Calcium/Calmodulin-Dependent Protein Kinase Kinase: Identification of Regulatory Domains†

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ABSTRACT: We recently cloned a calmodulin-dependent protein kinase kinase (CaM-KK) which phosphorylates and activates CaM-KI and CaM-KIV [Tokumitsu, H., Enslen, H., and Soderling, T. R. (1995) *J. Biol. Chem. 270,* 19320−19324]. In the present study, we have identified its regulatory CaM-binding and autoinhibitory domains (CBD and AID, respectively) using a series of COOH-terminal truncations and site-directed mutants expressed in COS-7 cells. Truncation mutant CaM-KK$_{1-463}$ activated CaM-KIV and bound CaM similar to wild-type enzyme (CaM-KK$_{1-505}$); CaM-KK$_{1-448}$ did not bind CaM and was largely inactive; and CaM-KK$_{1-434}$ also did not bind CaM but activated a CaM-independent mutant of CaM-KIV in the absence of Ca^{2+}/CaM. Substitution of triple negative charges (Asp) at positions ^{455}RKR, ^{448}ILV, or ^{443}SWT blocked CaM binding and suppressed by 70−90% CaM-KK activities. Mutants ^{438}VKL and ^{435}KNS to DDD exhibited partial Ca^{2+}/CaM-independent activities. These results identify overlapping AID and CBD between residues 430 and 460 in CaM-KK, similar to other CaM-Ks. Consistent with this assignment, the synthetic peptide corresponding to residues 438−463 bound CaM in a Ca^{2+}-dependent manner with a K_d in the low nanomolar range. Furthermore, phosphorylation by cAMP-kinase of Ser^{458} at the COOH-terminus of the CBD in CaM-KK, which suppresses subsequent CaM binding [Wayman, G., Tokumitsu, H., and Soderling, T. R. (1997) *J. Biol. Chem. 272,* 16073−16076], was blocked by prior binding of Ca^{2+}/CaM to CaM-KK.

Protein kinase cascades, such as cAMP-kinase/phosphorylase kinase (*1*), the MAP kinases (*2*), and AMP-kinase (*3*), have become established as important pathways for mediating multiple signal transduction systems. The recent identification (*4*−*6*) and cloning (*7*) of a Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK),[1] which can phosphorylate and activate CaM-Ks I and IV, established the existence of a CaM-K cascade. Operation of this CaM-K cascade has been demonstrated in Jurkat cells where stimulation of the CD3 receptor produces rapid but transient phosphorylation and activation of CaM-KIV (*8, 9*) and in PC12 cells where depolarization produces activation of CaM-KI (*10*).

CaM-KIV (*11, 12*) and CaM-KK (*13*) have significant nuclear localization and are involved in Ca^{2+}-dependent regulation of gene transcription (*14*−*16*). Activation of CaM-KIV by CaM-KK results in a 10−20-fold increase in its V_{max} for phosphorylation of the transcription factor CREB on its activation site, Ser^{133} (*17*). Transfection of COS-7 cells with CaM-KK and CaM-KIV enhanced CREB-mediated transcription of a reporter gene 14-fold in comparison to transfection with either kinase alone (*7*). CaM-KIV also phosphorylates the serum responsive factor (*18*), and the CaM-K cascade can indirectly regulate transcription through activation of the MAP kinases ERK, JNK, and p38 (*19*). Transfection of PC12 cells with constitutively-active forms of CaM-KK and CaM-KIV results in 3−10-fold activations of the three MAP kinases and 20−50-fold enhancements of transcription mediated by elk-1, c-Jun, and ATF-2. These results implicate roles for the CaM-K cascade in Ca^{2+}-regulated cell growth and stress responses (*19*).

In light of these important physiological functions for the CaM-K cascade, it is important to further define in molecular terms the regulation of CaM-KK. The catalytic domain of CaM-KK (residues 121−408) has considerable identity (30−40%) with other members of the protein kinase family (*7*). CaM-KK contains a CBD since it is purified on CaM−Sepharose and binds Ca^{2+}/CaM in a gel overlay assay *(5, 7*). Recent studies have shown that for the CaM-K cascade to operate, Ca^{2+}/CaM must bind to both CaM-KK and CaM-KIV or CaM-KI *(20*−*22*). This is analogous to the dual requirement for AMP in the AMP-kinase cascade (*3*). The COOH-terminal sequence of CaM-KK probably contains the AID and CBD since truncation at residue 434 generates a constitutively-active form which no longer binds CaM (*21*). We therefore further characterized this regulatory portion of CaM-KK using additional truncations and site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials. CaM-KK cDNA (7; accession number L42810) was from a rat brain cDNA library. Recombinant mouse

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[1] Abbreviations: AID, autoinhibitory domain; CBD, CaM-binding domain; CaM-K, Ca^{2+}/CaM-dependent protein kinase; CaM, calmodulin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; CREB, cAMP response element binding protein; SRF, serum response factor.

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CaM-KIV was expressed in Sf9 cells and purified as described previously (*5*). Recombinant wild-type CaM-KK was expressed in *E. coli* and purified as described previously (*21*). CaM was purified from bovine brain (*23*), and biotinylated CaM was from Biomedical Technologies, Inc. Recombinant catalytic subunit of PKA was kindly provided by Dr. Richard Maurer (Department of Cell Biology). All other chemicals were from standard commercial sources.

Construction of Plasmids. Truncated CaM-KK mutants (1−463, 1−448, 1−434) were made as follows. The PCR fragments of each 3′-end of CaM-KK mutants, amplified using 5′-AAA GAC CTG ATC CTG AA-3′ and either 5′-GTG CGG CCG CTC AAA ATG GGT TTC CAA AGG AAC GCT-3′ (CaM-KK 1−463), 5′-ATA GCG GCC GCT CAG ATC ACA GTG GTC CAG CTG GGG ATG A-3′ (CaM-KK 1−448), or 5′-TTG GCG GCC GCT CAC ACC TCC TCC TCA GTC ACC TCT-3′ (CaM-KK 1−434) as primers, were digested with *Eco*RV(internal site) and *Not*I and replaced with the 3′-end (*Eco*RV−*Not*I fragment) of wild-type CaM-KK in pME18s vector (pME-CaM-KK, *7*). Site-directed mutants of CaM-KK were made using a site-specific plasmid DNA mutagenesis kit (5 Prime-3 Prime, Inc.) and pME-CaM-KK wild-type as a target plasmid with mutagenic oligonucleotides as follows: CaM-KK mt-1 (^{455}RKR-DDD), 5′-GTC TAT GCT GGA TGA TGA TTC CTT TGG AA-3′; CaM-KK mt-2 (^{448}ILV-DDD), 5′-CTG GAC CAC TGA CGA CGA CGT CAA GTC TAT G-3′; CaM-KK mt-3 (^{443}SWT-DDD), 5′-TCA GTC AAG CTT ATC CCC GAT GAT GAT ACT GTG ATC CTG-3′; CaM-KK mt-4 (^{438}VKL-DDD), 5′-GAG GTG AAG AAT TCA GAC GAC GAC ATC CCC AGC TGG-3′; CaM-KK mt-5 (^{435}KNS-DDD), 5′-ATG CTG AGA AAG CGC GCC TTT GGA AAC CCA-3′; CaM-KK ^{458}S-A, 5′-ATG CTG AGA AAG CGC GCC TTT GGA AAC CCA-3′. The nucleotide sequence of each mutant was confirmed by automated sequencing using an Applied Biosystems 373 DNA sequencer.

Transient Expression and Partial Purification of CaM-KK Mutants. COS-7 cells were maintained in Dulbeco's modified Eagle's medium containing 10% fetal calf serum. Cells were subcultured in 10-cm dishes 12 h before transfection. The cells were then transferred to serum-free medium and treated with a mixture of either 20 μg of pME18s plasmid DNA (DNAX Research Institute, Inc.) or CaM-KK cDNA containing plasmid DNAs and 80 μg of LipofectAMINE Reagent (Life Technologies, Inc.) in 6.8 mL of medium. After 32−48 h incubation, the cells were collected and homogenized with 1 mL of lysis buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine, 10 mg/L leupeptin, and 10 mg/L pepstatin A) using a Potter−Elvehjem homogenizer at 4 °C. After centrifugation at 15000*g* for 15 min, the supernatant was applied to a Q-Sepharose Fast Flow column (200 μL of resin, Pharmacia Corp.) and then washed with 5 mL of lysis buffer. Elution was carried out by adding 0.5 mL of lysis buffer containing 0.6 M NaCl.

In Vitro Assay of CaM-KK Activity. Partially-purified CaM-KK (1 μL) was incubated with recombinant CaM-KIV (wild-type or mutant ^{315}EFN to EDD, 5.4 μM) at 30 °C in 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM DTT, 400 μM ATP, and either 1 mM $CaCl_2$/10 μM CaM or 1 mM EGTA. The reaction was terminated after 10 min by a 25-fold dilution at 4 °C with 50 mM HEPES, pH 7.5, 2 mg/mL bovine serum albumin, 10% ethylene glycol, and 1 mM EDTA. CaM-KIV (43 nM) activity was measured at 30 °C in a 25 μL assay containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM DTT, 1 μM microcystin-LR, 400 μM [γ-^{32}P]ATP (1000−2000 cpm/pmol), 40 μM syntide-2, and 1 mM EGTA (Ca^{2+}/CaM-independent activity). The reaction was initiated by the addition of CaM-KIV and terminated at 5 min by spotting aliquots (15 μL) onto phosphocellulose paper (Whatman P-81) followed by washing in 75 mM phosphoric acid (*24*). CaM-KK activity is expressed in terms of its ability to increase the Ca^{2+}/CaM-independent activity (picomoles per minute) of CaM-KIV under the defined assay conditions.

CaM Binding by Peptide 438−463. Real time binding analyses between CaM and the CaM-KK peptide 438−463 were performed with a Pharmacia BIAcore 2000 biosensor (*25*). Biotinoylated CaM was immobilized on the streptavidin preimmobilized Sensor Chip SA (Pharmacia) by injecting 60 μL of biotinylated CaM (0.3 mg/mL) in a solution containing 0.2 M NaCl, 20 mM HEPES (pH 7.5), and 5% glycerol. The surface plasmon resonance signal indicated the binding level of the biotinoylated CaM was 575 resonance units (RU). Binding of peptide was performed at 25 °C with a flow rate of 20 μL/min by injecting 25 μL of various concentrations of peptide in 10 mM HEPES (pH 7.5) in 0.1 M NaCl, 0.005% Tween 20, and either 0.5 mM $CaCl_2$ or 3 mM EGTA. After each binding experiment, bound peptide was completely dissociated by injecting buffer containing 3 mM EGTA. Evaluation and calculation of binding parameters were carried out according to the BIAevaluation software 2.1 package (Pharmacia). For calculation of binding parameters, the nonspecific binding obtained under the same experimental conditions using nonimmobilized sensor chip was subtracted. Plots of dR/dt against R gave a linear relationship. Plotting the slope values obtained at different peptide concentrations gave a line with slope k_a. The dissociation phase is described by $dR/dt = -k_d R$ or $\ln(R_0/R_t) = k_d(t - t_0)$. A plot of $\ln(R_t/R_0)$ against $t-t_0$ is a straight line with slope of $-k_d$. K_D was calculated from the relationship between the rate constants ($K_D = k_d/k_a$).

Others. Western blotting was carried out using antiserum (1/1000 dilution) against a peptide corresponding to a conserved protein kinase motif (residues 132−146 of CaM-KII), and the biotinylated CaM overlay was done as previously described (*7*). Detection was carried out using chemiluminescence reagent (DuPont NEN).

RESULTS AND DISCUSSION

Since truncation of recombinant full-length CaM-KK (CaM-KK_{1-505}) at residue 434 (i.e., CaM-KK_{1-434}) generates a constitutively-active species that no longer binds Ca^{2+}/CaM (*21*), this is strong evidence that the AID and CBD are located in its COOH-terminus analogous to many other CaM-dependent kinases. To further localize these regulatory elements in CaM-KK, we generated a series of additional truncations (Figure 1) which were expressed in COS-7 cells and partially purified on ion-exchange chromatography for biochemical characterization. Expressed kinases were quantitated by Western analyses (Figure 2A, left panel); their abilities to bind Ca^{2+}/CaM were determined using the

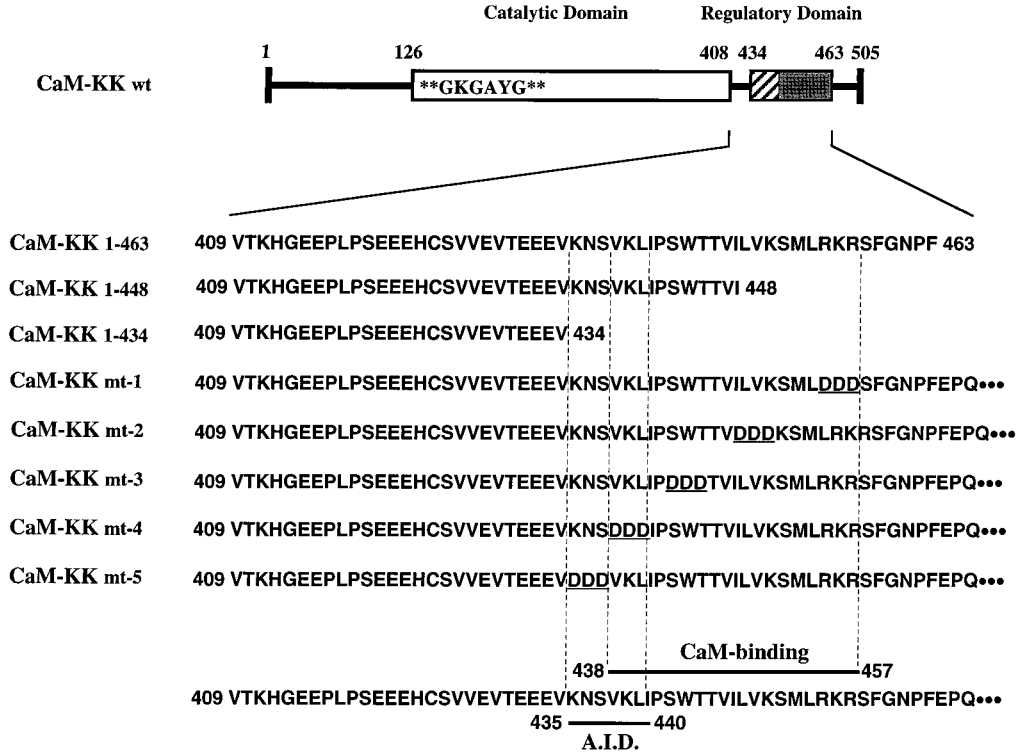

FIGURE 1: Schematic diagram of truncation and charge insertion mutants of CaM-KK. For the truncation mutants, stop codons were created by PCR at the indicated residues. For mt-1 to mt-5, the aspartates were introduced at the indicated positions by site-directed mutagenesis. See Experimental Procedures for mutagenesis details. The bottom schematic summarizes the assignments for the AID and CBD as determined by this study.

biotinylated CaM gel overlay technique (Figure 2A, right panel); and their kinase activities were assessed by their activation of either (1) wild-type CaM-KIV in the absence or presence of Ca^{2+}/CaM (Figure 2B) or (2) in the presence of EGTA using a mutant of CaM-KIV (^{315}EFN-EDD, ref *5*) which does not require Ca^{2+}/CaM for activity (Figure 2C). In the assays of Figure 2B, the substrate CaM-KIV requires binding of Ca^{2+}/CaM in order to be phosphorylated and activated by CaM-KK (*20*, *21*), so none of the CaM-KK truncations exhibit activity in the absence of Ca^{2+}/CaM. However, truncations which remove the AID and generate Ca^{2+}/CaM-independent CaM-KK should show activity in the presence of EGTA in the assays of Figure 2C.

Truncation mutant CaM-KK_{1-463} bound Ca^{2+}/CaM and exhibited kinase activity equal to or greater than wild-type enzyme (Figure 2). However, further truncations (i.e., CaM-KK_{1-448} and CaM-KK_{1-434}) resulted in loss of CaM binding. The first of these mutants, CaM-KK_{1-448}, was essentially inactive, whereas CaM-KK_{1-434} not only exhibited full activity in the presence of Ca^{2+}/CaM (Figure 2B) but also was largely active in the presence of EGTA (ref *21* and Figure 2C). The most likely interpretation of these results is that mutant CaM-KK_{1-448} retained an effective AID but lacked a functional CBD which prevented its activation. Although mutant CaM-KK_{1-434} also lacked a CaM-binding motif, it no longer had an effective AID and therefore exhibited constitutive activity. Collectively these results suggest that residues 435−448 contain autoinhibitory motifs and the sequence 449−463 contains functional elements of the CBD.

To further refine these assignments, we made a series of triple negative residue mutations, mt-1 through mt-5 (Figure 1), spanning residues 435−457. This strategy was chosen because our analyses of the AID in CaM-KII revealed the importance of positive charges (*26*), and insertion of negative charges into the AIDs of CaM-KII (*27*) and CaM-KIV (*5*) proved to be very effective in disrupting the AID and generating constitutive kinase activities. The first three of these CaM-KK mutations (^{455}RKR to DDD, mt-1; ^{448}ILV to DDD, mt-2; ^{443}SWT to DDD, mt-3) were in the putative CBD whereas the remaining two (^{438}VKL to DDD, mt-4; ^{435}KNS to DDD, mt-5) were in the probable AID. As expected, mt-1 through mt-3 had severely suppressed CaM binding as assessed by the gel overlay technique (Figure 3A, right panel). They also showed loss of kinase activity, determined in the presence of either Ca^{2+}/CaM (Figure 3B) or EGTA (Figure 3C), probably due to the presence of a functional AID plus an inability to be functionally activated by Ca^{2+}/CaM. Although mt-4 did not demonstrate CaM binding by the overlay technique, its kinase activity was stimulated to wild-type levels by high (10 μM) concentrations of Ca^{2+}/CaM (Figure 3B). mt-5 retained CaM binding (Figure 3A) and kinase activation (Figure 3B), and mutants 4 and 5 both exhibited approximately 20% constitutive activity (compare Figure 3C to Figure 2C). These results suggest that at least residues Val^{438} to Arg^{457} are directly involved in CaM binding. This sequence has a number of common features for CBDs including clusters of positively charged and hydrophobic amino acids (*28*). Furthermore, residues Lys^{435}−Leu^{440}, which partially overlap the CBD, appear to be important for autoinhibition in CaM-KK. However, mt-4 and mt-5, unlike CaM-KK_{1-434}, are not fully Ca^{2+}/CaM-independent, suggesting that some other element COOH-terminal of residue 440 also contributes to autoinhibition. We cannot exclude the possibility that the loss of activity of these mutants could be due to incorrect folding of the catalytic domain, but our interpetation of the results

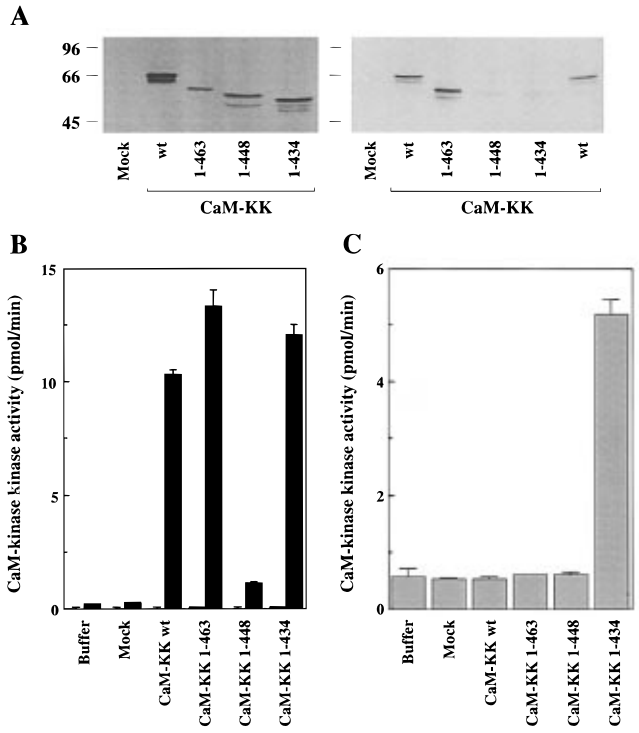

FIGURE 2: Characterization of truncated CaM-KK mutants. (A) Extracts from COS-7 cells that were either mock-infected or infected with the indicated CaM-KK truncation mutants (see Figure 1) were partially purified on Q-Sepharose. Twenty microliter aliquots of column eluants were subjected to SDS/10% PAGE and then transferred onto Immobilon (Millipore Corp.). Membranes were analyzed by Western blotting (left panel) using a protein kinase peptide antiserum or by biotinylated CaM overlay (right panel) as described under Experimental Procedures. (B) Activation of wild-type CaM-KIV by CaM-KKs. Recombinant wild-type CaM-KIV (5.4 μM) was incubated with either buffer, mock-transfected, or Q-Sepharose-purified CaM-KK (1 μL) in the absence (left bar of each pair) or presence (right bar of each pair) of Ca^{2+}/CaM at 30 °C for 10 min in a CaM-KIV activation reaction mixture as described under Experimental Procedures. After terminating the reaction, the Ca^{2+}/CaM-independent activity of CaM-KIV was measured. (C) Ca^{2+}/CaM-independent activation of constitutively-active CaM-KIV by CaM-KKs. Recombinant CaM-KIV (mutant ^{315}EFN-EDD, 5.4 μM) was incubated with either buffer, mock-infected, or Q-Sepharose-purified CaM-KK (1 μL) in the presence of 1 mM EGTA at 30 °C for 10 min in a CaM-KIV activation reaction mixture as described under Experimental Procedures. After terminating the reaction, the Ca^{2+}/CaM-independent activity of constitutively-active CaM-KIV was measured.

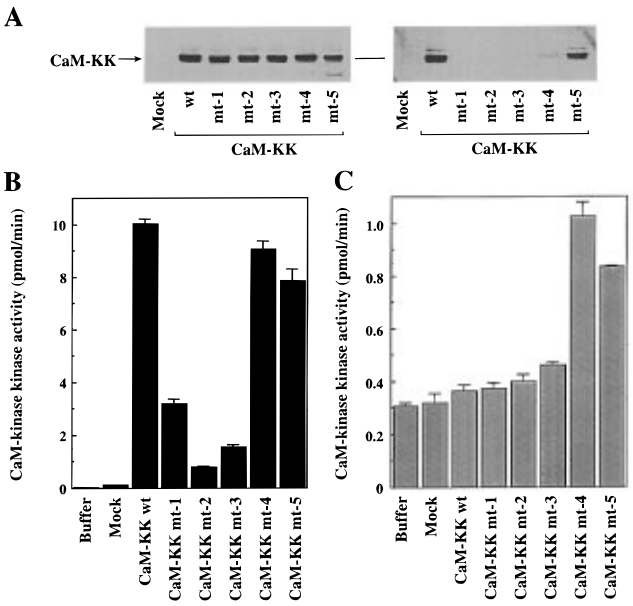

FIGURE 3: Characterization of charge insertion mutants of CaM-KK. (A) Extracts of COS-7 cells that were either mock-infected or infected with the indicated CaM-KK mutant (see Figure 1) were partially purified as in Figure 2. Twenty microliters of the Q-Sepharose eluants was subjected to SDS/10% PAGE, transferred onto Immobilon (Millipore Corp.), and analyzed by Western blotting (left panel) or with biotinylated CaM overlay (right panel) as in Figure 2. (B) Activation of wild-type CaM-KIV by CaM-KKs. Recombinant CaM-KIV was incubated with either buffer, mock-transfected, or partially-purified CaM-KK (1 μL) in the presence of Ca^{2+}/CaM at 30 °C for 10 min in a CaM-KIV activation reaction mixture as in Figure 2. After terminating the reaction, the Ca^{2+}/CaM-independent activity of CaM-KIV was measured. (C) Ca^{2+}/CaM-independent activation of constitutively active CaM-KIV by the indicated CaM-KK mutants was determined as in Figure 2C.

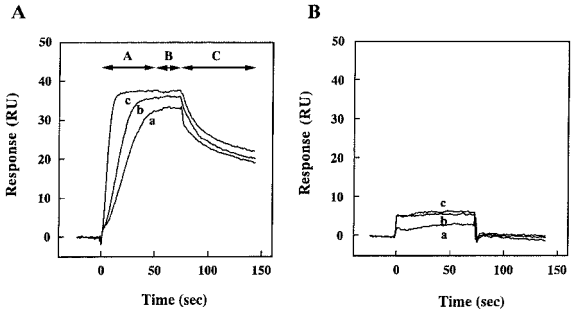

FIGURE 4: Binding kinetics of CaM-KK peptide 438−463 to CaM. Representative overlaid sensograms illustrating the real-time binding of CaM-KK 438−463 peptide (a, 33.1 nM; b, 49.5 nM; c, 132.5 nM) to immobilized CaM in the presence of 0.5 mM $CaCl_2$ (panel A) or 3 mM EGTA (panel B) (see Experimental Procedures). Arrows indicate phase of association (A), equilibrium (B), and dissociation (C). RU, resonance unit.

in terms of an AID is consistent with other CaM-kinases (*5*, *27*).

We directly analyzed the binding kinetics and affinity for CaM of a synthetic peptide which corresponds to residues 438−463 in CaM-KK. Figure 4 shows an example of the overlaid sensogram at three different concentrations of peptide in the presence of either Ca^{2+} (Figure 4A) or EGTA (Figure 4B). Binding of the peptide to CaM was completely dependent on Ca^{2+}, and analyses of the binding curves indicated an equilibrium dissociation constant in the low nanomolar range, consistent with of other high-affinity CaM-binding proteins (*28*). In analogy with other CBDs (*29*), it is likely that Trp^{444} interacts with the COOH-terminus of CaM, and multiple basic residues in the peptide may interact with glutamates in the NH_2-terminal domain of CaM. Based on the assignment of the AID to residues 435−440 (Figure 1), one would predict that peptide 438−463 contains part of the AID and should therefore be a weak inhibitor of CaM-KK. When CaM-KK_{1-434} was assayed for its ability to activate CaM-KIV (mutant ^{315}EFN to EDD) in the presence of EGTA, an IC_{50} of 75 μM for peptide 438−463 was obtained (not shown).

There is a consensus phosphorylation site ($RKRS^{458}$) for cAMP-dependent protein kinase (PKA) at the carboxyl terminus of the CBD (Figure 1), and we recently showed that Ser^{458} is phosphorylated by PKA (*30*). This phosphorylation suppresses subsequent binding of Ca^{2+}/CaM. Because CBDs contain basic residues which are specificity motifs for several protein kinases, there are numerous CBDs in proteins which are negatively regulated by phosphorylation

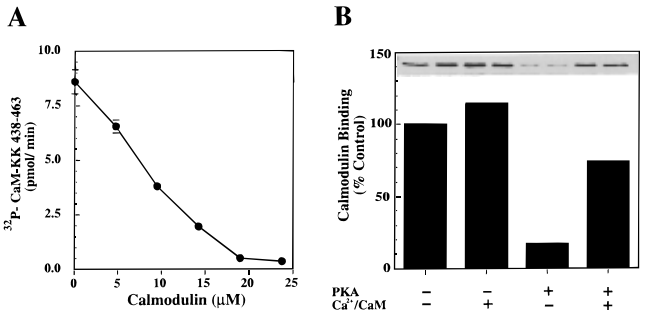

FIGURE 5: Binding of Ca^{2+}/CaM blocks phosphorylation of the CBD. (A) Phosphorylation of peptide 438−463 (20 μM) by PKA (14.5 units, 1 unit = 1 pmol of phosphate incorporated per minute into casein) was determined at 30 °C for 10 min in the presence of 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mM $CaCl_2$, 1 mM DTT, 0.2 mM [γ-^{32}P]ATP, and the indicated concentrations of CaM. Results are mean ± SE for triplicates. (B) Immunoprecipitated recombinant CaM-KK was phosphorylated by PKA for 20 min in the absence or presence of 25 μM CaM (1 mM $CaCl_2$), run on SDS−PAGE, and analyzed for binding of biotinylated CaM as described (*30*). The bars represent the average of the two illustrated overlays determined by densitometric scan.

(*31*−*35*). Generally, prior binding of Ca^{2+}/CaM prevents subsequent phosphorylation of these sites, and this was also true for peptide 438−463 as well as the native CaM-KK (Figure 5). Introduction of negative charge through phosphorylation of Ser^{458} is analogous to the introduction of negative charge in mutant mt-1 (^{455}RKR to DDD) which also resulted in loss of CaM binding. It is noteworthy that Ser^{458} is conserved in the human homologue and other rat brain isoforms of CaM-KK but is deleted in the *C. elegans* homologue (*6*, *36*).

In summary, by using truncated and site-directed CaM-KK mutants, we identified the regulatory domain of CaM-KK and demonstrated that the AID of CaM-KK overlaps with the NH_2-terminus of the CBD (Val^{435} to Arg^{457}) whose carboxy-terminal Ser^{458} is phosphorylated and regulated by PKA. Currently, we are investigating potential upstream and/or downstream regulatory components of the CaM-KK cascade, and these truncated and full-length mutants will be useful for these studies.

NOTE ADDED IN PROOF

The sequences containing the AID and CBD are conserved in the recently published CaM-KKβ clone (*36*).

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