Activation of a Ca^{2+}-Dependent Protein Kinase Involves Intramolecular Binding of a Calmodulin-like Regulatory Domain†

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ABSTRACT: Ca^{2+}-dependent protein kinases (CDPKs) are regulated by a C-terminal calmodulin-like domain (CaM-LD). The CaM-LD is connected to the kinase by a short junction sequence which contains a pseudosubstrate autoinhibitor. To understand how the CaM-LD regulates a CDPK, a recombinant CDPK (isoform CPK-1 from *Arabidopsis*, accession no. L14771) was made as a fusion protein in *Escherichia coli*. We show here that a truncated CDPK lacking a CaM-LD (e.g. mutant ΔNC-2_{6H}) can be activated by exogenous calmodulin or an isolated CaM-LD ($K_{act} \approx 2\ \mu$M). We propose that Ca^{2+} activation of a CDPK normally occurs through intramolecular binding of the CaM-LD to the junction. When the junction and CaM-LD are made as two separate polypeptides, the CaM-LD can bind the junction in a Ca^{2+}-dependent fashion with a dissociation constant (K_D) of 6×10^{-6} M, as determined by kinetic binding analyses. When the junction and CaM-LD are tethered in a single polypeptide (e.g. in protein JC-1), their ability to engage in bimolecular binding is suppressed (e.g. the tethered CaM-LD cannot bind a separate junction). A mutation which disrupts the putative CaM-LD binding sequence (e.g. substitution LRV-I444 to DLPG) appears to block intramolecular binding, as indicated by the restored ability of a tethered CaM-LD to engage in bimolecular binding. This mutation, in the context of a full-length enzyme (mutant $KJM4_{6H}$), appears to block Ca^{2+} activation. Thus, a disruption of intramolecular binding correlates with a disruption of the Ca^{2+} activation mechanism. CDPKs provide the first example of a member of the calmodulin superfamily where a target binding sequence is located within the same polypeptide.

Ca^{2+}-dependent protein kinases (CDPKs) provide the basis for a novel Ca^{2+}-mediated signal transduction pathway in plants and protists (Harper et al., 1991; Zhao et al., 1993). Although CDPKs are closely related to calmodulin-dependent protein kinases, they are distinguished by a unique structural arrangement. Within a single polypeptide, a calmodulin-like regulatory domain (CaM-LD) is tethered to the kinase by a short junction sequence (approximately 31 residues). CDPKs can function as monomeric enzymes (Putman-Evans et al., 1990) and are activated when Ca^{2+} binds to their CaM-LD (Harper et al., 1994).

In a model plant system, *Arabidopsis*, CDPKs are encoded by more than 12 isoforms (Hrabak et al., 1996). Different isoforms vary in predicted size from 55 to 72 kDa, due primarily to a variable N-terminal domain (21−185 residues). Isoform CPK-1 is used here as a prototype to understand how Ca^{2+} regulates CDPK activity. This isoform has been expressed as a fusion protein in *Escherichia coli*, and it displays activity similar to that of a native isoform purified from soybean (Harper et al., 1994).

CDPKs are normally kept in a basal state of low activity by a pseudosubstrate autoinhibitor located in the junction domain (Harper et al., 1994; Harmon et al., 1994). A mutation which disrupts the autoinhibitor generates a constitutively active kinase. The autoinhibitor is located in a position similar to that of calmodulin-dependent protein kinases, such as CaMKII and MLCK. Here we present biochemical and genetic evidence that CDPKs are activated by intramolecular binding between the junction and CaM-LD.

MATERIALS AND METHODS

DNA fragments encoding fusion proteins were subcloned and expressed in *E. coli* strains DH10α or XL-1 Blue (Stratagene, La Jolla, CA). All standard molecular techniques were performed according to Maniatas et al. (1982). Protein concentrations were measured using the BCA (bicinchoninic acid) reagent (Pierce, Rockford, IL) or the BioRad protein assay reagent (BioRad, Hercules, CA), with bovine serum albumin (BSA) as a standard. Amylose resin was obtained from New England Biolabs (Beverly, MA), glutathione-Sepharose 4B from Pharmacia (Piscataway, NJ), and nickel resin (ProBond) from Invitrogen (San Diego, CA). Calmodulin and calmodulin−agarose were obtained from Sigma (St. Louis, MO), and biotinylated calmodulin was obtained from GIBCO/BRL (Grand Island, NY), Syntide-2

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[1] Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; CPK-1, CDPK isoform-1 from *Arabidopsis*, also called AK1; GST, glutathione *S*-transferase; MBP, maltose binding protein; $AK1_{6H}$, CPK-1 fusion protein sandwiched between a GST at the N terminus and a six-histidine motif at the C terminus; CaM-LD, calmodulin-like domain; K_D, dissociation constant; CaMK, calmodulin-dependent protein kinase; skMLCK, skeletal muscle myosin light-chain kinase; smMLCK, smooth muscle myosin light-chain kinase; BSA, bovine serum albumin.

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from Bachem (Torrance, CA), protease-free BSA from U.S. Biochemicals (Cleveland, OH), and [γ-^{32}P]ATP from Amersham (Arlington Heights, IL).

Fusion Protein Constructs. The following constructs encode fusion proteins derived from CPK-1 (previously named AK1). A prefix p denotes the plasmid construct. Unless noted, constructs were made with the vectors pGEX-2T (Smith & Johnson, 1988) or pMAL-CR1 (New England Biolabs), providing either glutathione *S*-transferase (GST) or maltose binding protein (MBP) as N-terminal affinity tags, respectively. Some fusion proteins were engineered with an additional six-histidine affinity tag at the C-terminal end (GST-6H and MBP-6H fusions).

Proteins ΔNC-1 and ΔNC-2_{6H}, encoded by pΔNC-1 and pΔNC-2_{6H}, respectively, are GST fusions which contain CPK-1 sequence M125−A454 (i.e. N-terminal and CaM-LD deletions). ΔNC-2_{6H} contains a six-histidine tag at its C-terminal end (see Figure 1). The C-terminal end of ΔNC-1 has the sequence A445-ESLSEEEIAP.

The GST−junction protein, encoded by pJ-6H/KG, is a GST-6H fusion protein based in the vector pGEX-KG (Guan & Dixon, 1991). It contains a GST N-terminal affinity tag, a linker sequence GSPGISGGGGGILDSMGRLDGIEGRIDL (starting at the vector's *Bam*HI site), the CPK-1 sequence from residue D411 to A454 (primarily junction sequence), and a C-terminal six-histidine motif (as shown in Figure 1 for ΔNC-2_{6H}).

The CaM-LD protein, encoded by pCam-1, was made as an MBP fusion protein containing the CPK-1 sequence from A445 to L610. After Factor Xa cleavage, the predicted sequence preceding the start of the CaM-LD is ISEFGSSREG-A445. For activation of ΔNC constructs, a non-MBP version of the CaM-LD (CaM-LD 6H/ST) was made as a fusion protein containing an N-terminal six-histidine motif and Factor Xa site (MGGSHHHHHHGMASLEIEGRICSA-A445) and a C-terminal c-myc epitope and StrepTag (L600-EAAAPEQKLISEEDLPDPSAWRHPQFGG*). The parent vector for this construct was pTrcHisA (Invitrogen). The protein was purified by a sequential affinity purification for the six-histidine tag and StrepTag (Schmidt & Skerra, 1993) and was used in activation assays without proteolytic processing.

JC proteins, JC-1, JC-1_{6H}, and JC-4_{6H}, encoded by pJC-1, pJC-1_{6H}, and pJC-4_{6H}, respectively, were made as MBP fusion proteins containing the CPK-1 sequence starting from residue V410 and ending at L610 (JC-1) or ending with the sequence L600-DPGMHHHHHHGS (JC-1_{6H} and JC-4_{6H}). JC-4_{6H} is identical to JC-1_{6H}, except for a substitution of LRV-I444 to DLP-G444 encoded by the DNA sequence dA/GAT/CTC/CCG/GGT which contains *Bgl*II and *Xma*I diagnostic sites. After Factor Xa cleavage, the predicted N-terminal sequence of all processed JC proteins is ISEFGSSR-V410.

KJM4_{6H}, encoded by pKJM4-6H, is a GST-6H fusion protein containing the CPK-1 sequence from residue M1 to L600, with a substitution of LRV-I444 to DLP-G444 in the junction.

p*Camk-2*(1-T359) (mp#22) encodes a GST-6H fusion containing the sequence from residue M1 to T359 of the calmodulin-dependent protein kinase II β (*Camk-2*) from mouse (Karls et al., 1992). This protein lacks the C-terminal 182 residues of *Camk-2*, from R360 to Q542, and terminates with two c-myc epitope tags and a six-histidine motif. To construct this fusion, a *Camk-2* cDNA was PCR amplified and cloned into the vector pGEX-KG. The 5′ side primer was dAGAATTCCGCCATGGCCACCACGGTGAC (*Eco*RI site underlined), and the 3′ side primer was dGCTGTCCGGAGTGCTGTTTGTCTGGGGCTTGAC (*Bsp*EI site underlined). The template was mouse RNA provided by C. Evans (The Scripps Research Institute) primed with oligo(dT) and reverse transcribed with AMV reverse transcriptase. The PCR product was cloned as an *Eco*RI/*Bsp*EI fragment into the parent clone, pGFP-3TAG (mp#34). The reading frame at the *Bsp*EI is T/CCG/GA. A unique *Spe*I site follows the six-histidine motif with the reading frame of dA/CTA/GT. A stop codon is provided by the vector following the end of the polylinker (i.e. immediately after the unique *Hin*dIII site). The *Camk-2*(1-T359) protein was purified by sequential six-histidine and GST affinity chromatography. This kinase was stimulated 60-fold by Ca^{2+}/calmodulin to a specific activity of 0.6 μmol min^{-1} mg^{-1}.

Protein Expression and Purification. All protein purification procedures were carried out at 4 °C. GST-6H fusion proteins were expressed in *E. coli* and purified as previously described (Harper et al., 1994). Fusion proteins with only GST were purified with a similar procedure without a prior selection for a six-histidine affinity tag. MBP fusion proteins were purified according to recommendations by New England Biolabs. Briefly, *E. coli* cells were lysed by incubation for 30 min with lysozyme (2 mg mL^{-1}) in lysis buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride], followed by mild sonication. After centrifugation for 20 min at 12000*g*, the supernatant was incubated with amylose resin on a rocking platform for 20 min. The resin was pelleted and washed extensively with lysis buffer. Fusion proteins were eluted with 10 mM maltose in lysis buffer. Proteins were concentrated by ultrafiltration with Centricon-30 units (Amicon, Beverly, MA).

For binding studies, the N-terminal MBP affinity tags were proteolytically removed from fusion proteins for CaM-LD, JC-1, JC-1_{6H}, and JC-4_{6H}. The MBP fusion protein was bound to amylose resin, equilibrated with Factor Xa buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM NaN_3], and incubated with protease Factor Xa (1 μg of protease per 100 μg of sample) at 4 °C overnight with continuous agitation. The suspension was pelleted by a 1000*g* spin and the supernatant loaded on a Q-Sepharose column. The loaded column was washed extensively with 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl and eluted with 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl. The eluate was desalted by dialysis in 10 mM Tris-HCl (pH 7.5), concentrated by ultrafiltration in a Centricon-10 unit, and stored at −20 °C.

Kinase Assay. Protein kinase activity was assayed using a 0.05 μg sample of enzyme in a 50 μL reaction volume with 300 μM [γ-^{32}P]ATP (40 Ci/mol), 100 μM Syntide-2, 20 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1 mM EGTA, and 0.5 mg mL^{-1} BSA, with or without 1.1 mM $CaCl_2$. Reaction mixtures were incubated for 15 min at 22 °C and reactions terminated by spotting 20 μL onto phosphocellulose filter paper (P81, from Whatman) and immediately immersing in 75 mM phosphoric acid. Filters were washed twice with the same solution and twice with H_2O at 22 °C with gentle agitation for 5 min.

Calmodulin Gel Overlays. Calmodulin gel overlay assays were conducted according to Billingsley et al. (1985). Purified fusion proteins were separated by electrophoresis through a 12% (w/v) SDS−PAGE gel (Laemmli, 1970), electrotransferred to a nitrocellulose membrane, and incubated for 1 h in blocking solution [50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 0.1% (v/v) Tween 20, and 1% (w/v) BSA]. Blots were incubated for 1.5 h with 0.5 μg mL^{-1} biotinylated calmodulin in blocking solution with 5 mM $CaCl_2$ (plus Ca^{2+} conditions) or 5 mM EGTA (minus Ca^{2+} conditions). After being washed in blocking solution (without BSA), blots were incubated with avidin peroxidase conjugate (1:15000 dilution) (Boehringer Mannheim Biochemical, Indianapolis, IN). Bound calmodulin was detected by enhanced chemiluminescence (ECL) and exposure to X-ray film (Hyperfilm ECL) (Amersham).

Molecular Size Exclusion Chromatography. A 15 μg sample of JC-1 (Factor Xa cut and purified from its MBP affinity tag) was subjected to size exclusion FPLC (fast protein liquid chromatography) on a Superose HR 12 column (1.0 × 30 cm) (Pharmacia) in 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM $CaCl_2$. Fractions (0.5 mL each) were collected at a flow rate of 0.5 mL min^{-1}. The molecular mass standards were BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Fifteen microliters from each fraction was analyzed by SDS−PAGE.

Kinetic Binding Assays. Real-time binding analysis between immobilized GST fusion proteins and soluble calmodulin or a CaM-LD was performed using a plasmon resonance-based technology (Fägerstam et al., 1992; Johnsson et al., 1991) on a BIAcore TM2000 instrument (Pharmacia Biosensor AB). All procedures, including immobilization of anti-GST antibody, capturing of GST fusion proteins, and regeneration of the anti-GST antibody surface, were done as recommended by Pharmacia Biosensor AB. Briefly, goat anti-GST antibody was immobilized on a CM5 sensor chip through primary amine group coupling. The sensor chip was first activated by injecting 30 μL of 0.05 M *N*-hydroxysuccinimide and *N*-ethyl-*N*-[3-(diethylamino)propyl]carbodiimide with a continuous flow rate of 5 μL min^{-1}, before injecting 25 μL of anti-GST antibody (at 30 μg mL^{-1}) in 10 mM sodium acetate (pH 5.0). Unreacted surfaces were deactivated by injection of 30 μL of ethanolamine. The anti-GST antibody-coupled sensor chip was then used to capture purified GST fusion proteins (0.3−0.5 μg) injected in binding buffer [25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM $CaCl_2$] at a flow rate of 5 μL min^{-1}.

Kinetic binding experiments were performed at 25 °C by injecting successive 2-fold dilutions of calmodulin or CaM-LD (5−80 μM) in binding buffer at a flow rate of 20 μL min^{-1} for 1 min. The surface was regenerated between cycles by injecting 20 μL of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EGTA.

The association and dissociation kinetics were analyzed on sensorgrams using the BIAevaluation 2.1 software package. Briefly, informative data points were chosen from the ln[abs(d*Y*/d*X*)] plot and fit using different models: simple association, A + B ⇔ AB; parallel association, A + B1 + B2 ⇔ AB1 + AB2; simple dissociation, AB ⇔ A + B; and heterogeneous dissociation, A*i*B*j* ⇔ A*i* + B*j*.

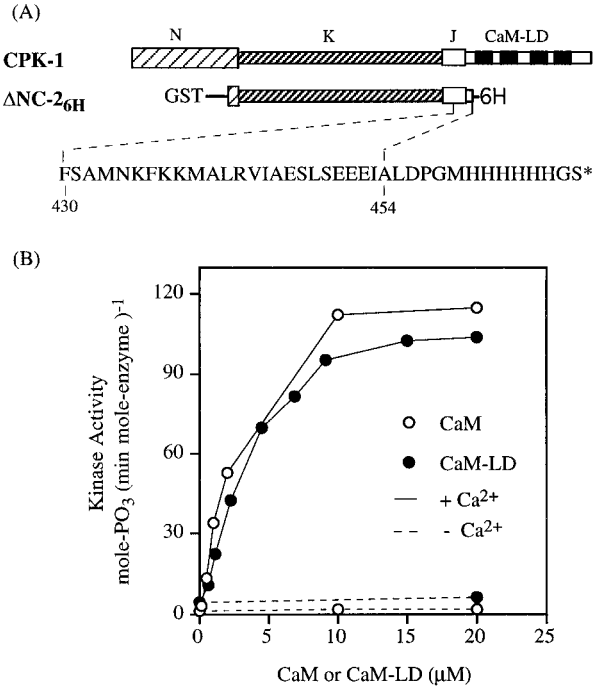

FIGURE 1: Activation of ΔNC-2_{6H} by exogenous calmodulin and an isolated CaM-LD. (A) Diagram of truncated ΔNC-2_{6H} in comparison to CPK-1. Four Ca^{2+}-binding EF hands are indicated by black boxes in the CaM-LD: N, N-terminal domain (residues 1−149); K, catalytic domain (residues 150−413); J, junction domain (residues 414−444); and C, CaM-LD (residues 445−610). The sequence at the C-terminal end of ΔNC-2_{6H} is shown. Numbered residues correspond to positions in the CPK-1 sequence. A454 is the last residue from CPK-1 and is followed by a C-terminal extension which includes a six-histidine affinity tag. (B) Stimulation of ΔNC-2_{6H} kinase activity by exogenous calmodulin and an isolated CaM-LD.

RESULTS

Reconstitution of CDPK as Two Components. A critical prediction based on a model in which CDPKs are activated by intramolecular binding between the junction and CaM-LD is that an isolated CaM-LD should be able to bind and activate a truncated CDPK in which the CaM-LD has been deleted (i.e. the enzyme can be separated into two components). To test this hypothesis, we constructed two mutants, ΔNC-2_{6H} (Figure 1) and ΔNC-1, which had their CaM-LD deleted and replaced by slightly different C-terminal extensions. The N-terminal domain was also deleted in these mutants to avoid any potential complications resulting from a second calmodulin binding sequence (Ca^{2+}-independent) located in this region (see Figure 2). However, analysis of mutants with intact N-terminal domains indicated that this second calmodulin binding site is not required for activation of the full-length enzyme or truncation mutants (data not shown). ΔNC-2_{6H} and ΔNC-1 were purified to approximately 90% homogeneity and analyzed for kinase activity. Consistent with previous results (Harper et al., 1994), these ΔNC mutants displayed a low level of basal activity and were not stimulated by Ca^{2+}. However, significant activation was achieved when assays included either Ca^{2+}/calmodulin, an isolated Ca^{2+}/CaM-LD, or an anti-junction antibody.

In the presence of 10 μM Ca^{2+}/calmodulin, the kinase activities of ΔNC-2_{6H} and ΔNC-1 were both stimulated 30−40-fold (Table 1). The calmodulin activation of ΔNC-2_{6H} was shown to be dose-dependent (Figure 1) and reached a maximum at around 10 μM calmodulin with an apparent K_{act}

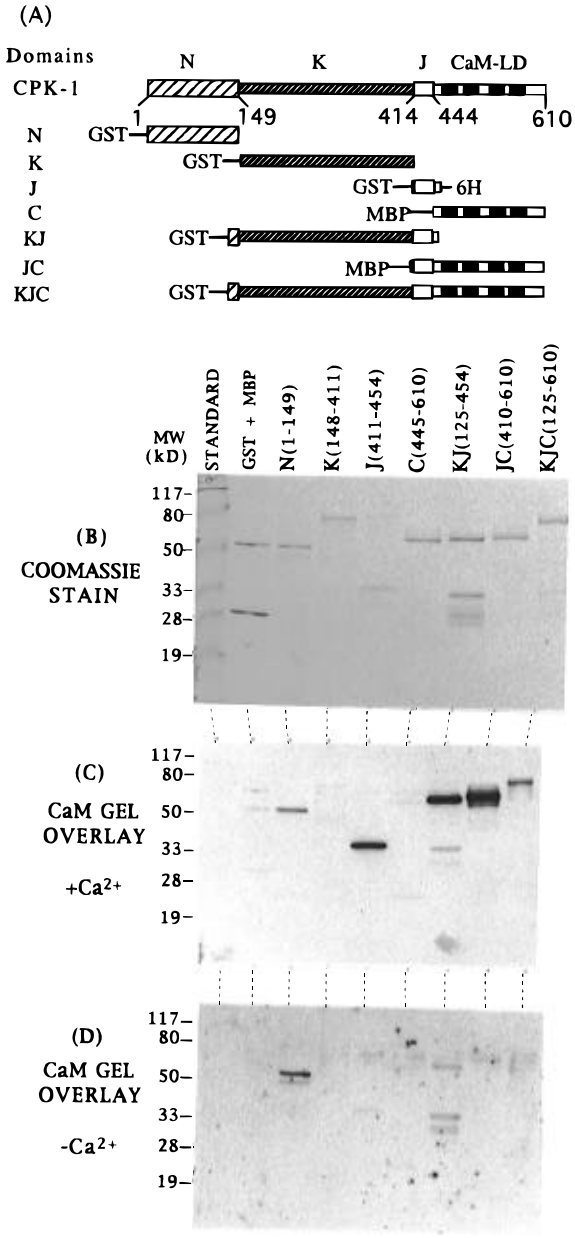

FIGURE 2: Identification of putative CaM-LD binding sequences by calmodulin (CaM) gel overlays. (A) Diagrams of GST and MBP fusion proteins containing various regions of CPK-1. The actual CPK-1 sequence present in each fusion protein is indicated in the parentheses above gel lanes in panel B. (B) Fusion proteins separated by a 12% (w/v) SDS−PAGE gel and stained with Coomassie blue. Gels were loaded to provide an equal amount of the major fusion protein band (approximately 1 μg of each fusion protein). (C and D) Calmodulin gel overlay analysis in the presence and absence of free Ca^{2+}, respectively. The gel overlays were performed with gels electrophoresed in parallel to the one shown in panel B.

of 2 μM. An equivalent activation was observed using an isolated CaM-LD (CaM-LD-6H/ST). While activation by both calmodulin and an isolated CaM-LD required Ca^{2+}, we did not test for a potential difference in the minimum Ca^{2+} level required for full activation.

The maximum specific activity observed for ΔNC-2_{6H} after stimulation by either Ca^{2+}/calmodulin or Ca^{2+}/CaM-LD was approximately 50% of that of a wild-type enzyme activated by Ca^{2+}. Since the truncations and wild-type enzymes differ in size, this specific activity was calculated on the basis of molar equivalents. This 50% relative activation level was the maximum found in multiple ΔNC−enzyme preparations.

Table 1: Activation of Mutant Kinases

	specific activity[a] [mol of PO_3 min^{-1} (mol of enzyme)$^{-1}$]				
	− calmodulin		+ calmodulin (10mM)		+ IgG-T857 [b]
enzyme	− Ca^{2+}	+ Ca^{2+}	− Ca^{2+}	+ Ca^{2+}	− Ca^{2+}
$AK1_{6H}$	8.2	299	6.5	355	42
ΔNC-1	3.2	3.4	3.4	104	−
ΔNC-2_{6H}	4.5	5.0	4.5	173	186
$KJM4_{6H}$	1.8	3.6	−	−	25

[a] Specific activities are expressed as moles of PO_3 per minute per mole of enzyme instead of micromoles of PO_3 per minute per milligram to normalize for the differences in molecular weights of enzymes assayed (the size of ΔNC-truncated enzymes are 68% of that of $AK1_{6H}$ and $KJM4_{6H}$). For $AK1_{6H}$, a specific activity of 299 mol of PO_3 min^{-1} (mol of enzyme)$^{-1}$ is equal to 3.2 μmol of PO_3 min^{-1} mg^{-1}. The Ca^{2+}-dependent specific activity of $AK1_{6H}$ is the average of eight enzyme preparations, which varied from 170 to 380 mol of PO_3 min^{-1} (mol of enzyme)$^{-1}$. Other specific activities are averaged from assays of at least two enzyme preparations. Calmodulin activation of ΔNC-1 was highly variable between different enzyme preparations [ranging from 50 to 122 mol of PO_3 min^{-1} (mol of enzyme)$^{-1}$]. The specific activities reported here were the average of the two most recent enzyme preparations conducted with extra care to minimize proteolysis. Calmodulin activation of ΔNC-2_{6H} was more consistent between different enzyme preparations [ranging from 155 to 200 mol of PO_3 min^{-1} (mol of enzyme)$^{-1}$]. [b] IgG-T857 is an anti-junction antibody which stimulates latent kinase activity in recombinant CDPKs without Ca^{2+} stimulation (Harper et al., 1994).

A similar 40-fold activation of ΔNC-2_{6H} was also achieved by the addition of a polyclonal anti-junction antibody. This antibody is thought to activate both truncated and full-length CDPKs through binding to the junction and blocking the action of the autoinhibitor (Harper et al., 1994).

Potential CaM-LD Binding Sequences Detected by a Calmodulin Probe. The ability of Ca^{2+}/calmodulin to activate a truncated CDPK lacking a CaM-LD suggests that calmodulin can be used as a tool to understand how the CaM-LD functions. Therefore, to identify potential intramolecular binding sequences for the CaM-LD, we used biotinylated calmodulin as a probe. Individual domains from CPK-1 were made and purified as fusion proteins and analyzed by calmodulin gel overlays (Figure 2).

The junction was the only region of CPK-1 which bound calmodulin in a Ca^{2+}-dependent manner (e.g. lane J). No significant binding was observed to an isolated CaM-LD (lane C) or kinase domain (lane K). Calmodulin binding to the N-terminal domain was observed (lane N) under both plus and minus Ca^{2+} conditions.

The Junction Region Contains a Binding Sequence for the CaM-LD. To verify that the junction would also bind to a CaM-LD, we conducted binding studies between a GST−junction protein and an isolated CaM-LD using the highly sensitive plasmon resonance-based technology.

The rate constants determined for binding of calmodulin or the isolated CaM-LD to different immobilized GST fusion proteins are shown in Table 2. Calmodulin and CaM-LD bound to the GST−junction with estimated K_Ds of 5 and 6 μM, respectively. These values are similar to the K_{act} determined above for the activation of ΔNC-2_{6H} by calmodulin and CaM-LD. The sensorgrams which recorded the real-time association and dissociation reactions displayed a best fit with binding kinetics comprised of both high- and low-affinity binding interactions. The high-affinity interaction dominates and is shown as the estimated K_D in Table 2. The reason for two component binding kinetics is unclear.

Table 2: Kinetic Binding Parameters from Biosensor Analysis

immobilized protein	injected protein (5−80 μM)	k_{ass} (M^{-1} s^{-1} × 10^3)	k_{diss} (s^{-1})	estimated K_D (M × 10^{-6})
GST	calmodulin	–	–	>500
GST	CaM-LD	–	–	>500
GST−junction	calmodulin	4.4	0.02	5
GST−junction	CaM-LD	3.3	0.02	6
ΔNC-1	calmodulin	6.7	0.01	2
ΔNC-1	CaM-LD	3.9	0.01	3
Camk-2(1-T359)	calmodulin	–	–	>500
Camk-2(1-T359)-P[b]	calmodulin	4.5	5.6×10^{-4}	0.12

[a] Association and dissociation rate constants were calculated from a best fit of data points to kinetic models describing parallel association and heterogeneous dissociation. The k_{ass} and k_{diss} shown represent the faster of two association rate constants and the slower of two dissociation rate constants, respectively. A dash indicates that no detectable binding was observed and that the estimated K_D is greater than 500 μM. [b] *Camk-2*(1-T359) was preincubated with 1 μM calmodulin and 300 μM ATP before immobilization. Bound calmodulin was removed by washing with a buffer containing 1 mM EGTA before binding analysis.

It is possible that both association and dissociation phases are the result of two events, such as an ordered binding in which one lobe of the CaM-LD binds before the other, as proposed for calmodulin binding to smMLCK (Meador et al., 1992). Alternatively, it may reflect a binding to two different sequences.

To verify that calmodulin and the isolated CaM-LD could bind the junction sequence of a ΔNC protein, we assayed their binding to ΔNC-1 (Table 2). Calmodulin and the CaM-LD bound ΔNC-1 with a K_D equivalent to that observed for binding to a GST−junction protein. This result confirms that a calmodulin/CaM-LD binding segment is accessible in a ΔNC protein.

As a control for our kinetic binding assays, we measured the K_D between calmodulin and a recombinant calmodulin-dependent protein kinase, **CamK-2*(1-T359) from mouse (Table 2). As expected, calmodulin bound this protein with high affinity (K_D = 130 nM). However, this binding appeared to require that the enzyme be autophosphorylated. Autophosphorylation of CaMKII from rats has been shown to increase the binding affinity for calmodulin by slowing the dissociation rate (Meyer et al., 1992), a phenomenon described as "calmodulin trapping". A parallel experiment using "prestimulated" ΔNC-1 failed to change the binding constant measured between ΔNC-1 and calmodulin (not shown).

LRV-I444 to DLPG Substitution Disrupts the Binding Sequence. To begin a more precise localization of the CaM-LD binding sequence, we used a gel overlay analysis to compare the binding of a calmodulin probe to a wild-type junction sequence (contained in JC-1_{6H}) and two mutant junctions (contained in JC-3 and JC-4_{6H}) (Figure 3A). The most severe disruption of calmodulin binding occurred with the DLP-G444 substitution contained in JC-4_{6H} (Figure 3B). With JC-3, calmodulin binding was detectable, but significantly reduced.

Bimolecular Binding Is Suppressed when a Junction and CaM-LD Are Tethered. The following binding studies were conducted to determine if JC-1 could still engage in bimolecular binding to either (i) a GST−junction protein, (ii) calmodulin, or (iii) another JC-1 protein.

In the first binding study, the CaM-LD in JC-1 was tested for Ca^{2+}-dependent binding to a junction column (GST−junction protein immobilized on a glutathione-Sepharose matrix). As shown in Figure 4, the JC-1 protein did not bind to the column whereas both calmodulin and an isolated

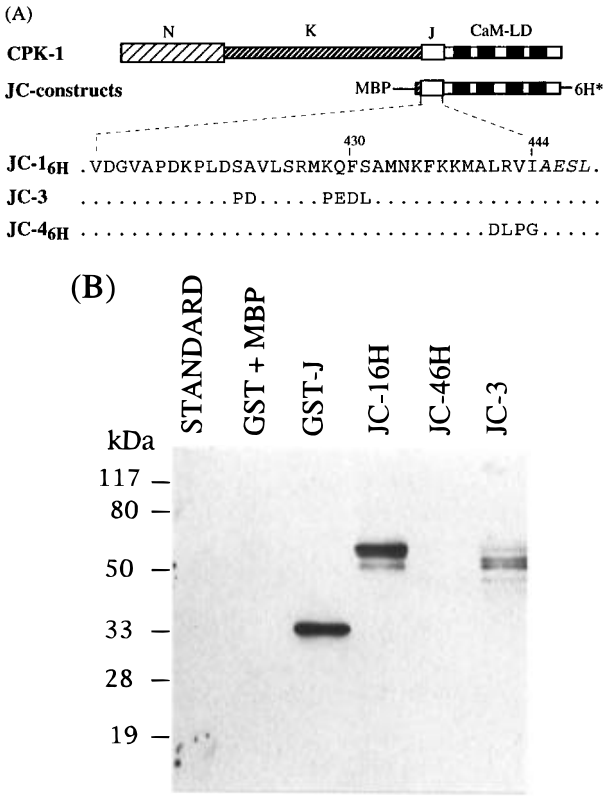

FIGURE 3: Disruption of the putative CaM-LD binding sequence in two junction mutants, as analyzed by a calmodulin gel overlay. (A) Diagram of JC proteins used for gel overlay analysis. For comparison, a diagram of the full-length CPK-1 is shown on top. All JC proteins are MBP fusions containing a tethered junction and CaM-LD. The 6H* in the diagram is to indicate that JC-1_{6H} and JC-4_{6H}, but not JC-3, have been modified with a C-terminal six-histidine affinity tag. This six-histidine modification does not affect calmodulin binding (not shown). The sequence shown for JC-1_{6H} corresponds to the junction and the first four residues of the CaM-LD. The substitutions in JC-3 and JC-4_{6H} are noted below. Identical residues are indicated by dots. (B) Calmodulin gel overlay analysis. A parallel gel was stained with Coomassie blue to show that each JC protein was present in an equal amount (not shown). Lane GST + MBP provides a negative control showing that calmodulin does not bind to the affinity tags. Lane GST-J contains the GST−junction protein as a positive control. Lanes JC-1_{6H}, JC-3, and JC-4_{6H} correspond to proteins diagrammed in panel A. No signal was detected under the minus Ca^{2+} condition (not shown).

CaM-LD bound to the column in a Ca^{2+}-dependent fashion (i.e. they were eluted by an EGTA wash).

In a second binding study, the junction in JC-1 was tested for binding to a calmodulin−agarose column (results are

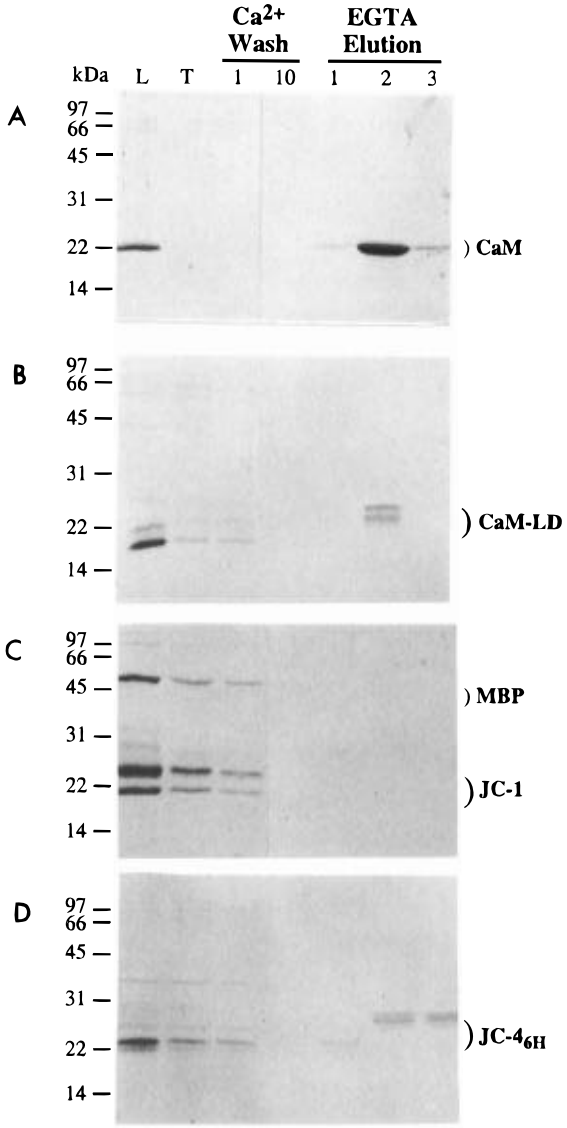

FIGURE 4: Binding of calmodulin, CaM-LD, JC-1, and JC-4_{6H} proteins to junction columns. Columns were prepared with a GST−junction fusion protein captured on glutathione-Sepharose. Identical columns with 0.1 mg of GST−junction protein in a 400 μL bed volume were equilibrated with 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM $CaCl_2$. Columns were run in parallel and loaded with 50 μg of calmodulin (CaM) (A), CaM-LD (B), JC-1 (C), or JC-4_{6H} (D). Columns were washed with 10 × 1 mL of equilibration buffer and eluted with 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM EGTA. Eluate was collected as 300 μL fractions. Samples of each fraction (15 μL) were electrophoresed through a 12% (w/v) SDS−PAGE gel and stained with Coomassie blue: lane L, loaded protein samples; lane T, through fraction (material not bound to the column); and lanes Ca^{2+} wash 1 and 10, samples from the first and last plus Ca^{2+} wash. Note that there was no detectable protein eluting in the 10th wash. Lanes EGTA elution 1−3, samples from the first three fractions eluted with an EGTA-containing buffer. Note that the CaM, CaM-LD, and JC-4_{6H} all show upward mobility shifts when free Ca^{2+} is chelated by EGTA. In panel C, the residual MBP affinity tag provides an internal control which shows the same retention and elution profile as the "nonbinding" JC-1 protein.

summarized in Figure 5). In controls, a GST−junction protein was shown to bind to this column in a Ca^{2+}-dependent fashion. However, in a parallel assay, the JC-1 protein did not bind (data not shown).

A third binding study was conducted to determine if JC-1 formed dimers (or multimers) as a result of the CaM-LD from one polypeptide binding to the junction of another. If this occurred, it would provide a potential explanation for

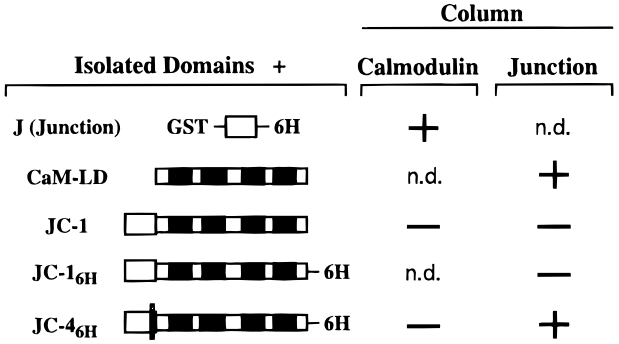

FIGURE 5: Summary of bimolecular binding properties for GST−junction, isolated CaM-LD, JC-1, JC-1_{6H}, and JC-4_{6H} proteins. The DLP-G444 substitution in JC-4_{6H} is indicated by a line through the end of the junction. Ca^{2+}-dependent binding is indicated by a +; − indicates absence of any detectable binding, and n.d. indicates that assays have not been conducted.

why the JC-1 protein failed to bind to a junction or calmodulin column in the first three studies. However, when a JC-1 protein was subjected to size exclusion chromatography, it eluted in a fraction consistent with its expected monomeric size of 26 kDa (data not shown).

While the absence of bimolecular binding in JC-1 is consistent with the hypothesis that its junction and CaM-LD are sequestered by intramolecular binding, it is also possible that the JC-1 protein is dysfunctional due to improper folding (i.e. "dead"). Although it is difficult to prove that the JC-1 protein has a normal junction and CaM-LD, we conducted a number of controls which indicate that the two domains are "functional". First, the JC-1 protein was shown to contain a binding sequence by a calmodulin gel overlay analysis. Second, JC-1 demonstrated a Ca^{2+}-induced mobility shift on both native and SDS−PAGE (data not shown). This mobility shift indicates that our JC-1 protein was able to bind Ca^{2+}. Furthermore, circular dichroism studies showed that an isolated JC-1 protein contained a high percentage of α-helical structure, similar to that observed for an isolated CaM-LD (J.-F. Huang and J. F. Harper, unpublished).

LRV-I444 to DLPG Substitution Disrupts Junction−CaM-LD Interaction. To confirm that the CaM-LD in JC-1 was interacting with its tethered junction domain, a mutant protein (JC-4_{6H}) in which the CaM-LD binding sequence was disrupted was tested for a restored bimolecular binding potential of the CaM-LD. The protein JC-4_{6H} is similar to JC-1 but contains a DLP-G444 substitution in the junction which appears to disrupt the CaM-LD binding sequence (Figure 3). JC-4_{6H} was tested for its binding to a junction column, in parallel with two controls, JC-1 and JC-1_{6H}. As shown in Figure 4D, JC-4_{6H} displayed Ca^{2+}-dependent binding, whereas no binding was observed for JC-1 (Figure 4C) or JC-1_{6H} (not shown). The second control protein, JC-1_{6H}, is identical to JC-1 except that it has a C-terminal six-histidine motif identical to that found in JC-4_{6H}. It was used as a control to verify that the binding of JC-4_{6H} to the column was not due to the addition of a C-terminal six-histidine motif. Thus, in the mutant JC-4_{6H}, a disruption of the intramolecular binding sequence allows its CaM-LD to bind a separate junction sequence.

LRV-I444 to DLPG Substitution Disrupts Ca^{2+} Activation. The kinase activity of the mutant KJM4_{6H} was evaluated to determine if a disruption of the CaM-LD binding sequence

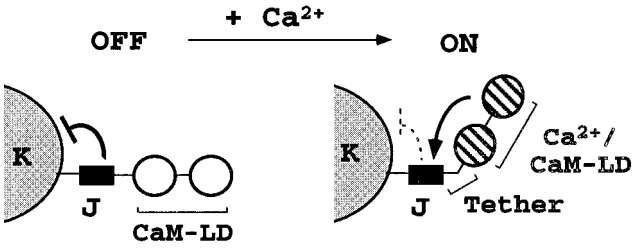

FIGURE 6: CDPK activation model. (Off) Kinase activity is normally turned off by a pseudosubstrate autoinhibitor located in the junction (J) that interacts with the kinase domain (K). (On) In the presence of Ca^{2+}, the CaM-LD binds to the junction and disengages the autoinhibitor. The tether here is shown as a hinge, but it may also exist as a rigid joint.

would also disrupt the Ca^{2+} activation mechanism. $KJM4_{6H}$ is a full-length enzyme which contains the DLP-G444 substitution. This enzyme was made and purified to near homogeneity as a GST-6H fusion protein. The basal kinase activity of $KJM4_{6H}$ was low and displayed a marginal 2-fold stimulation by Ca^{2+} (Table 1). However, a 15-fold stimulation was observed with the addition of an anti-junction polyclonal antibody (T857). Antibody activation was dose-dependent and closely paralleled the antibody activation observed with a full-length wild-type fusion, $AK1_{6H}$. This antibody activation indicates that the $KJM4_{6H}$ enzyme has latent activity even though it cannot be activated by Ca^{2+}.

DISCUSSION

Activation Model. Two models for CDPK activation have been proposed (Harper et al., 1994; Harmon et al., 1994). In both models, the CaM-LD undergoes a Ca^{2+}-induced conformational change which somehow disengages the autoinhibitor. In the first model, the CaM-LD binds to a sequence in the junction which overlaps or lies adjacent to the autoinhibitor. This model is similar to calmodulin-dependent protein kinases in which activation occurs through binding of calmodulin to the autoinhibitory region (Soderling et al., 1990; Kemp & Pearson, 1991; Means et al., 1991; Hanson & Schulman, 1992; Knighton et al., 1992; Hu et al., 1994). In the second model, the CaM-LD allosterically alters the conformation of the junction without binding to it.

Here we provide the following four lines of evidence supporting the first model in which activation involves a Ca^{2+}-induced intramolecular binding of the CaM-LD to the junction (Figure 6).

(1) A truncated CDPK, in which the CaM-LD has been deleted (e.g. construct $\Delta NC\text{-}2_{6H}$), can be activated through a binding interaction with exogenous calmodulin or an isolated CaM-LD ($K_{act} \approx 2\ \mu M$).

(2) A CaM-LD binding sequence is present in the junction and can bind to an isolated CaM-LD with a K_D of approximately 6 μM.

(3) When the junction and CaM-LD are tethered in the same polypeptide (e.g. JC-1), they appear to bind each other in an intramolecular fashion.

(4) A mutation which disrupts intramolecular binding (e.g. LRV-I444 to DLPG) also disrupts the Ca^{2+}-induced activation of a full-length enzyme ($KJM4_{6H}$).

While the evidence is most consistent with the first model, we have not formally disproven a second alternative model in which activation occurs without the CaM-LD binding to the junction. The primary argument against this alternative model is that a CDPK can be reconstituted as two components, with the CaM-LD functioning as a separate activator which binds to the junction of a ΔNC mutant. However, this reconstitution experiment does not prove that an analogous intramolecular binding event actually occurs in a full-length CDPK. Since activation of a truncated CDPK can also be achieved with anti-junction antibodies, it is possible that many different binding interactions can artificially stimulate a truncated CDPK. Likewise, the finding that a calmodulin/CaM-LD binding sequence is present in the junction does not prove that it is used as a target for intramolecular binding in a full-length enzyme. Its presence may be fortuitous, or an evolutionary remnant from CDPK's putative origin as a calmodulin-regulated kinase (Harper et al., 1991).

The critical supporting argument for our model is the evidence that the junction is actually used as a target for intramolecular binding (summarized in Figure 5). The central observation for this contention is that a polypeptide containing a tethered junction and CaM-LD (JC-1 protein) does not bind to a separate junction or calmodulin, as would be expected on the basis of the bimolecular binding properties of an isolated CaM-LD or junction domain. This result is consistent with the hypothesis that an intramolecular interaction between a junction and its tethered CaM-LD dominates over potential bimolecular interactions. To confirm that the tethered domains bind each other, we showed that this intramolecular interaction could be disrupted by a mutation which destroys the CaM-LD binding sequence (as shown with the $JC\text{-}4_{6H}$ polypeptide). This mutation restored the bimolecular binding potential of the tethered CaM-LD and allowed it to bind to a seperate GST−junction protein.

Intramolecular Binding Sequence. The precise location of the CaM-LD binding sequence has not been determined. However, the core binding sequence probably lies downstream of S431 since a mutation including residues from S421 to S431 reduced, but did not eliminate, binding by a calmodulin probe in a gel overlay analysis. In contrast, the LRV-I444 to DLPG substitution at the C-terminal end of the junction completely abolished calmodulin binding. This end of the junction contains several overlapping sites (e.g. F436−I444 and F430−I444) which have features similar to the calmodulin binding domains present in MLCK and CaMKII (Harper et al., 1994; and Harmon et al., 1994).

The structures of the calmodulin binding domains from MLCKs and CaMKII have been characterized as calmodulin−peptide complexes using NMR and X-ray crystallography (Ikura et al., 1992; Meador et al., 1992, 1993). These structures indicate that a typical binding segment forms a basic amphipathic helix. The N- and C-terminal hydrophobic residues in these helices provide "anchors" which help lock the peptide into the C- and N-terminal lobes of calmodulin, respectively. The target binding sequences in MLCK and CaMKII differ in size (14 and 10 residues, respectively), indicating that the length of a binding segment can be variable. We speculate that in CPK-1 a nine-residue segment from F436 to I444 forms an amphipathic basic helix and provides the core CaM-LD binding sequence.

Intramolecular Binding Affinity. While the calmodulin/CaM-LD binding sequence in CPK-1 has similarities with typical calmodulin binding sequences found in Ca^{2+}/calmodulin-regulated enzymes, it is distinct since its binding

affinity for an isolated CaM-LD or calmodulin is relatively poor. The binding affinity between an untethered junction and CaM-LD ($K_D \approx 6$ μM) is 1 order of magnitude weaker than that observed from binding of calmodulin to targets such as *CamK-2*(1-T359) ($K_D \approx 120$ nM). The weaker affinity of the CaM-LD−junction interaction appears to be an intrinsic property of the binding sequence (and not the CaM-LD) since calmodulin binds to this junction sequence with a similar K_D. This relatively weak binding affinity is consistent with the high concentrations of calmodulin which were required to activate ΔNC proteins (e.g. ΔNC-2_{6H}, $K_{act} \approx 2$ μM).

We suggest two reasons why a low-affinity calmodulin/CaM-LD binding sequence may be essential for CDPKs. First, a high-affinity binding sequence may result in a kinase with higher basal activity. The reason is that, in the context of an intramolecular reaction, a high-affinity binding sequence will shift the "on−off" equilibrium in the direction of the bound state. This may result in a significant increase in the rate of enzyme activation, even in the absence of a Ca^{2+} stimulation.

The second reason is that a high-affinity binding sequence may result in an inappropriate activation by ectopic binding of calmodulin. Since calmodulin binding can activate a truncated CDPK, it is reasonable to speculate that calmodulin binding could also activate a native enzyme. To ensure that *in vivo* regulation of CDPK is uncoupled from calmodulin stimulation, some mechanism must exist to prevent significant levels of ectopic binding. One potential strategy is to have a junction with a relatively low affinity for calmodulin (i.e. $K_D = 10^{-6}$ M). This would require that calmodulin be present at micromolar levels to bind and activate a CDPK. Although micromolar calmodulin may exist in some subcellular locations, the average concentration for a whole cell has been estimated as 0.27 and 0.045 nM for guard cells and epidermal cells, respectively (Ling & Assmann, 1992). Even if one assumes that the cytoplasmic volume of a guard cell is only 1% of that of the total cell, average calmodulin concentrations are probably not high enough to activate a CDPK. Thus, a low-affinity binding sequence may be optimally suited for activation by intramolecular binding and provide a mechanism to uncouple CDPKs from inappropriate activation by calmodulin.

N-Terminal Domain. An unexpected finding in our search for calmodulin/CaM-LD binding sequences in CPK-1 was that the N-terminal domain bound calmodulin under plus and minus Ca^{2+} conditions. The N-terminal domains of CDPKs are highly variable, and the presence of similar binding motifs in other isoforms is not known. However, Ca^{2+}-independent binding of calmodulin has been observed with other proteins, such as neuromodulin, brush border myosin-I, and phosphorylase kinase (Houbre et al., 1991; Alexander et al., 1987; Andreasen et al., 1983; Cohen et al., 1978). In the case of phosphorylase kinase, the Ca^{2+}-independent interaction may be important for maintaining the association of subunits in the holoenzyme.

In CPK-1, the significance of the Ca^{2+}-independent calmodulin-binding sequence is unknown. The sequence is apparently not required for the Ca^{2+} activation mechanism, since the entire N-terminal domain in CPK-1 can be deleted without causing detectable changes in kinase activity, as determined by *in vitro* kinase assays on recombinant enzymes (Harper et al., 1994). We have not tested whether the CaM-LD, like calmodulin, can bind to the N-terminal domain in CPK-1.

Tether. The feature of our model which distinguishes CDPK from a calmodulin-dependent enzyme is that the CaM-LD binding is an intramolecular event. Thus, an important structural feature of a CDPK is the connection between the junction and CaM-LD. We propose that this connection be designated as the "tether" and be defined as the region between the C-terminal end of the CaM-LD binding sequence and the beginning of the first putative α-helix in the first EF hand of the CaM-LD.

We offer two models to describe the tether's structure. First, it may provide a flexible hinge which allows the entire CaM-LD to move into a binding position. Alternatively, it may form a rigid joint which keeps the N-terminal lobe of the CaM-LD in a fixed position relative to the binding sequence. The tether's structural properties appear to be important since multiple mutations in this region have been shown to specifically disrupt the Ca^{2+} activation mechanism (J.-F. Huang and J. F. Harper, unpublished).

Tethered Modules. CDPKs provide a prototype for an emerging class of bimodular proteins which appear to be fusions between two formerly separate but interacting proteins, in this case calmodulin and a calmodulin-dependent protein kinase. Other examples may include the yeast and plant histidine kinases, SLN1 and ETR1, respectively, which appear to be fusions of bacterial two-component regulators (Ota & Varshavsky, 1993; Chang et al., 1993). For CDPKs, we have shown that the two modules can be experimentally separated (i.e. "untethered") and reconstituted as a two-component enzyme with Ca^{2+}-dependent kinase activity.

For CDPKs, an important consequence of having the CaM-LD tethered to the kinase is that the two modules can evolve as a unit, independent of a separate activator such as calmodulin. Since calmodulin is coupled to the activation of multiple enzymes, there is a constraint on its freedom to evolve new properties. Hence, calmodulin is highly conserved across phylogenetic kingdoms. In contrast, a CaM-LD from CDPK provides a less constrained evolutionary substrate since each CaM-LD is coupled to a single enzyme. An extensive divergence in CaM-LDs has probably occurred. For example, two CDPK-related kinases have been reported with highly divergent CaM-LDs, one from lily (*Lilium longiflorum*, isoform CCaMK) which has a CaM-LD with only three EF hands (Patil et al., 1995) and another from carrot (*Daucus carota*, isoform CRK) in which the CaM-LD contains four nonconsensus EF hand motifs (Lindzen & Choi, 1995).

Different CaM-LDs with different Ca^{2+} binding properties may account for the wide range of Ca^{2+} concentrations reported to activate different CDPKs (Roberts & Harmon, 1992). Most CDPKs are reported to be activated by Ca^{2+} concentrations in the micromolar range. For example, recombinant CPK-1 is 50% activated by 15 μM Ca^{2+} (Binder et al., 1994). In contrast, calmodulin is activated by submicromolar levels of Ca^{2+}. The relatively high Ca^{2+} activation thresholds for some CDPKs is probably a result of their CaM-LDs having weaker Ca^{2+}-binding affinities. For example, recombinant *Pf*CPK (from *Plasmodium falciparum*) is 50% activated by approximately 15 μM Ca^{2+}, and its CaM-LD binds Ca^{2+} with a mean K_D of 80 μM, which is 40-fold higher than the mean K_D for Ca^{2+} binding to calmodulin

(Zhao et al., 1994). Thus, an apparent evolutionary consequence of a tethered CaM-LD is that the Ca^{2+} activation properties of CDPKs have been free to diverge from those of calmodulin-dependent protein kinases, giving rise to a kinase family with the capacity to decode different Ca^{2+} signals.

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REFERENCES

Alexander, K. A., Cimler, B. M., Meier, K. E., & Storm, D. R. (1987) *J. Biol. Chem. 262*, 6108−6113.
Andreasen, T. J., Leutje, C. W., Heideman, W., & Storm, D. R. (1983) *Biochemistry 22*, 4615−4618.
Billingsley, M. L., Pennypacker, K. R., Hoover, C. L., Brigati, D. J., & Kincaid, R. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7585−7589.
Binder, B., Harper, J. F., & Sussman, M. R. (1994) *Biochemistry 33*, 2033−2041.
Chang, C., Kwok, S. F., Bleecker, A. B., & Meyerowitz, E. M. (1993) *Science 262*, 539−554.
Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., & Nairn, A. C. (1978) *FEBS Lett. 92*, 287−293.
Fägerstam, L. G., Frostell-Karlsson, Å., Karlsson, R., Persson, B., & Rönnberg, I. (1992) *J. Chromatogr. 597*, 397−410.
Guan, K., & Dixon, J. E. (1991) *Anal. Biochem. 192*, 262−267.
Hanson, P. I., & Schulman, H. (1992) *Annu. Rev. Biochem. 61*, 559−601.
Harmon, A. C., Yoo, B.-C., & McCaffery, C. (1994) *Biochemistry 33*, 7278−7287.
Harper, J. F., Sussman, M. R., Schaller, E. G., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991) *Science 252*, 951−954.
Harper, J. F., Binder, B. M., & Sussman, M. R. (1993) *Biochemistry 32*, 3282−3290.
Harper, J. F., Huang, J.-F., & Lloyd, S. J. (1994) *Biochemistry 33*, 7267−7277.
Houbre, D., Duportail, G., Deloulme, J.-C., & Baudier, J. (1991) *J. Biol. Chem. 266*, 7121−7131.
Hrabak, E. M., Dickmann, L. J., Satterlee, J. S., & Sussman, M. R. (1996) *Plant Mol. Biol. 31*, 405−412.
Hu, S.-H., Parker, M. W., Lei, J. Y., Wilce, M. C. J., Benian, G. M., & Kemp, B. E. (1994) *Nature 369*, 581−584.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632−638.
Johnsson, B., Löfås, S., & Lindquist, G. (1991) *Anal. Biochem. 198*, 268−277.
Karls, U., Müller, U., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., & Harbers, K. (1992) *Mol. Cell. Biol. 12*, 3544−3652.
Kemp, B. E., & Pearson, R. B. (1991) *Biochim. Biophys. Acta 1094*, 67−76.
Knighton, D. R., Pearson, R. B., Sowadski, J. M., Means, A. R., Ten Eyck, L. F., Taylor, S. S., & Kemp, B. E. (1992) *Science 258*, 130−135.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lindzen, E., & Choi, J. H. (1995) *Plant Mol. Biol. 28*, 785−797.
Ling, V., & Assmann, S. M. (1992) *Plant Physiol. 100*, 970−978.
Maniatis, T., Fritsch, E. F., & Sambroo, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1992) *Science 257*, 1251−1255.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1993) *Science 262*, 1718−1721.
Means, A. R., Bagchi, I. C., VanBerkum, M. F., & Kemp, B. E. (1991) *Adv. Exp. Med. Biol. 304*, 11−24.
Meyer, T., Hanson, P. I., Stryer, L., & Schulman, H. (1992) *Science 256*, 1199−1202.
Ota, I. M., & Varshavsky, A. (1993) *Science 262*, 566−569.
Patil, S., Takezawa, D., & Poovaiah, B. W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4897−4901.
Putnam-Evans, C., Harmon, A. C., & Cormier, M. J. (1990) *Biochemistry 29*, 2488−2495.
Roberts, D. M., & Harmon, A. C. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 43*, 375−414.
Schmidt, T. G. M., & Skerra, A. (1993) *Protein Eng. 6*, 109−122.
Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31−40.
Soderling, T. R., Fukunaga, K., Rich, D. P., Fong, Y. L., Smith, K., & Colbran, R. J. (1990) *Adv. Second Messenger Phosphoprotein Res. 24*, 206−211.
Zhao, Y., Kappes, B., & Franklin, R. M. (1993) *J. Biol. Chem. 268*, 4347−4354.
Zhao, Y., Pokutta, S., Maurer, P., Lindt, M., Franklin, R. M., & Kappes, B. (1994) *Biochemistry 33*, 3714−3721.

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