Calcium and Lipid Regulation of an *Arabidopsis* Protein Kinase Expressed in *Escherichia coli*†

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ABSTRACT: Calcium-dependent protein kinases (CDPKs) represent a new family of protein kinases which are proposed to contain, in a single polypeptide, both a kinase domain and an adjoining calmodulin-like domain with four calcium-binding EF-hand motifs [Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991) *Science 252*, 951–954]. DNA cloning and Western blot analysis indicate that multiple CDPK isoforms are present in the model plant system *Arabidopsis thaliana*. One CDPK gene called AK1 was isolated from *Arabidopsis* as a full-length cDNA. The predicted AK1 protein has a M_r of 72 645 and is 116 amino acid residues longer at the amino terminus than the prototype CDPKα gene previously identified in soybean. The most highly conserved region between these two CDPKs is a region of 31 amino acids that joins the kinase and calmodulin-like domains. To verify the kinase activity of the enzyme encoded by AK1, a fusion of an amino-terminally truncated AK1 to the C-terminus of glutathione S-transferase was expressed in *Escherichia coli*. The fusion protein was purified and displayed a maximum kinase activity of 40 nmol of phosphate/(min·mg), using histone IIIs as a substrate. The enzyme activity was stimulated 3–6-fold by calcium and 2–5-fold by crude lipid. However, a synergistic stimulation of 16–30-fold was observed by the addition of both calcium and crude lipid. Lipid stimulation was specific for lysophosphatidylcholine and phosphatidylinositol and did not occur with the addition of phosphatidylserine or phosphatidylcholine. The calcium and lipid stimulation properties of this isoform are most similar to a calcium-dependent protein kinase associated with the plasma membrane in oat roots [Schaller, G. E., Harmon, A. C., & Sussman, M. R. (1992) *Biochemistry 31*, 1721–1727].

Protein kinases are known to act as pleiotropic regulators of cell physiology (Hunter, 1987; Hanks et al., 1988). In higher plants, several genes predicted to encode protein kinases have been cloned [examples include Suen and Choi (1991), Stein et al. (1991), Guilluy et al. (1991), Alderson et al. (1991), Lin et al. (1991), Ferreira et al. (1991), Feng and Kung (1991), Hirt et al. (1991), Hata (1991), Feiler and Jacobs (1990), Lawton (1990), Walker and Zhang (1990), Lawton et al. (1989)]. However, only one protein kinase has been biochemically characterized and purified to homogeneity from extracts of higher plants (Harmon et al., 1987). This enzyme, calcium-dependent protein kinase (CDPK) is present at significant levels in root and shoot tissues. There is biochemical and immunocytochemical evidence that CDPKs are associated with the plasma membrane (Schaller et al., 1992) and cytoskeletal system (Putnam-Evans et al., 1989). The biological functions of CDPKs are unknown but could include a broad spectrum of phenomena which implicate free calcium (Ca^{2+}) as a second messenger, such as gravitropism, thigmomorphogenesis, cytoplasmic streaming, stress adaptation, and pathogen response (Roberts & Harmon, 1993, Trewavas & Gilroy, 1991).

CDPKs were first proposed as a new family of protein kinases on the basis of analysis of a purified soybean protein kinase (Putnam-Evans et al., 1990). This kinase was shown to bind Ca^{2+} directly as a binary complex without lipids and its activity was stimulated by Ca^{2+} in the absence of calmodulin. These features indicated that CDPKs were distinct from protein kinase C, which binds Ca^{2+} as a Ca^{2+}/lipid ternary complex, and from calmodulin-dependent protein kinases, which are activated by a Ca^{2+}/calmodulin ternary complex.

The first gene predicted to encode a CDPK was cloned from soybean, on the basis of protein sequence information derived from proteolytic fragments of a purified CDPK isoform (Harper et al., 1991). This gene was called CDPKα and its predicted protein sequence indicated a unique structural feature distinct from other protein kinases; in a single polypeptide, a kinase domain is followed by a sequence with 39% identity to calmodulin. The calmodulin-like domain of CDPKα has four stereotypical Ca^{2+}-binding EF-hand motifs which provide a probable molecular basis for direct binding of Ca^{2+}.

The purpose of the research presented here was to test the hypothesis that the unique structural theme identified in the soybean CDPKα gene actually encodes a calcium-dependent protein kinase activity. Evidence that CDPKα encodes such an activity is indirect and disputable. The hypothesis is based on the presence of structural features of the predicted CDPKα protein which are consistent with those of a Ca^{2+}-regulated kinase (Harper et al., 1991). In addition, there is strong similarity between two peptide sequences identified in the purified CDPK and predicted for the cloned CDPKα. However, these two enzymes are not identical; the two sequenced peptides share only 15 of 20 and 23 of 33 identical residues. Since these peptides correspond to regions within the kinase domain, it is possible that they are common to different families of plant protein kinases. To provide rigorous proof that the novel structural theme identified in CDPKα encodes a calcium-dependent protein kinase activity, we have expressed a putative CDPK in *Escherichia coli* and biochemically characterized its activity.

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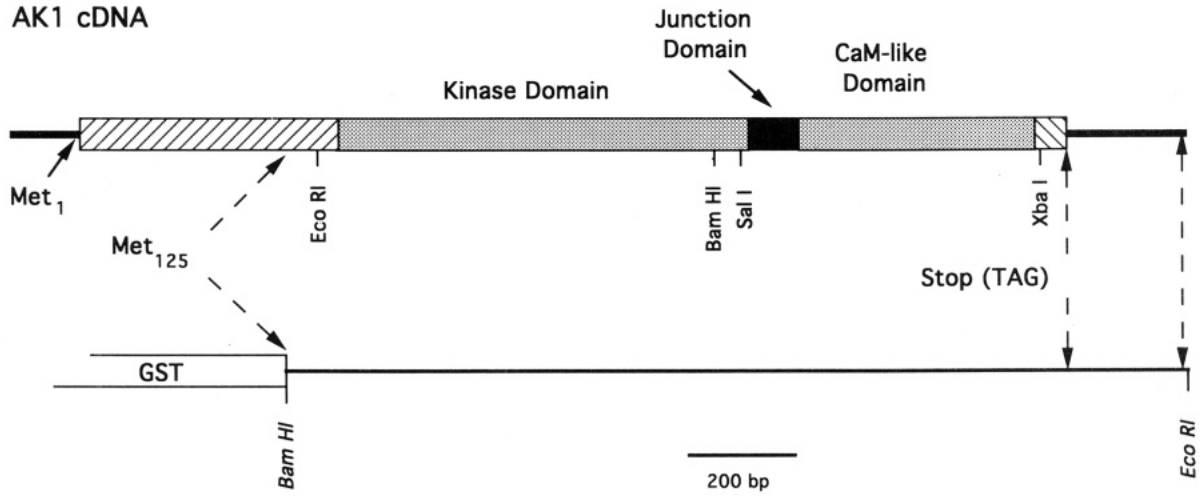

FIGURE 1: Diagram of the AK1 cDNA and the KGF-1 fusion. Important restriction enzyme sites are marked; sites marked in italics represent engineered sites. The position of the predicted start site, Met_1, and the position of Met_{125} used for the initiation of the KGF-1 fusion are marked. The position of the natural stop codon is marked (TAG). GST is the abbreviation for the glutathione S-transferase gene used in the fusion construct.

The results presented here confirm that a gene, designated as a member of the CDPK family because of adjoining kinase and calmodulin-like domains, actually encodes a calcium-dependent protein kinase. The CDPK isoform characterized in this study was cloned from the model higher plant, *Arabidopsis thaliana*, and is called AK1 (*Arabidopsis* kinase 1). The predicted M_r of AK1 is approximately 15 000 Da larger at the amino terminus than the prototype soybean CDPKα, suggesting that AK1 and soybean CDPKα may encode isoforms which are biochemically distinct. To obtain functional CDPK enzyme for biochemical analysis, AK1 was expressed in *E. coli* as an amino-terminally truncated fusion protein with glutathione S-transferase. The AK1 fusion was purified and its kinase activity was shown to be stimulated by Ca^{2+}. An additional characteristic of the AK1 fusion is that its kinase activity is stimulated by lipid and Ca^{2+} in a synergistic fashion, when histone is used as a protein substrate. Lipid stimulation has also been observed for a partially purified CDPK-like enzyme obtained from oat roots (Schaller et al., 1992). In higher plants, AK1 is the first gene to be identified which encodes a protein kinase with lipid stimulated activity in vitro. It is not known if the activity of a native AK1 with its endogenous substrate is regulated by lipid second messengers in vivo.

MATERIALS AND METHODS

The plant material used for protein and nucleic acid analysis was *Arabidopsis thaliana*, ecotype Columbia. The *E. coli* bacterial host for growing DNA plasmids and expression of fusion protein was XL1-Blue (Stratagene) and the *E. coli* host for bacteriophage λ production was LE392.

cDNA Analysis. A diagram of the full-length cDNA is shown in Figure 1. A 1.35-kbp partial cDNA clone for AK1 (an internal fragment lacking both 5′ and 3′ ends) was isolated by screening 140 000 plaques of an amplified λ-GEM2 *Arabidopsis* cDNA library (Promega Biotech) with a radioactive 151-bp PCR fragment used to identify the soybean CDPKα (SK5) gene (Harper et al., 1991). The 1.35-kbp AK1 insert was subcloned as a *Eco*RI/*Xba*I fragment in pBluescript (SK^-) (Stratagene) and called pAK1.

The 5′ and 3′ ends of the AK1 cDNA were cloned from a PCR amplification which used G-tailed first strand cDNA as template. First strand cDNA was prepared from *Arabidopsis* mRNA with reverse transcriptase and oligo(dT) primer using a cDNA synthesis kit (Bethesda Research Labs). A poly-(dG) tail was added to the 3′ end of the first strand cDNA using terminal transferase and dGTP (Harper et al., 1990).

The 3′ end of the gene was amplified from this first strand cDNA by an anchored PCR method using a 3′-side primer (called AN3) containing poly(dA) and an *Xho*I linker (Harper et la., 1991) and a 5′-side internal primer with a *Bam*HI linker (called AK10) which corresponds to base pair position +1852 to +1875. The PCR product was cloned as a *Bam*HI/*Xho*I insert in pBluescript (SK^-). The cDNA sequence presented is based on sequence from six unique clones derived from two independent first-strand cDNA and PCR reactions. All clones showed 100% identity in a 75-base pair overlap with gene sequence present in the original λ-cDNA clone.

The 5′ end of the cDNA (+1 to +651 bp) was amplified in four steps by an anchored PCR method (Harper et al., 1990). The template was G-tailed first-strand cDNA. The 5′ side primer was oligo(dC) with a *Not*I linker. A series of four 3′-side (internal) primers were used in the walk. Each internal primer was based on the longest extension obtained in a previous step. Each progressive step contained at least 18 base pairs of overlapping sequence to confirm that the series of clones obtained after amplification represented contiguous cDNA sequence. Representative clones spanning this region are pSS111 (+1 to +238), pSS32 (+115 to +294), pLL23 (+276 to +591), and p477 (+509 to +789).

The sequence of the 5′ region was confirmed in two ways. First, each fragment of the walk from +98 to +789 was verified by sequencing multiple clones obtained from PCR amplification of three independent first-strand cDNA reactions. Second, cDNA sequence was confirmed by comparison to genomic sequence obtained from PCR amplification. Genomic sequence corresponding to +168 to +591 was PCR amplified, cloned, and sequenced. The origin of one representative clone called pKME-3 is as follows: PCR amplification was performed with 1 μg of genomic template, a 5′-side primer with a *Bam*HI linker (called AK21) corresponding to DNA sequence +150 to +168 and a 3′-side primer (called AKr14) corresponding to sequence +753 to +770. An *Eco*RI site located at +591 was used to clone the amplified genomic fragment as an *Eco*RI/*Bam*HI insert in pBluescript II (KS^+). In addition, genomic sequence corresponding to +20 to +591 was PCR amplified, cloned, and sequenced. One representative clone called pKSE-15 was obtained as just described, except that the 5′-side primer with a *Bam*HI linker (called AK23) corresponded to DNA sequence +1 to +19. Genomic

sequence obtained from PCR amplifications was verified by analyzing clones from three independent PCR reactions.

All DNA sequence presented was sequenced on both strands using double-stranded DNA template and the Sequenase dideoxy sequencing kit (United States Biochemicals). DNA sequence computer analysis was performed using DNA/STAR (Madison, WI) software.

Fusion Protein Constructs. The AK1 fusion construct pKGF-1 represents a truncated AK1 which starts at amino acid Met_{125} and is fused to the C-terminal end of glutathione S-transferase (Figure 1). The AK1 sequence is cloned into the *Bam*HI/*Eco*RI site of the vector pGEX-2T (Pharmacia). The construct was made in two cloning steps. First, sequence from +522 to +591 was PCR amplified and subcloned into the vector as a *Bam*HI/*Eco*RI fragment. The DNA template used for PCR amplification was clone p477. The 5′-side primer for this reaction (called AK15) included a *Bam*HI linker (in the context ggatccATGaag) immediately preceding the Met_{125} codon, and the 3′-side primer was AKr14. The *Eco*RI site used for cloning this fragment occurred naturally at nucleotide position +591. The cloned fragment was sequenced to confirm the absence of PCR mistakes. In a second cloning step, the remainder of the kinase was inserted as an *Eco*RI fragment obtained by cutting the clone pAK-NX-25 at the natural *Eco*RI site at position +591 and adding an *Eco*RI linker to the *Xho*I cloning site located at the 3′ end of the gene. Clone pAK-NX-25 contains the majority of the kinase as a *Not*I/*Xho*I insert (5′ to 3′ orientation) in pBluescript II (KS^-) and was assembled from clones p851 (3′ end PCR fragment), pAK1 (1.35-kbp λ-cDNA insert), and p477 (5′ PCR fragment corresponding to base pairs $^+522$ to $^+767$).

Arabidopsis Extracts. Arabidopsis seeds were sterilized by washing in 70% (v/v) ethanol for 2 min and 30% (v/v) bleach with 1% (v/v) Triton X-100 for 30 min. Seeds were thoroughly rinsed with sterile H_2O and grown in liquid Gamborg's medium (Gibco) supplemented with 2% (w/v) sucrose and 0.5 g/L MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.7, at 24 °C, with constant shaking under lights. After four weeks, plant material was harvested, frozen in liquid nitrogen, and pulverized with mortar and pestle. The frozen powder was thawed in buffer containing 40% (w/v) sucrose, 25 mM EDTA, 0.27% (v/v) β-mercaptoethanol, 5 mM PMSF (phenylmethanesulfonyl fluoride) and 0.25 M Tris-HCl, pH 8.5, and maintained at 4 °C during sample processing. The homogenate was strained through cheesecloth and centrifuged at 7500*g* for 15 min to remove cellular debris. The supernatant, referred to as a crude extract, was separated into microsomal membranes and soluble fractions by centrifugation at 37000*g* for 75 min at 4 °C.

Fusion Protein Purification. Fusion protein KGF-1 was expressed and purified from *E. coli* by a modification of a previously described method (Smith & Johnson, 1988). *E. coli* was grown at 25 °C and expression was induced by the addition of 1 mM IPTG (isopropyl thio-β-D-galactoside) for 1 h. Cells were harvested, resuspended in ice-cold lysis buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3), and lysed by passage through a French pressure cell. Cellular debris was removed by centrifugation for 15 min at 7500*g* at 4 °C and fusion protein purified by affinity chromatography with glutathione–agarose beads. The fusion protein was eluted from the beads with buffer containing 50 mM Tris-HCl, pH 7.5, and 5 mM glutathione and stored at −70 °C until used. Protein concentrations were determined by Bradford assays (Bradford, 1976).

Protein Kinase Assay. Protein kinase activity was measured according to Schaller et al. (1992). Assay volumes were 50 μL and contained 0.5 μg of the purified fusion protein preparation in 20 mM Tris (pH 7.5), 6 mM $MgCl_2$, with 50 μg of histone type IIIS (lysine-rich fraction) (Sigma) as exogenous substrate. Control levels of phosphorylation were determined by chelating free Ca^{2+} with 1 mM EGTA. All lipids were obtained from Sigma. Crude soybean lipid was type IV-S phosphaditylcholine. Lipids were prepared by dissolving in chloroform/methanol (2:1), drying under a stream of nitrogen, adding the appropriate assay buffer and sonicating with a probe sonicator.

To test for the effect of Ca^{2+} and lipids, assays without EGTA were supplemented with 100 μM $CaCl_2$ or lipids or both. It was shown that EGTA (1 mM) can be included in all assays with similar results if an excess of $CaCl_2$ (1.1 mM) is added. Reactions were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (20 μM final ATP concentration) and incubated for 20 min at room temperature. Reactions were terminated by adsorption to Whatman No. 3 MM filters followed by immersion in ice-cold 10% (w/v) trichloroacetic acid with 10 mM disodium pyrophosphate. Filters were washed with the above solution four times for 15 min each, followed by 15 min in 95% (v/v) ethanol and 15 min in ether. All washes were carried out with constant shaking at room temperature. Filters were dried and Cerenkov radiation measured with a liquid scintillation counter. Background blanks were treated exactly as the samples above except that they lacked added histone. Control experiments established that kinase activity was linear with respect to enzyme concentration under our assay conditions. In some experiments, rabbit muscle cAMP-dependent protein kinase (Sigma) was used instead of the above plant kinase fusion proteins.

Western Blot. Proteins were separated by SDS–PAGE (Laemmli, 1970) and transferred onto Immobilon-P membranes (Millipore) using a Semi-phor transfer apparatus (Hoefer Instruments) for 1.5 h at 100 mA. The transfer buffer contained 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, 0.01% (w/v) SDS, pH 8.6. Primary monoclonal antibodies raised against purified soybean CDPK (Putnam-Evans et al., 1989) were kindly supplied by A. Harmon. Three antibody sera were pooled with the following final dilutions: 14G5 (1:2000), 12G8 (1:1000), and 3E8 (1:1000). Incubation of sera with blots followed a standard protocol with BSA as a blocking agent (Harlow & Lane, 1988). Immunodecorated protein bands were visualized using a goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma).

RESULTS

Isolation and Sequence Analysis of AK1. The full-length nucleotide sequence for CDPK isoform AK1 is shown in Figure 2. To determine this sequence, a 1.35-kbp partial cDNA was first isolated from a cDNA library by hybridization with a fragment of a soybean CDPK. The missing 3′ and 5′ ends of the cDNA were obtained by anchored PCR amplification from cDNA. The 3′ end shown is based on the longest PCR fragment, clone pAK858. The site of a poly(A) tail in five additional independent 3′ end clones followed nucleotide positions +2066, +2111, +2204 (three representatives), and +2209. The 5′ end of the cDNA was refractory to amplification by anchored PCR, and thus, it was necessary to compile the final 651 bp of sequence from the products of four sequential amplification steps. pSS111 represents the longest extension product and is used to start the cDNA sequence at +1. The next longest extensions from three independent

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1                                                GAGAAGTCCGAGGTTTGATGTCTGATGGC

30    TTTAAGATCGAAAGGATTTGGCTTTTATCAGATTAGTAATGAGATTAGATTGTTTTTGAG

90    GTTACTGTAATCAGATCTTGATGCTATTTCTGGTGAAAGAGATTTAAGACAAGTTAGGGA

150   ATGGGTAATACTTGTGTTGGACCAAGCAGAAATGGCTTCTTGCAATCTGTTTCAGCTGCA
      m  g  n  t  c  v  g  p  s  r  n  g  f  l  q  s  v  s  a  a

210   ATGTGGCGGCCAAGAGATGGAGATGATTCGGCTTCCATGAGTAATGGAGATATTGCAAGT
      m  w  r  p  r  d  g  d  d  s  a  s  m  s  n  g  d  i  a  s

270   GAAGCTGTTTCGGGAGAGCTTCGATCTCGATTATCTGATGAAGTCCAGAATAAACCTCCT
      e  a  v  s  g  e  l  r  s  r  l  s  d  e  v  q  n  k  p  p

330   GAACAGGTCACAATGCCGAAGCCAGGGACTGACGTCGAGACCAAGGACAGAGAGATTCGA
      e  q  v  t  m  p  k  p  g  t  d  v  e  t  k  d  r  e  i  r

390   ACTGAAAGCAAGCCTGAAACGCTAGAGGAGATAAGCCTTGAGTCCAAACCAGAGACTAAG
      t  e  s  k  p  e  t  l  e  e  i  s  l  e  s  k  p  e  t  k

450   CAGGAGACCAAGTCAGAGACCAAGCCGGAGTCAAAACCTGATCCTCCAGCTAAACCTAAG
      q  e  t  k  s  e  t  k  p  e  s  k  p  d  p  p  a  k  p  k

510   AAGCCTAAACACATGAAGAGAGTGTCAAGTGCAGGGCTTAGGACTGAGTCAGTGTTGCAG
      k  p  k  h  m  k  r  v  s  s  a  g  l  r  t  e  s  v  l  q

570   AGGAAGACTGAAAACTTCAAGGAATTCTATTCCTTGGGAAGGAAACTCGGACAAGGGCAA
      r  k  t  e  n  f  k  e  f  y  s  l  g  r  k  l  g  q  g  q

630   TTTGGGACGACTTTTTTATGTGTCGAGAAGACTACGGGGAAGGAGTTTGCCTGCAAGTCG
      f  g  t  t  f  l  c  v  e  k  t  t  g  k  e  f  a  c  k  s

690   ATTGCTAAGAGGAAGCTATTGACTGATGAGGACGTTGAGGATGTGAGAAGAGAAATTCAG
      i  a  k  r  k  l  l  t  d  e  d  v  e  d  v  r  r  e  i  q

750   ATAATGCATCACTTGGCTGGTCACCCTAATGTCATCTCCATCAAAGGAGCTTATGAGGAT
      i  m  h  h  l  a  g  h  p  n  v  i  s  i  k  g  a  y  e  d

810   GTTGTGGCAGTGCACCTTGTAATGGAGTGTTGTGCAGGCGGCGAGCTTTTTGACAGGATC
      v  v  a  v  h  l  v  m  e  c  c  a  g  g  e  l  f  d  r  i

870   ATTCAACGCGGTCACTACACAGAGAGGAAAGCGGCTGAGCTCACTAGAACCATTGTTGGG
      i  q  r  g  h  y  t  e  r  k  a  a  e  l  t  r  t  i  v  g

930   GTTGTAGAGGCTTGCCATTCTCTTGGTGTTATGCATCGAGACCTCAAGCCCGAGAATTTT
      v  v  e  a  c  h  s  l  g  v  m  h  r  d  l  k  p  e  n  f

990   CTGTTTGTCAGTAAACACGAAGATTCCCTCTTGAAGACGATTGATTTTGGACTCTCCATG
      l  f  v  s  k  h  e  d  s  l  l  k  t  i  d  f  g  l  s  m

1050  TTCTTTAAACCAGACGATGTTTTTACAGATGTTGTTGGTAGCCCATATTATGTTGCCCCA
      f  f  k  p  d  d  v  f  t  d  v  v  g  s  p  y  y  v  a  p

1110  GAAGTTCTTCGAAAGCGTTATGGCCCTGAAGCTGATGTCTGGAGTGCTGGAGTGATTGTG
      e  v  l  r  k  r  y  g  p  e  a  d  v  w  s  a  g  v  i  v

1170  TACATTTTATTAAGCGGAGTTCCTCCATTCTGGGCTGAAACCGAACAAGGTATTTTCGAA
      y  i  l  l  s  g  v  p  p  f  w  a  e  t  e  q  g  i  f  e

1230  CAGGTACTCCACGGTGATCTTGACTTTTCGTCCGATCCATGGCCAAGTATATCTGAAAGT
      q  v  l  h  g  d  l  d  f  s  s  d  p  w  p  s  i  s  e  s

1290  GCAAAGGATTTAGTGAGGAAAATGCTTGTCAGGGATCCCAAGAAAAGGTTAACTGCCCAC
      a  k  d  l  v  r  k  m  l  v  r  d  p  k  k  r  l  t  a  h

1350  CAAGTATTATGTCATCCATGGGTTCAAGTCGACGGTGTGGCTCCAGACAAGCCTTTGGAT
      q  v  l  c  h  p  w  v  q  v  d  g  v  a  p  d  k  p  l  d

1410  TCTGCTGTTCTGAGCCGTATGAAGCAGTTTTCTGCAATGAACAAGTTCAAGAAAATGGCT
      s  a  v  l  s  r  m  k  q  f  s  a  m  n  k  f  k  k  m  a

1470  CTTAGAGTCATTGCTGAGAGCTTATCTGAAGAAGAAATCGCCGGCTTGAAAGAAATGTTT
      l  r  v  i  a  e  s  l  s  e  e  e  i  a  g  l  k  e  m  f

1530  AATATGATAGATGCGGACAAGAGTGGTCAGATAACTTTCGAAGAACTGAAAGCAGGACTA
      n  m  i  d  a  d  k  s  g  q  i  t  f  e  e  l  k  a  g  l

1590  AAACGAGTAGGGGCGAATCTCAAAGAGTCTGAAATTCTCGACTTGATGCAAGCTGCTGAT
      k  r  v  g  a  n  l  k  e  s  e  i  l  d  l  m  q  a  a  d

1650  GTGGACAACAGCGGAACAATAGATTACAAAGAGTTCATAGCTGCAACATTACATCTAAAC
      v  d  n  s  g  t  i  d  y  k  e  f  i  a  a  t  l  h  l  n

1710  AAAATAGAGAGAGAGGACCATTTGTTTGCAGCCTTTACATACTTTGACAAAGATGGGAGC
      k  i  e  r  e  d  h  l  f  a  a  f  t  y  f  d  k  d  g  s

1770  GGCTATATCACCCCAGACGAGCTTCAACAAGCTTGTGAGGAGTTTGGTGTTGAGGATGTC
      g  y  i  t  p  d  e  l  q  q  a  c  e  e  f  g  v  e  d  v

1830  CGCATAGAAGAACTGATGCGCGATGTTGATCAAGACAATGACGGGCGAATAGACTACAAC
      r  i  e  e  l  m  r  d  v  d  q  d  n  d  g  r  i  d  y  n

1890  GAGTTTGTGGCGATGATGCAGAAAGGAAGCATCACAGGAGGACCTGTGAAAATGGGTCTA
      e  f  v  a  m  m  q  k  g  s  i  t  g  g  p  v  k  m  g  l

1950  GAGAAAAGCTTTAGCATTGCTCTTAAACTCTAGTTTCTCAGTCTATTTAAAAGTACAAAA
      e  k  s  f  s  i  a  l  k  l  .

2010  CTAAAGCCAAGAAGACAAAAGAGTTTTCTGATAATATATACCATTTTGATTATGTTGAAG
2070  ACATATGCACACAGCCAAGATTCTCTCTTGCCCTCTTTTGGATTCTCTCAATCTTTTGGA
2130  TGAGGTTTTGTCTTAGACTTGTTTTGGAAACTTTGTTGTATTGTGTTACTTCTTGAGCAT
2190  ATATAGTCTTCTAACATTACAAACC-2209
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FIGURE 2: AK1 cDNA sequence and predicted amino acid sequence. The +1 start site was identified in the longest PCR extension. The first 19 base pairs have not been confirmed to be free of PCR mistakes using genomic or independently derived cDNA sequence. The predicted protein is indicated in one-letter code beneath the cDNA.

reactions all ended between position +98 and +115. The compiled PCR-amplified cDNA sequence was confirmed by amplifying the region +20 to +591 from genomic DNA template. A comparison of genomic DNA and cDNA

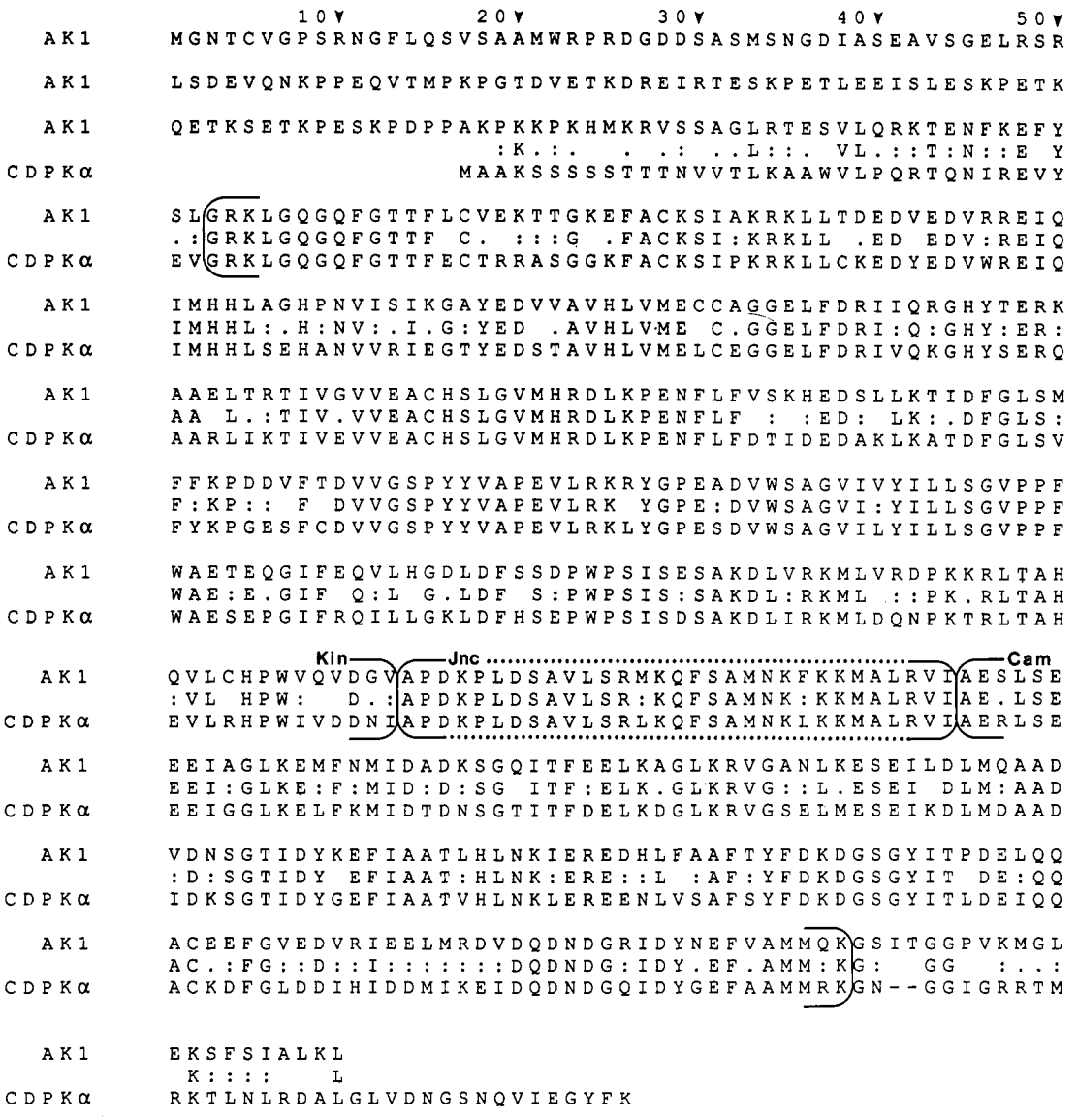

FIGURE 3: AK1 amino acid sequence compared to CDPKα. The kinase (Kin), junction (Jnc), and calmodulin-like (Cam) domains are bracketed. The amino acid sequences were compared with a PAM matrix (Lipman & Pearson, 1985) using DNA/Star software (Madison, WI). Identical residues are indicated between the aligned proteins. Amino acid substitutions that occur frequently in evolution are shown with a colon (high) or period (low). Substitutions with no significant relationship are shown as blanks.

sequence indicates that there are no introns between base pairs +18 and +591.

Protein Sequence Analysis. The predicted AK1 protein is shown in Figure 2 and its comparison to the prototype soybean CDPKα is in Figure 3. The longest open reading frame encodes a protein with a M_r of 72 645. The ATG start codon is located at base pair +150 and is followed by a TAG stop codon at +2130. Stop codons are present in all three reading frames upstream of the predicted ATG start codon. There are four short upstream reading frames present in the 5′ "untranslated" region.

The most striking difference between AK1 and CDPKα is that the amino-terminal domain of AK1 is 116 amino acid residues longer. This region has an abundance of negatively charged amino acids; however, no significant similarity of this amino-terminal region to known proteins could be found in a search of the EMBL database.

The proposed AK1 protein kinase catalytic domain, from residues Gly_{153} to Val_{413}, shows 70% identity to the same region of CDPKα and 63% identity to a partially sequenced CDPK clone (DcPK431) from carrot (Suen & Choi, 1991).

A 31 amino acid region referred to as the "junction domain" immediately follows the kinase domain, starting at Ala_{414}, and joins the kinase to the calmodulin-like domain. The junction domain of AK1 has 93% identity to the same region of CDPKα and 77% homology with that of the carrot homolog DcPK431. This is the most highly conserved stretch of 31 amino acids in a comparison of AK1 and CDPKα.

The calmodulin-like domain of AK1, from residues Ala_{445} to Lys_{588}, is 70% and 58% identical to CDPKα and a carrot homolog DcPK431, respectively. As in CDPKα, this domain contains four stereotypical EF-hand motifs which provide the potential molecular basis for binding Ca^{2+}.

A putative calmodulin binding site is present in AK1 from residues Ala_{432} to Ala_{455}. When this region is represented in a helical wheel diagram, it displays an amphipathic α-helix, with one side composed of hydrophobic residues, and the other containing three basic amino acids (data not shown). This structure has been recognized as a calmodulin-binding domain in rat brain CaMK II and many other calmodulin-regulated enzymes (O'Neil & DeGrado, 1990). Synthetic peptides containing this structure are potent inhibitors of rat brain CaMK II and addition of an excess of Ca^{2+}/calmodulin eliminates their effect. A second amphipathic α-helix that resembles recognized calmodulin-binding domains occurs in the last 18 amino acid residues at the carboxy terminus of

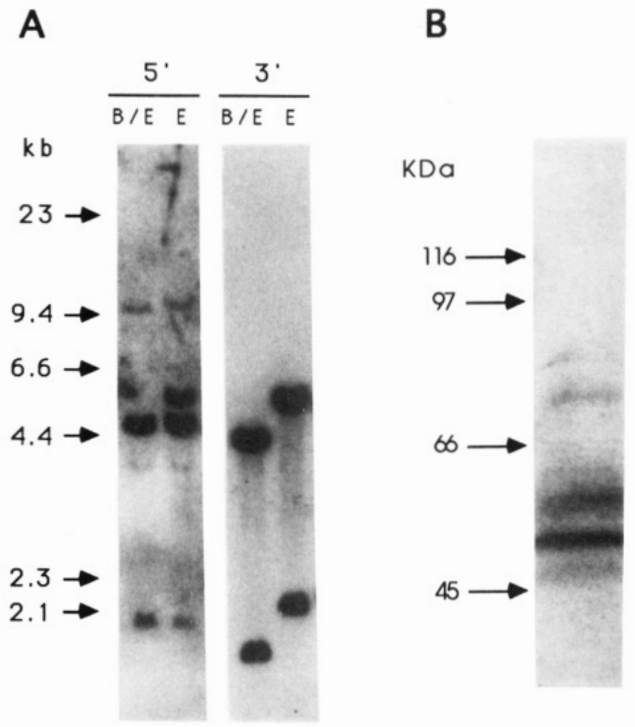

FIGURE 4: Evidence for multiple CDPK isoforms in *Arabidopsis*. (A) Southern blot of *Arabidopsis* genomic DNA probed with radioactive fragments from the 3′ and 5′ ends of AK1. Five micrograms of genomic DNA was digested with *Eco*RI or *Bam*HI and *Eco*RI, separated by 1% agarose gel electrophoresis, and transferred to nitrocellulose. Lanes marked 5′ were probed with a 5′-specific probe corresponding to coding sequence from base pair +509 to +805. This probe was generated by PCR amplification using primers AN1 and AK11 and template DNA from p477. Lanes marked 3′ were probed with 3′-specific probe corresponding to sequence from base pair +1852 to +2212. The probe was generated by PCR amplification using primers AN3 and AK10 and template DNA from p858. Probes were radiolabeled by incorporation of [^{32}P]-dCTP during PCR amplification. Hybridizations were performed as described (Harper et al., 1990). The autoradiogram shown was exposed for 16 h at −70 °C with an intensifying screen. (B) Western blot of crude protein extracts, prepared from rapidly frozen *Arabidopsis* plants, probed with a pool of three mouse monoclonal antibodies raised against a purified soybean CDPK.

AK1. CDPKα contains a similar motif, although its hydrophobic surface is disrupted with an arginine residue. It is not known if calmodulin binds to CDPKs at these sites or if they represent sites of "intraprotein binding" for the calmodulin-like domain.

Multiple Isoforms. CDPK appears to be encoded by a multigene family in *Arabidopsis*. Genes encoding two different CDPK isoforms in *Arabidopsis* have been identified by DNA cloning. The sequence of a cDNA encoding AK1 is presented here, and a genomic clone encoding a second isoform, AK2, has been partially sequenced and shown to have the characteristic structural theme of a kinase domain fused to a calmodulin-like domain (J. F. Harper, unpublished results).

Evidence for multiple CDPKs has been confirmed by Southern blot hybridizations. DNA fragments corresponding to AK1 and a second closely related isoform are detected on Southern blots hybridized with short fragments from the 3′ and 5′ ends of AK1 (Figure 4A). The two hybridization probes used correspond to highly diverged regions between CDPKα and AK1 isoforms and were designed to be "specific" probes for AK1. However, a high degree of identity between AK1 and AK2 is indicated by the cross-hybridization of these two probes. The pattern of restriction fragments detected in the genomic Southern is consistent with the pattern predicted for AK1 and AK2, on the basis of their restriction maps. For example, only two 3′ hybridizing fragments were observed in an *Eco*RI digest; the approximately 2.1 kbp fragment corresponds to a fragment present in the AK2 genomic clone and the approximately 5 kbp fragment is predicted to be diagnostic of AK1. Since AK1 has only been cloned as a cDNA, the corresponding AK1 genomic fragments can only be inferred by elimination of AK2 identified fragments. In addition to AK1 and AK2, other more diverged CDPK isoforms are indicated on the basis of a previous Southern blot analysis of genomic DNA using a more general probe (Harper et al., 1991).

The expression of multiple CDPK isoforms is consistent with evidence provided by Western blot analysis (Figure 4B). CDPK polypeptides were immunologically detected in Western blots of crude *Arabidopsis* plant extracts. A prominent band was observed at M_r = 58 000, and fainter bands at M_r = 47 000, 61 000, 72 000, and 78 000. The relatively faint 72 000-Da band corresponds most closely to the predicted molecular mass of AK1. However, caution is necessary in interpreting the number of isoforms as well as the relative abundance of each isoform on the basis of the bands' intensity. A careful immunological examination of CDPK-like enzymes extracted from oat roots suggests that there are two major isoforms present, one at M_r = 61 000 and a fainter band at M_r = 72 000 (Schaller et al., 1992). However, these proteins have been shown to be highly susceptible to posthomogenization proteolysis in crude extracts or in more purified samples during storage at −70 °C. A CDPK-like enzyme partially purified from oat roots migrates on SDS–PAGE as a M_r = 61 000 polypeptide and is associated with the plasma membrane. Proteolysis during purification and storage clips at one of the termini to generate an enzyme that is 3000 Da smaller and is no longer membrane associated. Although attempts were made to minimize proteolysis in the experiment shown in Figure 4B, it is not certain to what extent this artifact increases the multiplicity and changes the relative abundance of bands observed in *Arabidopsis* crude extracts.

E. coli Expression of an AK1 Fusion. A truncated AK1 cDNA was fused downstream of a glutathione S-transferase gene and the fusion protein expressed in *E. coli*. The AK1 portion of this fusion started at residue Met_{125}, which represents a deletion of the unique AK1 amino terminal end. Before a full-length cDNA sequence was obtained, the Met_{125} was thought to be the start methionine because it was located in the same proximity as the Met_1 in CDPKα. Therefore, the amino-terminal truncation of AK1 examined here is structurally similar to CDPKα.

The fusion protein was purified by affinity chromatography with agarose beads containing covalently attached glutathione (Figure 5). In this preparation, a single protein band, representing approximately 25–50% of the protein, corresponds to the major protein immunodecorated with a pool of monoclonal antibodies which recognize CDPKs. The yield of purified fusion protein varied from 5 to 100 μg of fusion protein/L of bacteria. This yield is more than sufficient for biochemical analysis but is relatively poor compared to an unfused glutathione S-transferase control which produced approximately 100-fold more purified protein. In comparison to the vector only control, bacterial growth after IPTG induction was severely retarded for the AK1 fusion. It is not known if the poor growth of the bacteria is due to aberrant kinase activity or a nonenzymatic property of the fusion protein.

Activity of the AK1 Fusion. The purified fusion protein had protein kinase activity which required both Ca^{2+} and crude lipid for maximal activity (Figure 6). When added

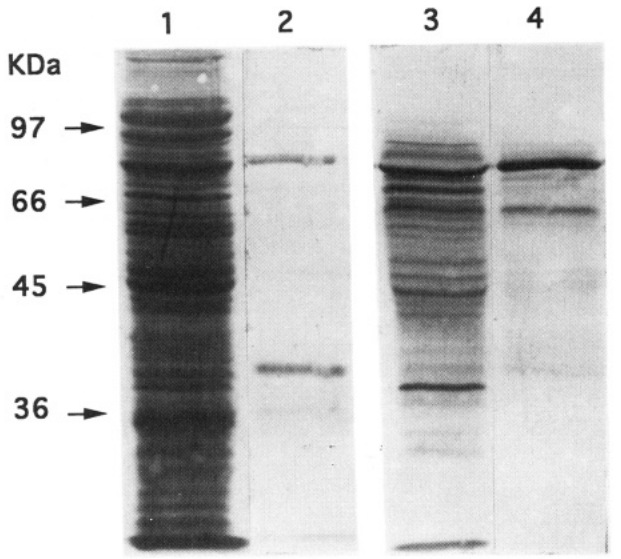

FIGURE 5: Purification of AK1 fusion (KGF-1). Proteins were resolved by SDS–PAGE and transfered to Immobilon-P. Transferred proteins stained with analine blue are shown for crude *E. coli* extract (lane 1) and purified KGF-1 fusion protein (lane 2). A Western blot of crude extract (lane 3) and purified KGF-1 (lane 4) is shown immunodecorated with a pool of three mouse anti-CDPK monoclonal antibodies.

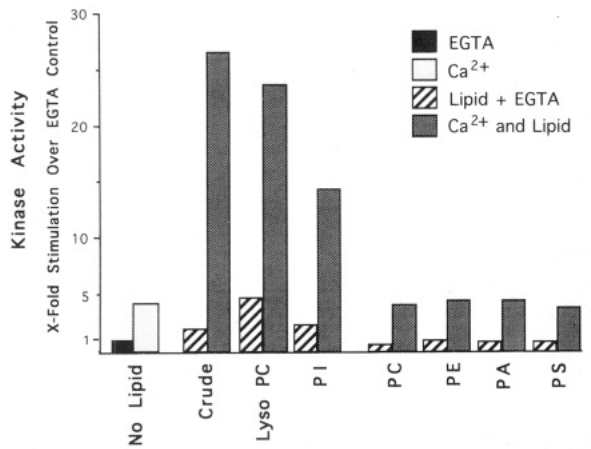

FIGURE 6: Stimulation of AK1 fusion protein (KGF-1) by Ca^{2+} and specific lipids. A control (no lipid) shows Ca^{2+} stimulation in the absence of added lipid. Basal activity was determined by adding EGTA to chelate any residual Ca^{2+}. Lipid stimulation was determined under conditions where Ca^{2+} was chelated by EGTA or where Ca^{2+} was added in excess to 100 μM. Stimulation by crude lipid (0.5 mg/mL) was compared to 0.25 mg/mL of other lipids, including lysophosphatidylcholine (lyso-PC), phosphatidylinositol (PI), purified phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), and phosphatidylserine (PS). Crude lipid was derived from soybean and contains 45% (w/v) phosphatidylcholine. Data represent the mean from two replicate samples. Similar results were obtained using purified lipids at 0.2–1mg/mL

individually, Ca^{2+} produced a 3–6-fold stimulation and lipid produced a 2–3-fold stimulation of activity relative to EGTA controls (minus Ca^{2+} activity). When lipid and Ca^{2+} are added together, they show a synergistic stimulation of at least 16–30-fold. Synergistic stimulation of kinase activity displayed saturating kinetics. Maximum stimulation was observed with the addition of 0.1 mg/mL to 4 mg/mL crude lipid (data not shown).

The specific activity of the AK1 kinase fusion was 40 nmol of phosphate/(min·mg of kinase) at 20 °C. The purity of this enzyme is estimated to be 25–50% (See Figure 5). This synergistic stimulation by lipid and Ca^{2+} has been confirmed for a full-length CDPK fusion, which shows a specific activity around 100 nmol of phosphate/(min·mg of kinase) (J. F. Harper and B. M. Binder, unpublished results). Histone (Sigma, type IIIS) was used as a substrate in our assays and was found to be a more reactive substrate than commercially available casein, cytochrome *c*, myosin light chains, and actin. Casein was the second best substrate, but its phosphorylation level was at least 10-fold lower than that of histone, when used at the same concentrations (w/v) (data not shown).

Table I: Side-by-Side Comparison of the Effect of Lipid on in Vitro Protein Kinase Activity with *A. thaliana* CDPK Isoform AK1 and Mammalian cAMP-Dependent Protein Kinase (Protein Kinase A)

	^{32}P incorporation (cpm)		
	–lipid	+lipid	% stimulation by lipid
CDPK AK1 (–Ca^{2+})	6008	8820	47%
CDPK AK1 (+Ca^{2+})	53764	143380	267%
protein kinase A (–Ca^{2+})	103150	105200	2%
protein kinase A (+Ca^{2+})	88146	98492	11%

Specificity of Lipid Stimulation. Stimulation of kinase activity was determined to be specific for certain lipids and not a general response to hydrophobic compounds. The crude lipid used was approximately 45% (w/v) phosphatidylcholine (PC) and was maximally active at a concentration as low as 0.1 mg/mL. However, purified phosphatidylcholine (PC) up to 1 mg/mL did not significantly stimulate kinase activity (Figure 6). Other inactive lipids included phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). All lipids were tested from 0.2 to 1 mg/mL. Two purified lipids which stimulated kinase activity were lysophosphatidylcholine (lyso-PC) and phosphatidylinositol (PI). This lipid specificity is similar to that observed with a partially purified CDPK-like enzyme isolated from the plasma membrane of oat roots (Schaller et al., 1992). Purified AK1 fusion was also tested in side-by-side assays with the catalytic subunit of rabbit muscle cAMP-dependent protein kinase (protein kinase A). In these assays, kinase activity of the AK1 fusion was stimulated by lipids whereas protein kinase A was not (Table I). This indicates that lipid-stimulated activity of the AK1 fusion is probably not an artifact of the histone preparation or assay conditions.

DISCUSSION

Definition of CDPK. This research demonstrates that the AK1 gene encodes an enzyme which has a kinase activity directly stimulated by Ca^{2+}. This kinase activity was predicted for the enzyme encoded by the soybean CDPKα gene (Harper et al., 1991), on the basis of its novel structural arrangement and its similarity to a biochemically characterized kinase purified to homogeneity from soybean (Putnam-Evans et al., 1990). This demonstration establishes the definition of a CDPK as an enzyme with Ca^{2+}-dependent kinase activity and a structural arrangement of a kinase domain with an adjoining calmodulin-like domain.

Kinase Activity of AK1 Fusion. To investigate the biochemical properites of the AK1 isoform, we expressed this isoform in *E. coli* as an amino-terminally truncated fusion to the C-terminus of glutathione S-transferase. The fusion protein was soluble and easily purified by affinity chromatography to glutathione–agarose beads. Analysis of the AK-1 fusion protein has generated two significant observations. First, the purified protein displays Ca^{2+}-dependent protein kinase activity, thus providing the first direct confirmation that a designated CDPK gene encodes a kinase that is directly regulated by Ca^{2+}. Second, the phosphorylation of histone by this enzyme in vitro is also stimulated by lipids.

The observation that kinase activity of an AK-1 fusion is synergistically stimulated by Ca^{2+} and lipid is a novel

characteristic which has not been reported for mammalian calmodulin-dependent protein kinases. Although we cannot be certain that kinase activity is lipid stimulated in vivo, our results suggest that this isoform could play an important role in signal transduction pathways that use lipid second messengers. It is unlikely that the observed lipid stimulation is an artifact of the assay conditions for two reasons. First, under identical conditions, phosphorylation of histone by a cAMP-dependent protein kinase is not stimulated by lipids. Second, the effect is very specific for certain lipids since lysophosphatidylcholine and phosphatidylinositol were effective whereas phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid were ineffective. Lipid stimulation does not appear to be an artifact of using a truncated fusion protein construct since an additional full-length fusion, which includes the unique amino terminus, still shows lipid stimulation (B. M. Binder and J. F. Harper, unpublished results).

A CDPK-like kinase with a similar pattern for lipid specificity has been characterized from oat root plasma membranes (Schaller et al., 1992). This argues that the observed lipid stimulation reflects a property of an endogenous CDPK isoform and is not an artifact of the *E. coli* fusion protein system. The failure of phosphatidylserine to stimulate kinase activity of the AK-1 fusion is noteworthy since this lipid activates mammalian protein kinase C. Lipid-stimulated kinase activity differing from protein kinase C has also been observed in zucchini membranes (Martiny-Baron & Scherer, 1989). Lipids such as lysophosphatidylcholine (Palmgren & Sommarin, 1989) as well as phosphoinositides have been reported to be activators of the plasma membrane proton pump (H^+-ATPase) in higher plants (Memon et al., 1989; Memon & Boss, 1990). It is thus possible that lipid activation of the pump is indirect, mediated by stimulation of this kinase. Although we have no direct evidence that phosphorylation stimulates the plant proton pump, such evidence does exist in yeast (Kolarov et al., 1988; Chang & Slayman, 1991). The plant proton pump appears to be a substrate for a CDPK-like enzyme in vitro (M. R. Sussman, unpublished), but it remains to be established whether this occurs in vivo.

Multiple Isoforms. CDPKs are encoded by a multigene family in *Arabidopsis*. Evidence from gene cloning, and confirmation by Southern blot analysis, demonstrates that there are at least two isoforms, AK1 and AK2. Additional related isoforms are also indicated by Southern blot analysis (Harper et al., 1991). The expression of multiple isoforms in *Arabidopsis* is consistent with evidence from Western blots decorated with monoclonal antibodies raised against a soybean CDPK. Multiple CDPK isoforms are also indicated for soybean (Harper et al., 1991). A CDPK multigene family raises the possibility that different isoforms will have unique biochemical properties. There is evidence for soluble (Harmon et al., 1987) and membrane-associated isoforms (Schaller et al., 1992). The presence of a large CDPK multigene family is analogous to the protein kinase C family whose members have diverse lipid and calcium stimulation properties as well as diverse substrate specificities (Bell & Burns, 1991).

The purified CDPK from soybean and the *Arabidopsis* AK1 expressed in *E. coli* have different specific activities, using histone as a substrate. The maximum specific activity of a truncated AK1 fusion, which is estimated to be 25–50% pure, is 40 nmol of phosphate/(min·mg), at 22 °C. In contrast, the reported specific activity of a CDPK purified to homogeneity from soybean was 1700 nmol of phosphate/(min·mg) at 30 °C (Harmon et al., 1987). The lower specific activity of the AK1 fusion does not appear to be a result of the truncation of the amino terminus since a full-length AK1 fusion displayed a similar specific activity of 100 nmol of phosphate/(min·mg) (J. F. Harper and B. M. Binder, unpublished results). It is possible that the higher specific activity reported for the soybean isoform reflects a real difference in substrate specificity or temperature optimum. It is likely that different CDPK isoforms will display a range of substrate specificities and kinetic properties, much like members of the protein kinase C family. Biochemical properties of the AK1 isoform characterized here are most similar to a native enzyme partially purified from oat root plasma membrane (Schaller et al., 1992), which shows a characteristic synergistic stimulation by calcium and specific lipid second messengers.

Unique N-Terminus. A potential function for the amino-terminal 116 amino acids of AK1, which are not present in CDPKα, has not been proposed, and no significant similarity to known proteins was detected in a search of the EMBL database. The amino terminus does not appear to be necessary for calcium or lipid stimulation, since the AK1 fusion characterized here is missing the amino-terminal 125 amino acids. A preliminary investigation to compare the in vitro activity of this truncated fusion with a full-length AK1 fusion has failed to identify any significant differences (J. F. Harper, unpublished). However, it is possible that in vivo regulatory properties unique to the amino terminus of AK1 remain undetected by these studies.

Junction Domain. The junction domain, which joins the kinase and calmodulin-like domains together, is the most highly conserved region between AK1 and CDPKα isoforms, with 93% identity over a region of 31 amino acids. In contrast, the kinase and calmodulin-like domains of AK1 and CDPKα share only 72% and 70% identity, respectively. The junction domain is located in the same position that corresponds to two important overlapping domains that have been identified in rat brain CaMK II: (i) an autoinhibitory domain and (ii) a calmodulin-binding domain (O'Neil & DeGrado, 1990). In studies on the rat brain CaMK II, synthetic peptides corresponding to the autoinhibitory domain are potent and specific inhibitors of kinase activity. These peptides appear to function by competitive inhibition with ATP and peptide substrates and support a model for CaMK II regulation where the enzyme is "activated" when a Ca^{2+}/calmodulin complex binds to the calmodulin-binding domain and prevents the adjacent autoinhibitory domain from blocking substrate binding sites. By analogy, we propose that the junction domain in CDPK functions as an autoinhibitor that is regulated by its neighboring calmodulin-like domain.

Usefulness of the Expression System. To formulate and test models of Ca^{2+} and lipid stimulation of CDPK kinase activity, it is now possible to analyze site-directed mutants which can be expressed and purified from *E. coli*. The *E. coli* expression system used here provides rapid purification based on the affinity of fusion proteins to glutathione–agarose beads. This procedure permits the same purification strategy to be used in the analysis of site-specific mutants which have different biochemical properties. The caveat of heterologous expression is that properties of a fusion protein may not exactly parallel those of the native enzyme because of the potential differences in posttranslational processing between plants and bacteria. For example, the maturtion of CDPK may require the plant cytoplasm to provide specific proteolysis, lipidation, or chaperone type processing for accurate and efficient three-dimensional folding. In addition, the fusion protein used here has a glutathione S-transferase attached to the amino terminus. Although the activity of the fusion was similar whether glutathione S-transferase was fused at position Met_1 or Met_{125}

(J. F. Harper and B. M. Binder, unpublished results), the fusions were refractory to thrombin proteolysis and it was not possible to remove the glutathione S-transferase domain (data not shown). However, even if the glutathione S-transferase domain could be removed from the fusion protein, it is not clear that it better represents the kinase produced in planta since it is not known what the natural amino terminus of the mature AK1 protein is.

It is not yet possible to make a direct comparison of the native and *E. coli* produced enzymes since the enzyme corresponding to AK1 has not been purified from *Arabidopsis*. Nevertheless, our investigation indicates that when AK1 is expressed as a fusion protein, its activity (using histone as a substrate) and its Ca^{2+} and lipid stimulation properties are very similar to a CDPK partially purified from oat root plasma membrane (Schaller et al., 1992). Therefore, it appears reasonable to use an AK1 fusion as a model system to conduct a structure and function investigation into the mechanisms by which Ca^{2+} and lipid could regulate a CDPK kinase.

There are several examples in which kinases from eukaryotic systems have been expressed as functional proteins in *E. coli*. For example, the crystal structure for a cyclic AMP-dependent protein kinase from mouse was recently solved using enzyme purified from an *E. coli* expression system (Knighton et al., 1991). An *E. coli* expression system has also been used to conduct a mutational analysis of a calmodulin-dependent protein kinase (Kapiloff et al., 1991).

Novelty of CDPKs. The discovery of the CDPK family of protein kinases indicates that plants possess a novel pathway for Ca^{2+}-mediated signal transduction. The structure of CDPKs and their mechanisms of regulation by lipid and Ca^{2+} make them distinct from other Ca^{2+}-dependent protein kinases characterized in animal systems. At present, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinases have not been conclusively demonstrated either biochemically in plant extracts or from sequence homology with cloned plant kinase genes. It is possible that CDPKs are multifunctional kinases and provide plants with an alternative to protein kinase C and Ca^{2+}/calmodulin-dependent protein kinases.

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