzymatic parameters of AK-1-6H. The addition of

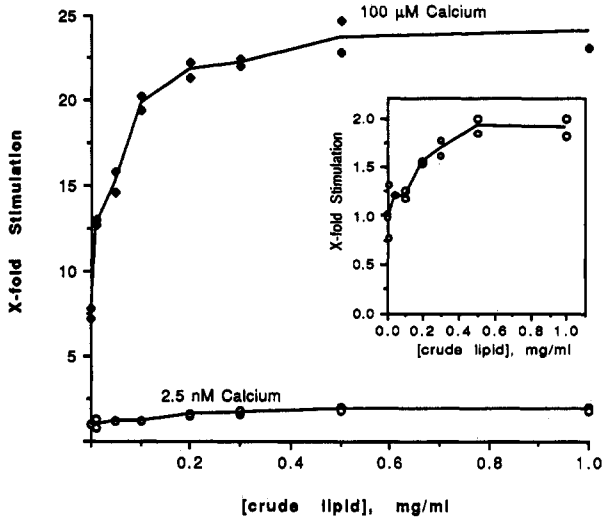

FIGURE 4: Lipid stimulation of AK-1-6H activity. The stimulation of histone phosphorylation by AK-1-6H with various amounts of crude phosphatidylcholine isolated from soybean was measured in the absence (○ and inset) or presence (●) of 100 μM $CaCl_2$. Under both conditions, maximum activity was observed with 0.2–0.3 mg/mL crude lipid. In this figure, the maximum stimulation with lipid at 2.5 nM calcium was 1.8-fold higher than without lipid added (inset). All data in this figure are normalized to phosphorylation levels in the absence of added lipid and at 2.5 nM calcium.

Table 2: Effects of Calcium and Lipid on the Apparent K_m and V_{max} of AK-1-6H[a]

conditions	apparent K_m for ATP[b] (μM)	apparent K_m for histone[c] (μg/mL)	V_{max} (pmol/min)
control	27.9 ± 10	37 ● 2	0.73 ± 0.33
calcium	5.9 ± 0.7	160 ± 50	6.7 ± 2.0
lipid	26.7 ± 9.0	40 ± 3	1.2 ± 0.2
calcium + lipid	3.8 ± 0.2	164 ± 15	12.9 ± 1.8

[a] Phosphorylation assays were carried out as described in Materials and Methods using histone IIIS as substrate and 100 ng of AK-1-6H. Samples with no added calcium were buffered at 2.5 nM with EGTA while samples with calcium added were buffered to 100 μM calcium with EGTA. Samples with added lipid were at 0.5 mg/mL crude soy phosphatidylcholine. [b] ATP concentration was varied between 1 and 100 μM. [c] Histone concentration was varied between 0.01 and 0.5 mg/mL.

calcium to AK-1-6H decreased its apparent K_m for ATP by 3–6-fold and increased its apparent K_m for histone by 2.4–6-fold. In addition, calcium increased the V_{max} of the kinase 5.5–20-fold. In contrast, addition of crude lipid had little measurable effect on the apparent K_m of ATP, histone, or syntide 2 but did increase the V_{max} of this kinase 1.4–2-fold using syntide 2 as substrate and 1.9–3-fold using histone as substrate (Table 2). This effect was most easily seen in the presence of calcium.

Like other CDPKs (Harmon *et al.*, 1987; Putnam-Evans *et al.*, 1990; Son *et al.*, 1993), AK-1-6H autophosphorylates in a calcium-dependent manner. Autophosphorylation is also augmented 2–11-fold by the presence of crude lipid (Figure 5), suggesting that lipid is interacting directly with the kinase.

(III) Lipid Specificity of AK-1-6H Activation. We previously showed that the N-terminally truncated AK-1 fusion protein was stimulated by phosphatidylinositol and lysophosphatidylcholine but not by other lipids such as phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine (Harper *et al.*, 1993). In the present study, we have tested the specificity of this response in more detail. In comparing phosphatidylinositol, phosphatidylinositol monophosphate, and phosphatidylinositol bisphosphate, we find that all three phosphoinositides stimulate AK-1-6H phosphorylation of

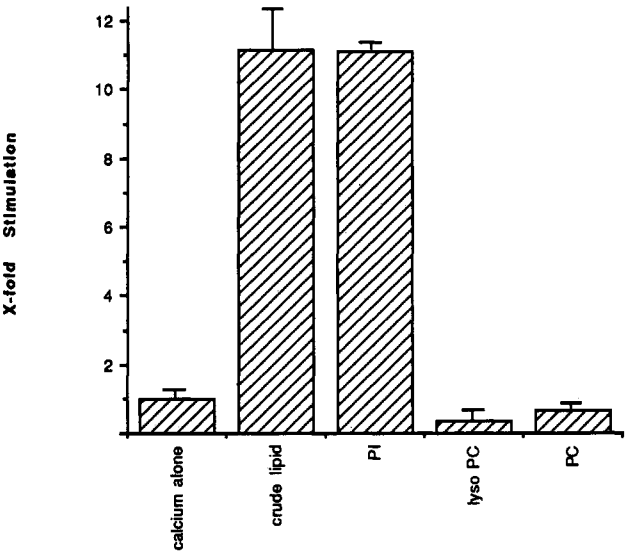

FIGURE 5: Lipid-specific stimulation of AK-1-6H autophosphorylation. The autophosphorylation of AK-1-6H was compared under five conditions: no added lipid (calcium alone), 0.5 mg/mL crude lipid (crude lipid), 200 μM phosphatidylinositol (PI), 200 μM lysophosphatidylcholine (lyso-PC), and 200 μM phosphatidylcholine (PC). All samples contained 100 μM calcium. Data represent the mean of two separate samples under each condition. Bars represent the range around the mean. All data in this figure are normalized to phosphorylation levels in the absence of added lipid and at 100 μM calcium.

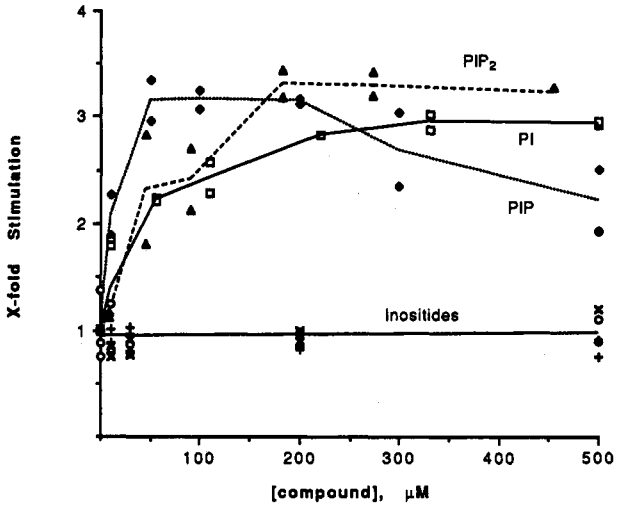

FIGURE 6: Effects of the phosphoinositides and inositides on AK-1-6H. Phosphatidylinositol (□, PI), phosphatidylinositol monophosphate (●, PIP), and phosphatidylinositol bisphosphate (▲, PIP_2) all stimulate AK-1-6H to a similar maximal activity with similar half-maximal concentrations. In contrast, the inositides—inositol (○), inositol monophosphate (+), and inositol trisphosphate (×)—were without measurable effect. In this figure histone IIIS was used as a phosphorylation substrate and data were normalized to activity observed using no added lipid in the presence of 100 μM calcium.

histone with half-maximal activation at concentrations of 18.3 ± 14.7 μM, 13.7 ± 10 μM, and 17.0 ± 4 μM, respectively (Figure 6). We tested whether the inositol head group alone is responsible for this effect and found that adding inositol, inositol monophosphate, or inositol trisphosphate at levels up to 500 μM was ineffective at stimulating AK-1-6H (Figure 6). Inositol bisphosphate at levels up to 100 μM was also without effect (data not shown). Another cyclic compound and putative plant second messenger, cAMP, at 100 μM had no measurable effect on AK-1-6H activity (data not shown).

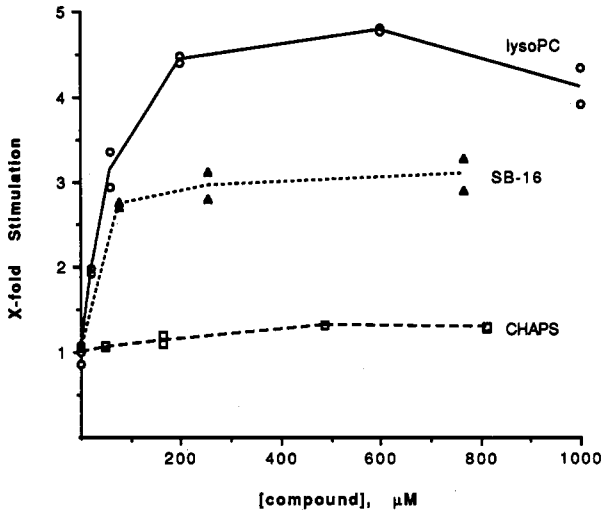

FIGURE 7: Stimulation of AK-1-6H by lysophosphatidylcholine and other detergents. AK-1-6H activity was stimulated by lysophosphatidylcholine (○, lyso-PC) and another zwitterionic detergent, SB-16 (▲), in a concentration-dependent manner, whereas another zwitterionic detergent, CHAPS (□), had little effect. In this figure histone IIIS was used as a phosphorylation substrate and data were normalized to activity observed using no added lipid in the presence of 100 μM calcium.

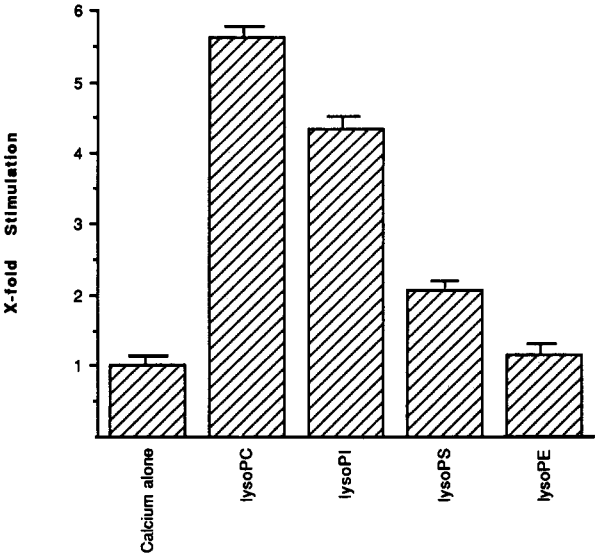

FIGURE 8: Comparison of various lysophospholipids. The stimulation by lysophosphatidylcholine (lyso-PC) was compared to that by other lysophospholipids. Of those tested, lysophosphatidylinositol (lyso-PI) stimulated AK-1-6H almost as well as lyso-PC. In contrast, lysophosphatidylserine (lyso-PS) and lysophosphatidylethanolamine (lyso-PE) had little or no effect. All lysophospholipids were tested at 700 μM (approximately 10-fold higher than the half-maximal concentration for activation by lyso-PC). In this figure histone IIIS was used as a phosphorylation substrate and data were normalized to activity observed using no added lipid in the presence of 100 μM calcium. Bars represent the range around the mean of two separate samples.

Lysophosphatidylcholine from egg yolk also stimulated AK-1-6H in a synergistic fashion with calcium and gives half-maximal stimulation at a concentration of 75 ± 26 μM using histone as substrate (Figure 7). Since lysophosphatidylcholine is a zwitterionic detergent, two other zwitterionic detergents were tested: 3-(*N*-hexadecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate (SB-16) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). SB-16 stimulated AK-1-6H approximately 3-fold (less than the stimulation induced by lysophosphatidylcholine), while CHAPS was inactive (Figure 7).

To determine whether the stimulation of AK-1-6H by lysophospholipids requires any specific headgroup, we compared the effects of various lysophospholipids on AK-1-6H activity. Lysophosphatidylinositol was almost as effective at stimulating AK-1-6H as lysophosphatidylcholine, while lysophosphatidylserine and lysophosphatidylethanolamine had little or no effect (Figure 8). A similar specificity is observed in a calcium- and lipid-stimulated protein kinase activity in crude membranes from zucchini (Martiny-Baron & Scherer, 1989). Lysophosphatidylinositol is not a zwitterionic detergent, again suggesting that some specific structural requirement rather than a general detergent effect is necessary to stimulate AK-1-6H.

In side-by-side experiments, we find that the phosphoinositides and lysophosphatidylcholine stimulate AK-1-6H to the same maximum level of activity as the crude mixture of lipids (data not shown). This is in contrast to a report on a membrane-associated, calcium-dependent kinase from oat root where individual, purified lipids were not as effective as the crude lipid mixture (Schaller *et al.*, 1992). The effects of these stimulatory lipids are not synergistic with each other. In other words, when maximum stimulation with lysophosphatidylcholine is achieved, further addition of a phosphoinositide is without effect (data not shown). This is similar to results studying the effects of individual lipids on the membrane associated, calcium-dependent kinase from oat roots (B. M. Binder, unpublished data). This means either that these lipids are acting via the same mechanisms or that the kinase is at maximal activity and cannot be stimulated further.

When we compared the stimulatory effects of phosphatidylinositol, phosphatidylcholine, and lysophosphatidylcholine, only phosphatidylinositol stimulated autophosphorylation (Figure 5), suggesting two mechanisms of lipid action on AK-1.

(IV) AK-1-6H Is Inhibited by Poly(L-*Arginine*) *and Poly*(L-*Lysine*). When histone or myelin basic protein levels are increased to approximately 5.0 mg/mL, AK-1-6H kinase activity is reduced to less than 10% of maximal activity whether lipid and calcium are present or not. This reduction in activity could represent a decrease in the availability of substrate to the kinase at high concentrations because substrate micelles form (Bazzi & Nelsestuen, 1987), or it could represent direct inhibition of the enzyme by these highly basic protein substrates. It has been suggested that protein kinase C activity might be regulated by its substrates too (Leventhal & Bertics, 1993). To test this idea, we added basic polypeptides [poly(L-lysine) and poly(L-arginine)] to our assay mixture using syntide 2 as peptide substrate. Calcium-dependent protein kinases from wheat and silver beet are inhibited by these basic polypeptides (Polya *et al.*, 1990) and with certain substrates these basic polypeptides also inhibit protein kinase C (Leventhal & Bertics, 1993).

We found that strongly basic polypeptides, such as poly(L-arginine) and poly(L-lysine), inhibit AK-1-6H phosphorylation of syntide 2 in a concentration-dependent manner. With 0.2 mg/mL syntide 2 as protein substrate, poly(L-lysines) with average M_r of ~3000, 20 000, or 37 100 required nanomolar concentrations to produce 50% inhibition (IC_{50}) of AK-1-6H. The free amino acid, L-lysine, had little or no effect on AK-1-6H.

The poly(L-lysine) with an average M_r of 37 100 was a mixed-type inhibitor of AK-1-6H activity that increased the apparent K_m for syntide and decreased the V_{max} of AK-1-6H (Table 3), suggesting that poly(L-lysine) interacts with AK-

Table 3: Effects of Poly(L-Lysine) and Lipid on the Apparent K_m for Syntide and V_{max} of AK-1-6H[a]

	calcium		calcium + lipid	
conditions	apparent K_m for syntide[b] (μM)	V_{max} (nmol/min)	apparent K_m for syntide (μM)	V_{max} (nmol/min)
no poly-(L-lysine)	44.0 ± 9.2	0.050 ± 0.02	44.2 ± 9.6	0.104 ± 0.07
+ poly-(L-lysine)	332.5 ± 59.9	0.019 ± 0.019	152.7 ± 94.7	0.029 ± 0.022

[a] All samples were buffered to 100 μM free calcium levels. Samples with lipid contained 0.5 mg/mL crude soybean phosphatidylcholine. Samples with poly(L-lysine) had 10 nM poly(L-lysine) (average M_r ~37 100) added. Each condition was repeated in two experiments with similar results. [b] Syntide 2 levels were varied between 13 and 660 μM.

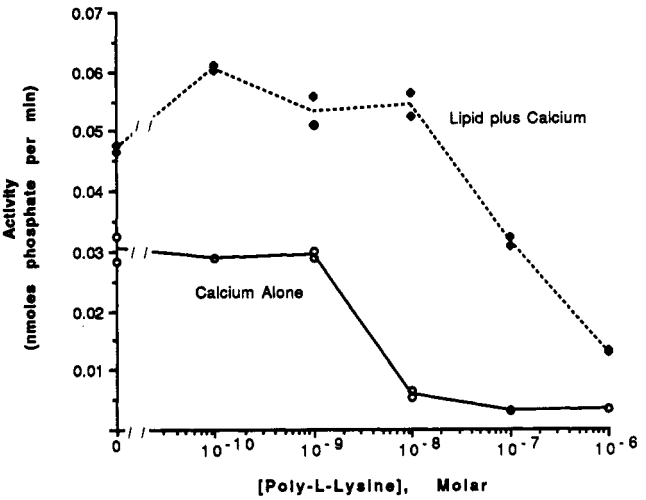

FIGURE 9: Poly(L-lysine) inhibition of AK-1-6H is reversed by lipid. The inhibition of AK-1-6H by various amounts of poly(L-lysine) (average M_r ~37 100) in the absence (○) and presence (●) of 0.5 mg/mL crude phosphatidylcholine is compared. Incorporation of radioactive phosphate into syntide 2 was measured as described in Materials and Methods. All assays included 100 μM calcium.

1-6H at two or more sites. This poly(L-lysine) had no effect on the apparent K_m for ATP (data not shown). With poly-(L-lysine) present, crude lipid both increased the V_{max} of AK-1-6H and decreased the apparent K_m for syntide. The IC_{50} of poly(L-lysine) (average M_r ~ 37 100) in the presence of 0.2 mg/mL syntide 2 increased from 2 nM in the absence of lipid to 341 nM in the presence of 0.5 mg/mL added crude soybean phosphatidylcholine (Figure 9). In the presence of poly(L-lysine), phosphatidylinositol stimulated AK-1-6H while lysophosphatidylcholine was without effect (data not shown).

DISCUSSION

In this study, we expanded on our initial finding that calcium and lipid act synergistically to stimulate a protein kinase with a calmodulin-like domain from *A. thaliana* (Harper *et. al.*, 1993). We observed a higher specific activity (typically 2000 nmol min^{-1} mg^{-1}) and a higher stimulation by calcium alone (up to 50-fold) in this study. The increase in specific activity probably reflects the higher purity of the kinase preparation. Since it is likely that truncated products of the kinase are active and calcium-independent, the apparent increase in stimulation by calcium is probably due to less background activity of these fragments in the current purification method. The high specific activity of AK-1-6H is comparable to that reported for an endogenous CDPK from soybean (Roberts & Harmon, 1992), suggesting that AK-1-6H expressed and purified from *E. coli* provides a good model for investigating the regulation of CDPKs.

Inhibition by Basic Proteins. An important finding of this study was that basic polypeptides such as poly(L-arginine) and poly(L-lysine) were potent, mixed-type inhibitors of AK-1-6H activity which decreased the V_{max} of AK-1-6H and increased the apparent K_m for syntide. However, from these experiments we cannot determine whether these highly basic polypeptides are interacting with the AK-1-6H or the syntide or both. Polylysine does not alter autophosphorylation (data not shown). Others have shown that these basic peptides inhibit calcium-stimulated protein kinase activity from several higher plants, suggesting that this may be a general property of CDPK kinases (Polya *et al.*, 1990; Putnam-Evans *et al.*, 1990). We found that a poly(L-lysine)polypeptide as small as ~3000 Da (or ~27 amino acid residues long) was as effective as longer poly(L-lysine) chains, although the free amino acid L-lysine was virtually without effect. Addition of crude lipid increased the IC_{50} of poly(L-lysine).

Most evidence indicates that CDPKs phosphorylate sites with a basic amino acid residue nearby (Roberts & Harmon, 1992). Since basic polypeptides inhibit AK-1-6H, it is possible that these basic amino acids in the substrate also inhibit the kinase. If this is so, lipid may act by alleviating inhibition by positively charged domains on substrate proteins. The stimulation of kinase activity by crude lipid was higher using histone as substrate, compared to syntide. This may reflect an inhibitory effect of the basic protein, histone, and its alleviation by lipids, similar to what was seen with syntide only in the presence of polylysine.

Lipid Activation of AK-1-6H. Our results suggest that there are two mechanisms by which lipids stimulate kinase activity in this assay. Phosphatidylinositol stimulated autophosphorylation while lysophosphatidylcholine did not, which strongly suggests that phosphatidylinositol is acting directly on AK-1. In addition, phosphatidylinositol increased the IC_{50} of poly(L-lysine), lysophosphatidylcholine was without effect. This is different from the effects of these lipids in the absence of poly(L-lysine) where both increase AK-1 activity as measured using exogenous substrates. It is possible that the phosphoinositides and basic proteins interact at the same site on AK-1 (see below), while lysophosphatidylcholine acts at another site. However, we do not know whether lipid is also interacting directly with poly(L-lysine) to block inhibition. From these data, it is unclear whether lysophosphatidylcholine acts directly on AK-1 or on substrate proteins or both.

We found that the specificity of lipid stimulation of histone phosphorylation showed no correlation with unit charge, detergent characteristics, or other obvious physical properties of these lipids. Of those tested, only the phosphoinositides, lysophosphatidylcholine, and lysophosphatidylinositol stimulated AK-1-6H. Since lysophosphatidylcholine is a zwitterionic detergent, we examined the effects of two other zwitterionic detergents, SB-16 and CHAPS. While SB-16 stimulated AK-1-6H, CHAPS was without measurable effect. This stimulation does not correlate with the critical micelle concentrations of these compounds. Both SB-16 and lysophosphatidylcholine appear to have similar concentrations of half-maximal activation (~75 μM) yet different critical micelle concentrations. The critical micelle concentration of SB-16 is 31 μM (Neugebauer, 1990), while egg yolk lysophosphatidylcholine mainly consists of 1-hexadecanoyl-2-lysophosphatidylcholine, with a critical micelle concentration of 7 μM (Stafford *et al.*, 1989), and 1-octadecanoyl-2-lysophosphatidylcholine, with a critical micelle concentration of 0.4 μM (Kemp *et al.*, 1984). Both detergents (lysophosphatidylcholine and SB-16) are stimulatory at concentrations

AK-1	112-121	K	.	P	D	P	P	A	K	P	K	K
	428-438	K	Q	F	S	A	M	N	K	F	K	K
Profilin	123-136	K	C	Y	E	M	A	S	H	L	R	R
Cofilin	13-22	K	.	V	F	N	D	M	K	V	R	K
Gelsolin	161-169	K	.	.	L	F	Q	V	K	G	R	R
Villin	137-145	R	.	.	L	L	H	V	K	G	K	R
Phospholipase C	451-468	K	.	.	.	I	L	V	K	N	K	K
Gelsolin	135-142	K	.	.	.	S	G	L	K	Y	K	K
Consensus		K/R	x	x	x	x	x	x	K/H	x	K/R	K/R

FIGURE 10: Comparison of putative phosphoinositide binding sites. Two regions of AK-1 with a basic amino acid motif are compared to similar motifs found in phosphoinositide-binding proteins. The numbers represent the amino acid residues in each protein. Comparison is made to human gelsolin (Yu *et al.*, 1992; Janmey *et al.*, 1992), chicken villin (Bazari *et al.*, 1988), porcine cofilin (Kwiatkowski *et al.*, 1986), human profilin (Ampe *et al.*, 1988), and rat phospholipase (Rhee *et al.*, 1989). The dots represent a gap in the amino acid sequence.

above their critical micelle concentrations. CHAPS, which has a critical micelle concentration of 6.5 mM (Neugebauer, 1990), was inactive even at concentrations of 10 mM (data not shown). It should be noted that phosphatidylcholine is also a zwitterion, yet it is without measurable effect (Harper *et al.*, 1993), and nondetergent zwitterions, such as the amino acids alanine, glycine, creatine, tryptophan, and lysine, at levels up to 1 mM did not stimulate AK-1-6H (data not shown). The fact that, under identical conditions, cAMP-dependent protein kinase is not stimulated by lipids (Harper *et al.*, 1993) suggests that the observed lipid stimulation of AK-1-6H is not an artifact of the assay conditions.

Possible Lipid Binding Sites on AK-1-6H. We find that phosphatidylinositol stimulates autophosphorylation, indicating that this phospholipid is interacting directly with the kinase. An amino acid motif containing basic amino acid residues has been identified in several cytoskeleton-associated proteins (gelsolin, gCap39, villin, cofilin, and profilin) which bind phosphoinositides. This amino acid sequence has also been identified in phospholipase C. A comparison of this basic amino acid motif from several of these proteins is made to two similar amino acid motifs in AK-1 (Figure 10). One of these is in the N-terminal domain (from Lys_{112} to Lys_{121}) and the other is in the junction domain (from Lys_{428} to Lys_{438}).

We propose that the phosphoinositides interact at these basic residues on the kinase to stimulate its activity (as measured with exogenous substrate or by autophosphorylation). We have made an N-terminally deleted AK-1 (Harper *et al.* 1993) that retains lipid-stimulated activity, suggesting that at least one lipid binding site is distal to Met_{125}. More work is required to determine which, if any, of these sites is the binding site for stimulatory lipids on AK-1. One model we have is that either the N-terminal domain or the junction domain or both have allosteric binding sites. When lipid binds, this allosteric binding is reversed and the kinase is activated. A similar phenomenon may occur with highly basic substrate proteins (such as histone) and with basic polypeptides where lipid removes inhibition caused by these positively charged molecules.

Do Lipids Regulate AK-1 in Vivo? Since there is a large amount of evidence that calcium levels change in plant cells upon stimulation by various environmental signals (Shacklock *et al.*, 1992; Knight *et al.*, 1991; 1992) and AK-1 has a calmodulin-like domain with four calcium-binding EF hand motifs (Harper *et al.*, 1993), it is likely that calcium is a major regulator of this kinase *in vivo*. The role of lipids in regulating AK-1 is less clear because the stimulatory effect of lipid was smaller than that of calcium. In order to test this point, it will first be necessary to demonstrate that levels of these lipids change in plant cells upon stimulation. Both the turnover of the phosphoinositides (Morré *et al.*, 1984; Kamada & Muto, 1991; Drøbak, 1992) and the activity of phosphatidylinositol and phosphatidylinositol phosphate kinase (Chen & Boss, 1990; Memon & Boss, 1990; Tan & Boss, 1992; Yang *et al.*, 1993) change in plant cells with certain stimuli. However, future studies are needed to determine whether these changes alter plant cell function *in vivo* and whether these effects are mediated by lipid-induced changes in CDPK activity.

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