Intramolecular Activation of a Ca^{2+}-Dependent Protein Kinase Is Disrupted by Insertions in the Tether That Connects the Calmodulin-like Domain to the Kinase[†]

Veronique Vitart, John Christodoulou, Jing-Feng Huang, Walter J. Chazin,[‡] and Jeffrey F. Harper*

Department of Cell Biology, BCC283, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received October 12, 1999; Revised Manuscript Received January 18, 2000

ABSTRACT: Ca^{2+}-dependent protein kinases (CDPK) have a calmodulin-like domain (CaM-LD) tethered to the C-terminal end of the kinase. Activation is proposed to involve intramolecular binding of the CaM-LD to a junction sequence that connects the CaM-LD to the kinase domain. Consistent with this model, a truncated CDPK (ΔNC) in which the CaM-LD has been deleted can be activated in a bimolecular interaction with an isolated CaM-LD or calmodulin, similar to the activation of a calmodulin-dependent protein kinase (CaMK) by calmodulin. Here we provide genetic evidence that this bimolecular activation requires a nine-residue binding segment from F436 to I444 (numbers correspond to CPK-1 accession number L14771). Two mutations at either end of this core segment (F436/A and VI444/AA) severely disrupted bimolecular activation, whereas flanking mutations had only minor effects. Intramolecular activation of a full-length kinase was also disrupted by a VI444/AA mutation, but surprisingly not by a F436/A mutation (at the N-terminal end of the binding site). Interestingly, intramolecular but not bimolecular activation was disrupted by insertion mutations placed immediately downstream of I444. To show that mutant enzymes were not misfolded, latent kinase activity was stimulated through binding of an antijunction antibody. Results here support a model of intramolecular activation in which the tether (A445 to G455) that connects the CaM-LD to the kinase provides an important structural constraint and is not just a simple flexible connection.

Ca^{2+}-dependent protein kinases (CDPKs)[1] provide the basis for a novel Ca^{2+}-mediated signal transduction pathway in plants and protists (*1*−*4*). They are most closely related to calmodulin-dependent protein kinases (CaMKs), on the basis of protein sequence identity (*1*). However, CDPKs are unique since they contain an intrinsic calmodulin-like regulatory domain (CaM-LD). The protein is organized with a kinase domain, a junction domain (with an autoinhibitor sequence), and a C-terminal CaM-LD. This unique structural organization probably arose early in evolution from a genetic recombination event that fused a CaMK with a calmodulin.

Similar to a CaMK (*5*, *6*), CDPKs are kept in a low basal state of activity by an "active site-directed, intrasteric autoregulatory sequence" located immediately downstream of the kinase domain (*7*, *8*). Activation of both CaMKs and CDPKs appears to result from a "release of autoinhibition" mechanism. In CaMKs, this release from inhibition occurs as a result of calmodulin binding to a region immediately downstream of an autoinhibitory sequence, in a Ca^{2+}-stimulated bimolecular interaction. In CDPKs, activation somehow occurs as a result of Ca^{2+} stimulating an intramolecular interaction between the kinase and its tethered CaM-LD. The precise nature of this intramolecular interaction is not known.

The simplest model for CDPK activation is based on a direct analogy to a CaMK, with the only distinction being that the CaM-LD binds to the CDPK in an intramolecular instead of bimolecular reaction. This analogy is supported by three lines of evidence. First, in CDPKs, a binding sequence for the CaM-LD was shown to be present in the junction domain, a position analogous to the calmodulin binding sites in CaMKs. This region can bind an isolated CaM-LD (or calmodulin) with a K_D of approximately 2−3 μM in a Ca^{2+}-dependent fashion (*9*). Second, a mutation in this binding sequence (LRV-I444/DLPG) disrupted both an apparent intramolecular binding by the CaM-LD and intramolecular activation of the kinase, consistent with an activation mechanism involving a binding event similar to that which activates CaMKs. Third, the activity of a truncated kinase (ΔNC) in which the CaM-LD was deleted was shown to be stimulated by an isolated CaM-LD or calmodulin, with half-maximal activation around 3 μM for both activators (*9*, *10*). This final evidence demonstrated that like the activation

[†] This research was supported by the National Science Foundation (NSF) (Grants MCB-9408101 and MCB-9723539), a joint grant from the National Aeronautics and Space Administration and NSF (Grant IBN-9416038), Novartis Agricultural Discovery Institute, a Wellcome Trust International Prize Traveling Research Fellowship to J.C., and travel funds from the tri-agency Kinase Collaboration Group organized by D. Randall and sponsored by the Department of Energy, United States Department of Agriculture, and the NSF.

* To whom correspondence should be addressed. Phone: (858) 784-2862. Fax: (858) 784-9840. E-mail: Harper@Scripps.edu.

[‡] Present address: Department of Biochemistry, Vanderbilt University, Nashville, TN 37232-0146.

[1] Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; CPK-1, CDPK isoform-1 from *Arabidopsis*, also called AK1; GST, glutathione *S*-transferase; AK1-6H, CPK-1 fusion protein sandwiched between a GST at the N-terminus and a six-histidine motif at the C-terminus; CaM, calmodulin; 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.

10.1021/bi992373m CCC: $19.00
Published on Web 03/14/2000

of a CaMK, the activation of a CDPK could be reconstituted as a bimolecular interaction with a separate calmodulin activator.

The structural feature that distinguishes a CDPK from a CaMK is the tether that physically connects the CaM-LD to the kinase (*9*). To identify the sequence comprising the tether, we first needed to determine the point where the functional CaM-LD binding site ended and the CaM-LD began. A CaM-LD binding site was identified here by testing the effect of mutations made in the junction domain for their ability to specifically disrupt the ability of an isolated CaM-LD (or calmodulin) to activate a truncated kinase (ΔNC-wt). On the basis of this bimolecular activation assay, a nine-residue binding segment was identified at the C-terminal end of the junction domain. Interestingly, insertion mutations made immediately downstream of the proposed CaM-LD binding site produced a dramatic disruption of intramolecular activation, but had no significant effect on the reconstitution of CDPK as two separate components. These results suggest potentially important differences between the intramolecular activation of a CDPK and the bimolecular activation of a CaMK. We present two alternative models of CDPK activation that illustrate how a structural constraint that accompanies the tethering of the CaM-LD to the kinase may impact the mechanism of intramolecular activation.

MATERIALS AND METHODS

DNA fragments encoding fusion proteins were subcloned and expressed in *Escherichia coli* strains DH10α or DH5α (Stratagene, La Jolla, CA). Protein concentrations were measured using the Bio-Rad protein assay reagent (Hercules, CA), with bovine serum albumin (BSA) as a standard. Glutathione−Sepharose 4B was purchased from Pharmacia (Piscataway, NJ), nickel resin (Ni−NTA agarose) from Qiagen (Santa Clarita, CA), calmodulin (bovine) from Sigma (St. Louis, MO), Syntide-2 from Bachem (Torrance, CA), protease-free BSA from U.S. Biochemicals (Cleveland, OH), and [γ-^{32}P]ATP (>5000 μCi/mmol) from Amersham (Arlington Heights, IL).

Fusion Protein Constructs. The following constructs encode fusion proteins derived from CPK-1 (previously called isoform AK1). A prefix of "p" denotes the plasmid construct that encodes a fusion protein. All constructs were made with the vector pGEX-2T (*11*) providing an N-terminal glutathione *S*-transferase (GST) affinity tag. In addition, a six-histidine tag was added to the C-terminal end to produce a kinase sandwiched between N- and C-terminal affinity tags (GST-6H fusion) (*7*).

Full-length mutants were made with the parent clone $AK1_{6H}$ (*7*), a full-length GST-6H fusion of isoform CPK-1. These mutants were termed KJMs (kinase junction mutations). Most mutations were made by PCR amplification of a region using a primer containing the mutant sequence, followed by the insertion of the modified fragment into a full-length clone. Some mutations were made by insertion of oligonucleotide adapters. All mutations and PCR-amplified sequences were confirmed by DNA sequencing at the Scripps Biotechnology Core Facility using an Applied Biosystems automated DNA sequencer. The following oligonucleotides were used as PCR primers to generate mutations: KJM-31 (F340/A), dTCTGCTGTTCTCTCGAGAATGAAGCAGGCTTCTGCA; KJM-46 (M433/A), dCATCTTAAGGCCATTTTCTTGAACTTGTTCGCTGCAGAAAACTG; KJM-45(F436/A),dACTCTTAAGGCCATTTTCTTGGCCTTGTTCATTGCAGAAAACTG; KJM-41 (V-I444/AA), dAAAATGGCTCTTAGAGCGGCCGCTGAGAGCTTATCT; KJM-30 (L448/A), dGGCCTTAAGAGTCATTGCTGAGAGCGCATCTGA; KJM-36 (E446/A), dGGCCTTAAGAGTCATTGCTGCTAGCTTATCTGAAGAAG; KJM-37 (A-E446/GG), dGGCCTTAAGAGTCATTGGTGGTAGCTTATCTGAAGAAG; KJM-32 (E-E451/AA), dGAGAGCTTATCTGCCGCGGAAATCGCCGGCTTG; KJM-34 (one-A insertion), dTTGCCGCGGAGAGCTTATCTGAAGAAG; KJM-40 (three-A insertion), dAAAATGGCCTTAAGAGTCATTGCCGCGGCCGCT; KJM-43 (five-A insertion), dAAAATGGCCTTAAGAGTCATTGCCGCGGCTGCGGCCGCT; and KJM-35 (three-G insertion), dGGCCTTAAGAGTCATTGGGGGCGGCGCCGAGAGCTTATCTGA. The KJM-42 (13-residue insertion) mutant was made through a series of site specific mutations and subcloning steps, and has the sequence starting with the codon for M439: ATGGCCTTAAGAGTCATTGCCGCGGCCCTCGAGATCGAGGGCCGCATATGCTCGGCCGCT, where GCT is the codon for A445.

ΔNC truncation mutants were made by subcloning mutant sequences into ΔNC-wt. ΔNC-wt has a truncation of the N-terminal and CaM-LD domains, resulting in a fusion protein with an N-terminal GST tag, the CPK-1 sequence from M125 to A454, and a C-terminal six-histidine tag (*9*). Two ΔNC mutants, ΔNC-44 and ΔNC-46, did not contain a C-terminal six-histidine tag and ended with the sequence A445-ESLSEEIA. This different C-terminal end did not affect enzyme activation, as indicated by a control NC-wt with the same C-terminal end (not shown). Mutants made in ΔNC constructs were also engineered into full-length enzymes and given the same number following a KJM prefix (e.g., ΔNC-31 and KJM-31) (see Figure 1).

The CaM-LD protein used for activation of ΔNC truncation mutants, CaM-LD 6H/ST, was made as a fusion protein containing an N-terminal six-histidine motif and a 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 CaM-LD protein was purified using Ni affinity chromatography as previously described (*7*). The CaM-LD was further purified by size-exclusion chromatography on a Sephacryl S-100 column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, and 1.1 mM $CaCl_2$. This purification step was efficient in removing the CaM-LD dimer and higher-order aggregates from the monomeric protein. The purity was >80% as assayed by SDS−PAGE. Ca^{2+}/CaM-LD was concentrated by centrifugation in a Centricon-10 filtration unit and stored at −20 °C.

Expression and Purification of Mutant CPK-1 Enzymes. The wild type and mutant CPK-1 enzymes were purified from *E. coli* as GST-6H fusion proteins as previously described (*7*). SDS−PAGE analysis and Coomassie blue staining were used to check the purity and relative concentration of the different enzyme preparations.

Kinase Assay. Protein kinase activities were assayed with 0.05 μg of enzyme in a 50 μL reaction mixture with 300 μM ATP spiked with [γ-^{32}P]ATP (40 Ci/mol), 100 μM

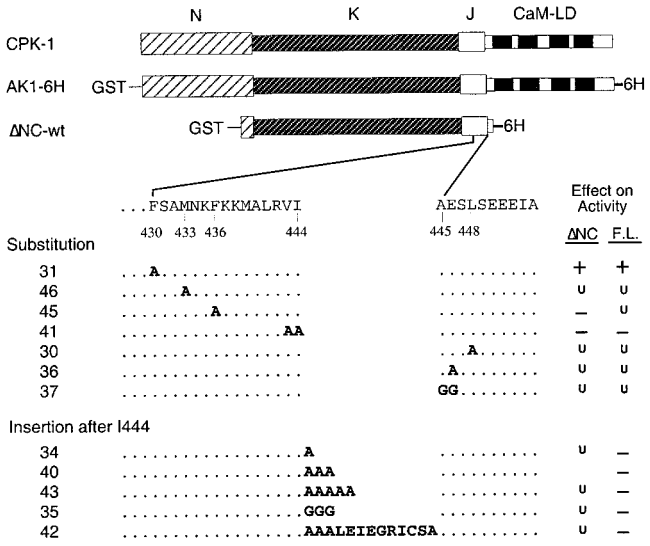

FIGURE 1: Diagram of CPK-1 showing the locations of mutations made in a full-length (KJM constructs) and truncated kinase (ΔNC constructs). N, N-terminal domain (residues 1−149); K, catalytic domain (residues 150−413); J, junction domain (residues 414−444); and C, CaM-LD (residues 445−610). Four Ca^{2+}-binding EF-hands are represented by black boxes in the CaM-LD. The sequence from F430 in the junction region to E452 in the CaM-LD is shown. The sequence shown from residue 436 to 444 corresponds to the proposed binding site, and the sequence from residue 445 to 455 identifies the proposed tether. Mutations are identified by numbers. Most mutations were made in both a ΔNC truncation construct (designated by the prefix ΔNC) and a full-length enzyme (designated by the prefix KJM which stands for kinase junction mutation). A summary is shown to the right indicating the effect of each mutation on the activation of the truncated (ΔNC) and full-length (F.L.) constructs: (+) constitutive deregulated activity, (u) undetectable change in activation, and (−) disrupted activation.

Syntide-2, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, and 0.5 mg/mL BSA, with or without 1.1 mM $CaCl_2$ (*7*). Reactions were carried out for 15 min at 22 °C and terminated by spotting 20 μL of the mixture onto phosphocellulose filter paper (P81 from Whatman) and immediately immersing it 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 for each wash. Antijunction IgG T857 was purified using a protein G affinity column (*7*) and added to kinase reaction mixtures for a 30 min incubation on ice prior to starting the reaction. Both the isolated CaM-LD and calmodulin were loaded with Ca^{2+} prior to addition to assays. Both activators were resuspended in a large volume of kinase buffer [20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, and 1.1 mM $CaCl_2$] such that the molar ratio of calmodulin to $CaCl_2$ was 1:4, followed by concentration by centrifugation in a Centricon-3 filtration unit and storage at −20 °C.

RESULTS

To delineate the intramolecular binding site for the CaM-LD, a series of mutations were made in the junction domain (Figure 1) and tested for their effect on the activation of both truncated (ΔNC-wt) and full-length (AK1-6H) versions of isoform CPK-1. The purity of all enzymes was judged to be within ±20% of that of the wild-type control, as indicated by evaluating a dilution series of both proteins by SDS−PAGE and Coomassie staining. Therefore, only differences in specific activity of >20% were considered potentially significant. All mutant enzymes showing a potential disruption of activity were confirmed by an analysis of at least two independent enzyme preparations.

ΔNC Mutants. All ΔNC mutants were initially tested for activation by 10 μM calmodulin (Table 1) and 10 μM isolated CaM-LD (Table 2). The control ΔNC-wt was fully activated by both activators, although calmodulin produced a 5-fold higher V_{max} compared to the isolated CaM-LD (Figure 2). In contrast to the activation of ΔNC-wt, two mutants, ΔNC-41 (VI444/AA) and ΔNC-45 (F436/A), showed a dramatic disruption of activation (5−30% of the ΔNC-wt activity). The most severe disruption was observed for mutant ΔNC-45 (F436/A), which showed no stimulation above basal activity, even at a concentration of the activator that was 4-fold higher (i.e., 40 μM calmodulin or CaM-LD) (Figure 2). Mutant ΔNC-41 (VI444/AA) showed a partial but very weak stimulation by both calmodulin and an isolated CaM-LD. The K_{act} for both calmodulin and CaM-LD was estimated to be >13 μM (Figure 2). This K_{act} was probably underestimated since the enzyme activity did not appear to reach a maximum even at the highest calmodulin concentration tested in our assay (i.e., 40 μM). Nevertheless, this value is still significantly higher than an approximate K_{act} of 2 μM observed with the activation of a control ΔNC-wt.

The observation that mutations F436/A and VI444/AA disrupted the activation of a truncated kinase is consistent with the hypothesis that these mutations disrupted a binding site, resulting in no significant binding (e.g., F436/A) or binding with a reduced affinity (e.g., VI444/AA). However, an alternative artifactual explanation is that the mutations caused the enzymes to be misfolded, thereby resulting in "dead" enzymes. To show that these mutants had latent activity (i.e., they were not simply dead), we tested for their potential to be activated by a purified antijunction polyclonal IgG (Table 2). This antiserum was previously shown to activate both a full-length and truncated kinase in the absence of Ca^{2+} or calmodulin, presumably by binding to a sequence within the junction and blocking its ability to function as an autoinhibitor. ΔNC-41 (VI444/AA) was fully activated by the antijunction IgG activation control, consistent with the hypothesis that the mutation specifically disrupted the ability of the enzyme to be activated by calmodulin or a CaM-LD. Although ΔNC-45 (F436/A) was only partially activated to 35% activity, this still represented a significant 7-fold stimulation. A potential explanation for a weaker response to antibody activation is that the F436/A mutation may also alter the recognition site for the antibody, which binds to an undetermined site(s) in the junction. Thus, the retention of latent activity in these mutants indicated that both mutations (VI444/AA and F436/A) specifically disrupted activation of the truncated kinase by either calmodulin or an isolated CaM-LD.

The results described above are consistent with the hypothesis that residues between F436 and I444 are part of a core CaM-LD binding sequence. Additional mutations made upstream (*2*) and downstream (*5*) appeared to a have significantly weaker effect or no effect at all on activation (Tables 1 and 2). In cases where a mutation showed a weak effect, such as the L448/A mutant that reduced activation levels by an isolated CaM-LD to 55%, there was no apparent change in the K_{act}, suggesting that the mutation in question did not weaken the affinity of the binding site. In addition,

Table 1: Calmodulin Activation of Truncated Kinase Mutants of CPK-1

enzyme	*n*	kinase activity[a] (μmol min^{-1} mg^{-1}) Ca^{2+}	Ca^{2+}/CaM (10 μM)	% of ΔNC-wt[b]	with antijunction IgG[c]	estimated K_{act}[d] (μM CaM)
ΔNC-wt	3	0.04	2.24	100	2.30	1.9 ± 0.5
substitution						
ΔNC-31 (F430/A)	3	1.00	3.10	138	nd[f]	<1.9[e]
ΔNC-46 (M433/A)	2	0.13	1.50	67	2.30	1.5
ΔNC-45 (F436/A)	2	0.14	0.12	5	0.83	NSA[g]
ΔNC-41 (VI444/AA)	2	0.06	0.67	30	2.60	14
ΔNC-30 (L448/A)	1	0.14	2.94	131	2.40	2.5
insertion after I444						
ΔNC-35 (+3 G)	2	0.10	2.50	112	2.60	2.1
ΔNC-34 (+1 A)	1	0.09	2.02	90	nd	nd
ΔNC-43 (+5 A)	2	0.04	2.20	98	1.60	3.0
ΔNC-42 (+13 residues)	1	0.07	2.00	89	nd	nd

[a] Specific activities were averaged from at least two assays of independent enzyme preparations (*n*). All enzyme preparations were initially tested for activation in the presence of 10 μM calmodulin (CaM) in a standard plus Ca^{2+} kinase assay mixture. [b] The % activity was based on reactions in the presence of 10 μM Ca^{2+}/calmodulin. [c] Sixty micrograms of antijunction IgG was used. [d] At least one preparation for each mutant was assayed in the presence of increasing amounts of calmodulin. K_{act} was calculated as the inverse of the slope from a line obtained by plotting the kinase specific activity vs the kinase specific activity divided by the CaM-LD concentration. Three assays of independent ΔNC-wt preparations showed a range of K_{act} values from 0.6 to 3.4. [e] For two independent ΔNC-31 preparations, maximal kinase activity was reached in the presence of 2 μM Ca^{2+}/calmodulin. [f] nd, not determined. [g] NSA, no significant activation.

Table 2: Calmodulin-like Domain Activation of Truncated Kinase Mutants of CPK-1

enzyme	*n*	kinase activity[a] (μmol min^{-1} mg^{-1}) Ca^{2+}	Ca^{2+}/CaM-LD (10 μM)	% of ΔNC-wt[b]	estimated K_{act}[c] (μM CaM-LD)
ΔNC-wt	2	0.05	0.44	100	3.8 ± 1.3
substitution					
ΔNC-31 (F430/A)	1	1.00	3.10	705	<1.9[d]
ΔNC-46 (M433/A)	1	0.11	0.48	109	3.2
ΔNC-45 (F436/A)	2	0.10	0.07	16	NSA[e]
ΔNC-41 (VI444/AA)	2	0.05	0.07	16	13
ΔNC-30 (L488/A)	1	0.04	0.24	55	3.3
insertion after I444					
ΔNC-35 (+3 G)	1	0.10	0.24	55	2.1
ΔNC-43 (+5 A)	1	0.03	0.20	45	0.5

[a] Specific activities were averaged from at least two assays of independent enzyme preparations (*n*). All enzyme preparations were initially tested for activation in the presence of 10 μM calmodulin-like domain (CaM-LD) in a standard plus Ca^{2+} kinase assay mixture. [b] The % activity was based on reactions in the presence of 10 μM Ca^{2+}/calmodulin. [c] At least one preparation for each mutant was assayed in the presence of increasing amounts of CaM-LD. K_{act} was calculated as the inverse of the slope from a line obtained by plotting the kinase specific activity vs the kinase specific activity divided by the CaM-LD concentration. [d] Maximal activity was reached in the presence of 2 μM Ca^{2+}/calmodulin. [e] NSA, no significant activation.

none of these "weak effects" were observed for activation by both calmodulin and an isolated CaM-LD, further supporting the hypothesis that a common functional binding site was not disrupted by these mutations.

The five mutations made on the downstream side of the proposed binding sequence included insertions immediately after the I444 position, such as a three-G insertion in mutant ΔNC-35. The three-G insertion was made to disrupt a potential extension beyond I444 of a putative amphipathic basic helix. The failure to disrupt activation with multiple insertion mutations at this location strongly supports the contention that a binding site does not extend downstream of I444.

Similarly, two mutations made upstream of F436 support the hypothesis that the core binding sequence does not extend further upstream. These additional mutations, M433/A and F430A, were made to disrupt large hydrophobic residues that may provide alternative anchor residues for the start of a typical calmodulin binding sequence. The nearest upstream mutation, M433/A, failed to produce any detectable effect on activation using a recombinant CaM-LD, although a slight inhibition was observed for activation by calmodulin (i.e., reduced to 67% activity compared to that of a wild-type control). Nevertheless, since the K_{act} for both CaM and an isolated CaM-LD was equivalent to that observed in the activation of the control ΔNC-wt, these mutations do not appear to disrupt a binding site.

The F430/A mutation (in ΔNC-31), which was made further upstream, also failed to change the apparent affinity of the binding site. However, this mutation was more difficult to evaluate since it also showed a partial disruption of the autoinhibitor. As a result, the ΔNC-31 mutant enzyme displayed high basal activity and was only stimulated 3-fold by calmodulin or a CaM-LD. Nevertheless, a 3-fold stimulation was sufficient to allow the K_{act} for both calmodulin and CaM-LD to be estimated to be 1.9 μM, equivalent to the K_{act} determined for the activation of the control ΔNC-wt.

Full-Length Mutants. Selected mutations were also examined in the context of a full-length enzyme (Table 3). The purpose was to test whether mutations would have similar effects on bimolecular and intramolecular activation.

In agreement with expectations, the VI444/AA mutation in the context of a full-length kinase (KJM-41) caused a significant disruption of intramolecular activation. Mutant KJM-41 (VI444/AA) was only partially activated by Ca^{2+} to a level that was approximately 56% of that of a control wild-type enzyme. As mentioned above, this mutation significantly reduced the K_{act} for the activation of truncated kinase by calmodulin or an isolated CaM-LD. Together, these results support the hypothesis that the VI444 position is part of an intramolecular binding site required for activation of a full-length kinase.

In contrast to expectations, the F436/A mutation (in mutant KJM-45) failed to show a significant disruption of intramolecular activation (i.e., its activity was 84%). This result was

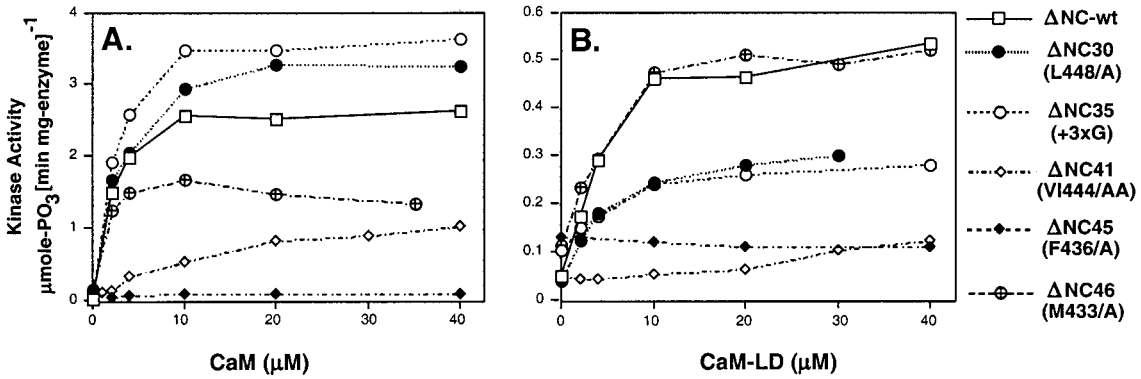

FIGURE 2: Activation of selected ΔNC constructs by calmodulin and an isolated CaM-LD. Kinase activities were assayed in the presence of increasing concentrations of calmodulin (A) or an isolated CaM-LD (B) under standard assay conditions with 0.1 mM free Ca^{2+} using Syntide-2 as a peptide substrate. Calmodulin and an isolated CaM-LD were preloaded with Ca^{2+} to keep the Ca^{2+} concentration constant for different titration points.

Table 3: Ca^{2+}-Dependent Kinase Activities of Full-Length Mutants of CPK-1

		kinase activity[a] (μmol min^{-1} mg^{-1})		
enzyme	*n*	without Ca^{2+}	with Ca^{2+}	% activity of AK1-6H (wt)[b]
AK1-6H (wt)	4	0.09	3.20	100
substitution				
KJM-31 (F430/A)	2	1.3	3.30	103
KJM-46 (M433/A)	3	0.10	2.86	89
KJM-45 (F436/A)	4	0.06	2.70	84
KJM-41 (V-I444/AA)	4	0.06	2.70	84
KJM-30 (L448/A)	1	0.06	2.58	81
KJM-36 (E446/A)	2	0.10	2.85	89
KJM-37 (A-E446/GG)	1	0.07	3.01	94
insertion after I444				
KJM-34 (+1 A)	3	0.05	0.88	27
KJM-40 (+3 A)	1	0.09	1.28	39
KJM-43 (+5 A)	2	0.04	0.04	11
KJM-35 (+3 G)	2	0.05	0.16	5
KJM-42 (+13 residues)	1	0.04	0.10	3

[a] Specific activities were averaged from at least two assays of independent enzyme preparations (*n*). For AK1-6H, independent enzyme preparations showed activities that varied within the range of 0.04−0.13 without Ca^{2+} and 3.05−3.4 with Ca^{2+}, while the extent of activation ranged from 24- to 76-fold. [b] The % activity was based on reactions in the presence of Ca^{2+}.

unexpected since the same mutation in the context of a truncated kinase resulted in a complete disruption of bimolecular activation, more severe even than the VI444/AA mutation. To be absolutely certain that the KJM-45 mutant enzyme still harbored the F436/A mutation, the mutation was confirmed by resequencing the plasmid construct isolated directly from a culture used for the expression and assay of this mutant kinase.

In agreement with expectations, additional substitution mutations made upstream and downstream of F436 and VI444, respectively, did not block intramolecular activation. The mutation F430/A, which disrupted the autoinhibitor in the truncated kinase, also disrupted the autoinhibitor in the full-length enzyme (KJM-31). In the absence of Ca^{2+}, KJM-31 displayed a basal activity that was 40% of that of a Ca^{2+}-activated wild-type enzyme. There was no indication that the F430/A mutation disrupted the intramolecular interaction with the CaM-LD since Ca^{2+} was still able to further stimulate KJM-31 to greater than 100% activity.

In contrast to expectations, the most dramatic disruption of intramolecular activation was observed for insertions made immediately after I444. These mutations had no significant effect on the activation of a truncated kinase by calmodulin, although there was a weak disruption of activation by an isolated CaM-LD. The most severe disruption of a full-length kinase occurred with a 13-residue insertion (KJM-42; 3% activity), while the least severe occurred with a three-A insertion (KJM-40; 39% activity). The length and amino acid content of the insertion sequences appeared to influence their degree of disruption. For example, increasing the insertion length from one A (KJM-34) to five A (KJM-43) made the disruption more severe, yielding activities of 27 and 11%, respectively. In addition, changing the amino acid composition of the insertion sequence from a three-A (KJM-43) to a three-G sequence (KJM-35) increased the level of disruption to yield 39 and 5% relative activities, respectively.

Two lines of evidence indicate that the reduced activity of these insertion mutants resulted from a specific disruption in their activation mechanism, rather than from a nonspecific artifact such as protein misfolding. First, all insertion mutants had a basal activity comparable to that of the wild-type enzyme. Second, with two representative insertion mutants, KJM-43 (plus five A) and KJM-35 (plus three G), we established that they could be further activated beyond their Ca^{2+}-stimulated levels by an antijunction antibody (to approximately 50% wild-type activity), as was done for selected ΔNC constructs (Table 3).

DISCUSSION

The initial objective of this study was to identify the binding sequence used by the CaM-LD during the Ca^{2+} activation of a CDPK. While our approach successfully identified a functional CaM-LD binding site required for a bimolecular activation mechanism, it also produced evidence that intramolecular activation may involve significantly different interactions.

Evidence for a Nine-Residue CaM-LD Binding Sequence. Three lines of evidence indicate that a nine-residue binding sequence from F436 to I444 (in isoform CPK-1) is required for activation of a truncated kinase by an isolated CaM-LD. First, two mutations (F436/A and VI444/AA) at opposite ends of this proposed binding sequence severely disrupted the potential activation of ΔNC constructs by either an isolated CaM-LD or calmodulin. Importantly, both of these

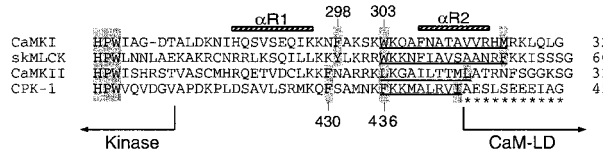

FIGURE 3: Sequence comparison between the junction and the regulatory domains of CaMKI (*23*), CaMKII (*24*), and skMLCK (*25*). The positions of two helices, αR1 and αR2, formed in the regulatory segment of CaMKI are indicated (*16*). CaM-binding domains in CaMKII and skMLCK and the putative CaM-binding domain in CaMKI are underlined. The position of the proposed tether in CPK-1 is denoted with asterisks.

mutant enzymes displayed latent kinase activity that could be stimulated by an antibody that binds to the junction (autoinhibitory) region. This control indicated that the mutant proteins were not misfolded. These results are consistent with a proposed CaM-LD binding site, including the sequence from F436 to I444.

Second, five mutations made downstream of the sequence from F436 to I444 failed to disrupt the activation of ΔNC constructs, including a three-G insertion made immediately after I444. The rationale for making a three-G insertion was disruption of an amphipathic basic helix that might extend downstream of I444. Amphipathic basic helices are characteristic of calmodulin binding sequences proposed for typical CaMKs. Since every downstream insertion and substitution failed to disrupt activation, as indicated by normal concentrations of an isolated CaM-LD or calmodulin required for half-maximal activity, a functional CaM-LD binding sequence does not appear to require specific residues downstream of I444.

Third, two mutations made upstream of F436 also failed to disrupt the activation of ΔNC constructs. These mutations were made as alanine substitutions of the first two upstream hydrophobic residues. The rationale was to disrupt hydrophobic residues that might serve as alternative hydrophobic anchor residues for the N-terminal end of a typical calmodulin binding sequence. Since both substitutions failed to disrupt activation, it appears that a CaM-LD binding sequence does not require additional hydrophobic residues upstream of F436.

Therefore, a core binding sequence for the CaM-LD can be functionally assigned to residues F436−I444. The same core sequence appears to be equally recognized by calmodulin and an isolated CaM-LD. Both activators produced half-maximal activation between 2 and 3 μM. This value is in close agreement with their measured binding affinities (K_d's of 2−3 μM), as determined by kinetic binding measurements using plasmon resonance technology (*9*). The ability of calmodulin to functionally interact with a CaM-LD binding sequence is not surprising since a functional full-length CDPK was successfully engineered by replacing the CaM-LD with a true calmodulin sequence (B.-C. Yoo and A. C. Harmon, 1996).

Similarities between a CDPK and CaMK. (1) CaM-LD Binding Sequence. The proposed CaM-LD binding segment is shown as an alignment with three calmodulin binding domains from related CaMKs (MLCK, CaMKII, and CaMKI) in Figure 3. In all three CaMKs, the calmodulin binding sequence is thought to form a basic amphipathic α-helix when bound by calmodulin (*12*−*14*). These calmodulin-binding domains, although variable in length, have bulky hydrophobic residues at their N- and C-terminal ends. Similarly, the proposed CaM-LD binding sequence here has the potential to form a basic amphipathic α-helix and is flanked by bulky hydrophobic residues (F and I).

The N-terminal anchor residue (F436) of the proposed binding CaM-LD binding site aligns closely with the N-terminal hydrophobic residues identified in MLCK (W583), CaMKII (L298), and CaMKI (303). For nmMLCK, an alanine substitution at this N-terminal position was shown to disrupt calmodulin binding and activation (*5*). Likewise, the corresponding mutation F436/A analyzed here in CPK-1 also disrupted the analogous bimolecular activation of a truncated kinase by calmodulin or an isolated CaM-LD.

The C-terminal anchor residue (I444) in the proposed binding site aligns one residue short of the analogous position in CaMKII. The relative shortness of the CaM-LD binding sequence may contribute to its 20-fold lower affinity for calmodulin (or a CaM-LD) compared to that of the binding site in CaMKII, as previously determined (*9*). This weaker affinity may be important in counterbalancing the increased driving force for binding that results from tethering the CaM-LD to the kinase which effectively increases the local concentration of activator.

(2) Autoinhibitor. In the process of evaluating hydrophobic residues for their contribution to a CaM-LD binding site, we observed that mutation F430/A disrupted the autoinhibitor in CPK-1. This mutation resulted in a partially deregulated enzyme with a basal activity that was approximately 40% of that of a fully activated wild-type enzyme. In CaMKII, the equivalent position (Figure 3) also appears to function in autoinhibition, as shown by a mutational analysis (*15*). Consistent with this genetic evidence, a crystal structure of CaMKI shows a corresponding Phe as part of a potential pseudosubstrate autoinhibitor structure that interacts with a hydrophobic pocket in the kinase domain (*16*). A large hydrophobic residue at this position was proposed to mimic the P-5 residue of a substrate sequence. Thus, there appears to be a very close correspondence in the position of autoinhibitors in both CDPKs and CaMKs.

In some CaMKs, such as nmMLCK, there is evidence that the core sequences for autoinhibition and calmodulin binding are nonoverlapping (*5*, *17*). Consistent with a similar separation of regulatory sequences, we failed to find any mutations that disrupted both the CaM-LD binding site and autoinhibitory sequences. The closest autoinhibitory mutation (F430/A) lies six residues upstream from the proposed CaM-LD binding site. This suggests that the primary sequence representing the core CaM-LD binding site is distinct from the autoinhibitor.

The results presented above indicate that CDPKs and CaMKs share significant similarities in the architecture of their autoinhibitory domains. Such parallels are not surprising if CDPKs actually evolved from a fusion of a CaMK and calmodulin.

Location of the Tether. The structural feature that makes a CDPK distinct from a CaMK is the tether that physically connects the CaM-LD to the kinase (*9*). In the simplest models of CDPK activation the tether was hypothesized to form a flexible connection that functioned as a hinge to allow the CaM-LD to interact with the upstream autoinhibitor (*7*, *8*).

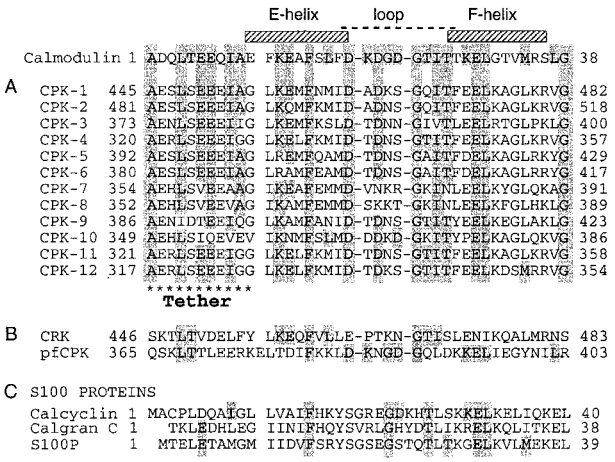

FIGURE 4: Sequence comparison between the N-terminal regions of CaM-LDs and calmodulin-related proteins. Residues conserved between calmodulin and other proteins are shaded. Numbers at the beginning and end of the sequence indicate the residue position in the complete protein sequence. The first sequence shows the 38 N-terminal amino acid residues of calmodulin. This region includes the first EF-hand, identified above as E-helix−loop−F-helix. A canonical EF-hand consists of a 10-residue α-helix (E-helix), a Ca^{2+}-binding loop, and an 11-residue α-helix (F-helix). In calmodulin, the α-helical nature of the E-helix extends in the N-terminal direction to at least E6 without any disruption. (A) Aligned below the calmodulin sequence are the CaM-LD sequences of 12 CDPK isoforms from *Arabidopsis*, CPK-1−12 (*26*). Regions identical to calmodulin are shaded. The proposed tether is denoted with asterisks. (B) The corresponding N-terminal regions for two highly divergent CDPKs are shown: isoform CRK from carrot (*21*) and isoform pfCPK-1 from plasmodium (*3*). (C) Three members of the S100 protein family are shown: Calgran C from porcine and Calcyclin and S100P from human [alignment for S100 proteins is from Potts et al. (27)].

We propose here that the tether is comprised of an 11-residue segment connecting the C-terminal end of the CaM-LD binding sequence to the first EF-hand in the CaM-LD (i.e., A445−G455). This region is shown in an alignment with other *Arabidopsis* CDPKs and calmodulin-like proteins in Figure 4. Two features of this region appear to be notable. First, this region is highly conserved between calmodulin and typical CDPKs, but less so with other calmodulin-like proteins. This similarity includes the retention of three or more acidic residues. This is of interest since calmodulin activation of MLCK appears to require an acidic motif (EEQ-8) in this region (*18*). Second, in contrast to a Glu in position 11 in calmodulin, a Gly occupies the corresponding position in 9 of 12 *Arabidopsis* CDPKs. This is of interest since a Gly in this position may help break an α-helix and provide a turn to help reorient the CaM-LD so it can interact with its upstream binding site.

The Sum of the Parts Does Not Equal the Whole. Despite the similarities between CDPKs and CaMKs, three results suggest important differences between intramolecular and bimolecular activation.

First, all five insertions made in the tether disrupted intramolecular but not bimolecular activation. This disruptive effect ranged from 95% disruption (three-Gly insertion) to 60% (three-Ala insertion). These results were unexpected since it was postulated that a flexible tether should be able to accommodate an insertion of three Gly or Ala residues. Importantly, these mutants were not misfolded, as indicated by their normal levels of basal activity and their ability to be artificially stimulated by an antijunction antibody. While it is not clear how these insertions disrupted activation, they suggest that the tether's length or secondary structure plays a critical role in either mediating "proper" intramolecular binding or somehow coupling the binding interaction to a "release of inhibition".

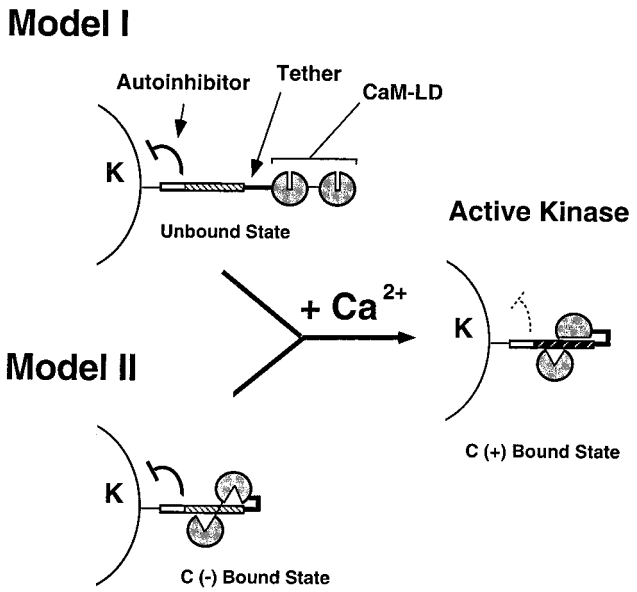

FIGURE 5: Two alternative models for CDPK activation. The interaction of the autoinhibitor with the kinase is indicated by a solid line (kinase off) or dotted line (kinase on). The tether functions as a flexible linker in model I (simple analogy to a CaMK), whereas in model II it forms a rigid structure that pre-positions the CaM-LD in alignment with the junction. In both models, a partial binding interaction is shown for the C-terminal lobe of the CaM-LD.

Second, the F436/A mutation in the proposed CaM-LD binding site failed to disrupt intramolecular activation but severely disrupted bimolecular activation. This is in contrast to the VI444/AA mutation at the opposite end of the binding site that disrupted both intra- and bimolecular activation. These results indicate that the contribution of F436 to an interaction with the CaM-LD is not equivalent in bimolecular and intramolecular activation mechanisms.

Third, despite the ability to reconstitute CDPK activity as two components, the highest V_{max} that was obtained was always less than 50% of that of a full-length kinase, after normalizing activities per mole of enzyme (*9*). This was true whether activation was reconstituted with either calmodulin or an isolated CaM-LD. A similar partial reconstitution was reported for a truncated soybean CDPK isoform (*10*). Together, these three results suggest that the physical connection between the binding sequence and the CaM-LD provides an important structural constraint that is required for optimal activation.

Alternative Models for Intramolecular Activation. Two general models for activation of a CDPK are presented in Figure 5. Both models involve intramolecular binding of the CaM-LD, consistent with prior evidence for intramolecular binding between the CaM-LD and junction (*9*). Model I provides the most direct analogy to a CaMK paradigm. In the apo state, the CaM-LD is unbound; activation occurs when Ca^{2+} induces the CaM-LD to bind to the junction. In this model, the tether functions as a simple flexible connection that allows the CaM-LD to freely bind and dissociate.

While the simplicity of this model is appealing, it is not easily reconciled with observations that the tether's function was disrupted by insertions that were expected to increase its flexibility, such as a three-G insertion. Model II illustrates an alternative model in which the tether functions as a rigid structure. In this model, a necessary consequence of a more rigid tether is a pre-positioning of an apo CaM-LD aligned with its target binding sequence. In this case, activation would result from Ca^{2+} inducing a subtle change in conformation as opposed to the "de novo formation" of the bound complex.

For both models shown here, the conformation of the final Ca^{2+}-activated state is the same. A subtle feature of that activated state illustrated in Figure 5 is the hypothesis that the two lobes of the CaM-LD are functionally nonequivalent. Prior mutational studies on a plasmodium CDPK (PfCPK-1) indicated that mutations in the C-terminal lobe of the CaM-LD (that disrupted the Ca^{2+} binding properties of EF-hands 3 and 4) had only minor effects on activation. In contrast, parallel mutations of EF-hands in the N-terminal lobe caused a severe disruption of activity (*19*). Results presented here provide complementary evidence showing that the C-terminal half of the target CaM-LD binding site is more important than the N-terminal half, as indicated by the greater disruption of intramolecular activation caused by the mutation VI444/AA compared to F436/A. Taken together, these results suggest an antiparallel mode of binding between the CaM-LD and junction, with activation mediated primarily through an interaction of the N-terminal lobe of the CaM-LD with the C-terminal end of the binding site. This feature may be significant in considering other CDPK-related kinases in which one or more EF-hands appear to be lost (*20*, *21*).

In summary, while the mechanism of intramolecular activation of a CDPK is significantly similar to the bimolecular activation of a CaMK, evidence presented here suggests caution in interpreting results based on separating CDPK into two components. The tether that normally connects a CDPK to its CaM-LD appears to function as more than a simple flexible connection, and its role in the activation mechanism is sensitive to insertions that are presumed to increase its length and flexibility. Whether a tethered CaM-LD confers CDPK with a unique biochemical and biological activity is still not clear. Nevertheless, this tethered arrangement has apparently allowed different CDPK isoforms to evolve with the potential to sense and respond to different Ca^{2+} concentrations (*22*).

REFERENCES

1. Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C. L., Charbonneau, H., and Harmon, A. C. (1991) *Science 252*, 951−954.
2. Roberts, D. M., and Harmon, A. C. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 43*, 375−414.
3. Zhao, Y., Kappes, B., and Franklin, R. M. (1993) *J. Biol. Chem. 268*, 4347−4354.
4. Satterlee, J. S., and Sussman, M. R. (1998) *J. Membr. Biol. 164*, 205−213.
5. Lukas, T. J., Mirzoeva, S., and Watterson, D. M. (1998) in *Calmodulin and Signal Transduction* (Van Eldik, L. J., and Watterson, D. M., Eds.) pp 66−167, Academic Press, San Diego.
6. Kobe, B., and Kemp, B. E. (1999) *Nature 402*, 373−376.
7. Harper, J. F., Huang, J.-F., and Lloyd, S. J. (1994) *Biochemistry 33*, 7267−7277.
8. Harmon, A. C., Yoo, B.-C., and McCaffery, C. (1994) *Biochemistry 33*, 7278−7287.
9. Huang, J. F., Teyton, L., and Harper, J. F. (1996) *Biochemistry 35*, 13222−13230.
10. Yoo, B.-C., and Harmon, A. C. (1996) *Biochemistry 35*, 12029−12037.
11. Smith, D. B., and Johnson, K. S. (1988) *Gene 67*, 31−40.
12. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) *Science 256*, 632−638.
13. Meador, W. E., Means, A. R., and Quiocho, F. A. (1993) *Science 262*, 1718−1721.
14. Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) *Science 257*, 1251−1255.
15. Cruzalegui, F. H., Kapiloff, M. S., Morfin, J.-P., Kemp, B. E., Rosenfeld, M. G., and Means, A. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 12127−12131.
16. Goldberg, J., Nairn, A. C., and Kuriyan, J. (1996) *Cell 84*, 875−887.
17. Shoemaker, M. O., Lau, W. l., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P. E., Guerra-Santos, L., Wilson, E., Lukas, T. J., Van Eldik, L. J., and Watterson, D. M. (1990) *J. Cell Biol. 111*, 1107−1125.
18. Persechini, A., Gansz, K. J., and Paresi, R. J. (1996) *J. Biol. Chem. 271*, 19279−19282.
19. Zhao, Y., Pokutta, S., Maurer, P., Lindt, M., Franklin, R. M., and Kappes, B. (1994) *Biochemistry 33*, 3714−3721.
20. Harmon, A. C., Gribskov, M., and Harper, J. F. (2000) *Trends Plant Sci.* (in press).
21. Lindzen, E., and Choi, J. H. (1995) *Plant Mol. Biol. 28*, 785−797.
22. Lee, J. Y., Yoo, B.-C., and Harmon, A. C. (1998) *Biochemistry 37*, 6801−6809.
23. Picciotto, M. R., Czemik, A. J., and Nairn, A. C. (1993) *J. Biol. Chem. 268*, 26512−26521.
24. Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N., and Kelly, P. T. (1987) *Science 237*, 293−297.
25. Takio, K., Blumenthal, D. K., Walsh, K. A., Titani, K., and Krebs, E. G. (1986) *Biochemistry 25*, 8049−8057.
26. Hrabak, E. M., Dickmann, L. J., Satterlee, J. S., and Sussman, M. R. (1996) *Plant Mol. Biol. 31*, 405−412.
27. Potts, B. C. M., Smith, J., Akke, M., Macke, T. J., Okazaki, K., Hidaka, H., Case, D. A., and Chazin, W. J. (1995) *Nat. Struct. Biol. 2*, 790−796.

BI992373M