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Articles

Calmodulin Kinase II Chimeras Used To Investigate the Structural Requirements for Smooth Muscle Myosin Light Chain Kinase Autoinhibition and Calmodulin-Dependent Activation†

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ABSTRACT: Segments of the autoregulatory domain of MK, a catalytically active fragment of the monomeric smooth muscle myosin light chain kinase (smMLCK) (residues 472−972), were replaced with their counterparts from a homologous but multimeric enzyme, calmodulin-dependent protein kinase II (CaM KII). Chimeric proteins in which both the autoregulatory and oligomerization domains of CaM KII (residues 281−478) were substituted for residues 781−972 of smMLCK, MK(CK281−478), or only the autoregulatory domain of CaM KII (residues 281−315) was exchanged for residues 781−813 of smMLCK, MK(CK281−315), exhibited significant enzymatic activity in the absence of Ca^{2+}/CaM. In contrast, both MK and a chimeric protein in which the C-terminal half of the autoregulatory domain of smMLCK was replaced with CaM KII residues 301−315, MK(CK301−315), were inactive in the absence of Ca^{2+}/CaM. These results indicate that the sequence of the N-terminal half of the autoregulatory domain of smMLCK is important for complete autoinhibition of its enzymatic activity. All proteins bound to Ca^{2+}/CaM, and the chimeric proteins MK(CK281−478) and MK(CK281−315) were activated by Ca^{2+}/CaM with activation constants (K_{CaM}) and maximal enzymatic activities comparable to those of the wild-type MK enzyme. This demonstrates that the entire autoregulatory domain of CaM KII can replace that of smMLCK in its ability to promote efficient CaM-dependent activation of the smMLCK enzyme. However, the inability of the chimeric protein MK(CK301−315) to be activated by Ca^{2+}/CaM suggests that replacement of only the C-terminal half of the autoregulatory domain of smMLCK, while still retaining the ability to bind Ca^{2+}/CaM, also substitutes residues that prevent activation of the enzyme by Ca^{2+}/CaM.

Calmodulin (CaM[1])-dependent protein kinases are critical regulators of several important cellular functions. Some examples of these enzymes include the smooth and skeletal muscle myosin light chain kinases (smMLCK and skMLCK) involved in changing cellular shape and movement and CaM

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[1] Abbreviations: CaM, calmodulin; smMLCK, smooth muscle myosin light chain kinase; skMLCK, skeletal muscle myosin light chain kinase; CaM KIIα, Ca^{2+}/calmodulin-dependent protein kinase II α; CaM KI, Ca^{2+}/calmodulin-dependent protein kinase I; CaM KIV, Ca^{2+}/calmodulin-dependent protein kinase IV; MK, catalytic fragment of smooth muscle myosin light chain kinase spanning residues 472−972; PCR, polymerase chain reaction; BSA, bovine serum albumin.

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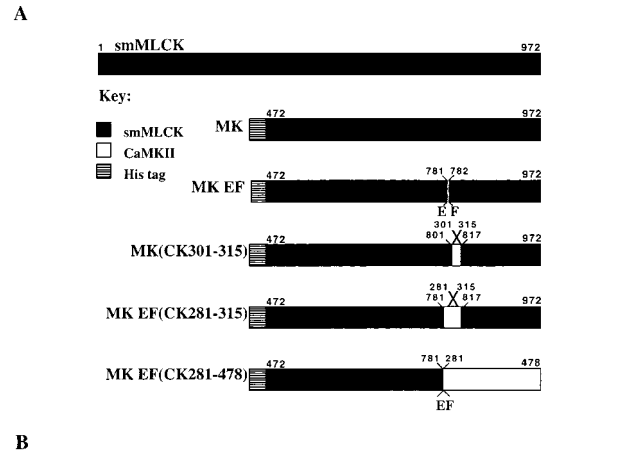

FIGURE 1: Structures of autoregulatory domains of smMLCK and chimeric proteins. (A) The domain organization of wild-type smMLCK, the catalytic fragments MK and MK EF, and chimeric proteins containing autoregulatory sequences of CaM KIIα. The N- and C-terminal sequence positions from the parental protein are listed above the chimeric fragment of each construct. (B) Amino acid sequences of the autoregulatory domains from the wild-type and mutant enzymes. The numbers above the wild-type proteins indicate their amino acid sequence positions. The CaM binding domains of the wild-type proteins are underlined, and the autoinhibitory domains are overlined. CaM KIIα sequences are bold.

kinases I, II, and IV (CaM KI, CaM KII, and CaM KIV, respectively) involved in learning and memory. The enzymatic activities of the CaM-dependent protein kinases are regulated by their respective autoregulatory domains, which are responsible for the dual functions of enzyme autoinhibition and CaM activation. The autoregulatory domains of the CaM kinases are contiguous with their catalytic domains. In the specific case of smMLCK, the kinase domain (residues 513−774) is immediately followed by its autoregulatory domain (residues 774−813) (Figure 1A). The autoregulatory domain of smMLCK can be further divided into two functional units: an autoinhibitory domain (residues 774−807) that is required for high-affinity inhibition of enzymatic activity (*1*) and an overlapping CaM binding domain (residues 796−813) that extends beyond the autoinhibitory domain (*2*) (Figure 1B).

Experiments in which the autoregulatory domain of smMLCK was targeted have started to define the relationship between the closely linked functions of enzyme autoinhibition and CaM-dependent activation. Fragments of smMLCK with C-termini at either A796, K799 (*3*), or I810 (*4*) are inactive in the absence of CaM and are also unable to be activated by CaM. However, smMLCK with C-termini between K779 (*5*) and K793 (*3*), as well as mutants with amino acid substitutions within the autoinhibitory domain (residues 794−796), exhibit constitutive activities in the absence of CaM (*3*). Studies focusing on activation by CaM indicate that W800, located in the N-terminal half of the CaM binding domain of smMLCK, plays an important role in CaM binding and hence in the subsequent activation of the enzyme (*6*, *7*). In addition, the substitution of other residues within the CaM binding domain severely interferes with the activation of smMLCK by CaM (*6*). Taken together, these results suggest that (a) residues located within the autoinhibitory domain of smMLCK (residues 774−807) do not simply behave as a spacer but appear instead to inhibit enzymatic activity in a sequence-specific manner, (b) residues in the N-terminal half of the CaM binding domain of smMLCK, such as W800, are important for CaM binding and subsequent enzyme activation, and (c) although the CaM binding sequence (residues 796−813) is not required for autoinhibition, many residues within this domain are required for CaM-dependent activation of smMLCK.

The structural analyses of related proteins have provided valuable insights into the mechanisms by which smMLCK is regulated. The crystallographic structure of the kinase domain of twitchin kinase, an enzyme that is homologous to smMLCK, shows an extensive interface between the autoregulatory and catalytic domains of this enzyme (*8*), in agreement with an earlier model building study of smMLCK (*1*). The three-dimensional structure of a closely related CaM-dependent protein kinase, CaM KI, provides additional evidence supporting a model of intrasteric autoinhibition (*9*). Here the homologous autoregulatory domain of CaM KI makes extensive intramolecular contacts with its kinase domain, thus blocking the access of the substrate to the enzyme active site. The structure also reveals that W303, in the N-terminal half of the CaM binding domain of CaM KI and the homologue of W800 of smMLCK, is unusually exposed to solvent, thus providing an accessible site for hydrophobic interactions with CaM.

Critical information about the specific interactions between smMLCK and CaM has been gleaned from the crystallographic structure of a complex between CaM and the CaM binding domain of smMLCK (*10*). In this complex, the CaM binding peptide of smMLCK (residues 796−813) assumes a helical structure and is engulfed by the two globular domains of CaM. The N-terminal half of the smMLCK peptide, including the residue corresponding to W800, makes hydrophobic interactions with the C-terminal domain of CaM, whereas the C-terminal half of the peptide interacts primarily with the N-terminal domain of CaM. The contacts observed in the crystal structure are consistent with the results from recent investigations with CaM. Mutating Met residues in the C-terminal domain of CaM, which interact with W800 of the smMLCK peptide, greatly reduces the affinity of CaM for the intact enzyme (*11*), whereas mutations at residues in the N-terminal domain of CaM, which interact with the C-terminal half of the smMLCK peptide, interfere with the activation of smMLCK, but not with CaM binding (*12*).

These results imply that the autoregulatory domain of smMLCK is specifically suited for efficient autoinhibition in the absence of CaM, and also for maximal activation in the presence of CaM. To further investigate the mechanisms of action of the autoregulatory domain of smMLCK, we have replaced all or a part of this domain with the autoregulatory domain of a related enzyme, CaM KII (residues 281−309) (Figure 1B). Similar to smMLCK, CaM KII is an enzyme that is autoinhibited in the absence of CaM and activated in the presence of CaM. However, in contrast to smMLCK, CaM KII is a multimeric enzyme that can remain active upon dissociation of CaM due to the autophosphorylation of Thr286. The evidence obtained from characterizing the resulting chimeric smMLCK−CaM KII enzymes supports

the idea that the native autoregulatory domain of smMLCK is most efficient at enzyme autoinhibition and CaM-dependent activation.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes were from Boehringer Mannheim and New England Biolabs. Taq polymerase was purchased from Boehringer Mannheim, and dNTPs were from Pharmacia. DNA sequencing was performed using Amersham's Sequenase kit. Bacterial media were from Difco, and Sf9 cell culture medium was from Life Technologies, Inc. FMC provided Seakem agarose (ME), and acrylamide was purchased from Serva. Nickel−NTA resin was obtained from Qiagen. Radiochemicals were purchased from Amersham. Myosin light chain and CaM were purified after expression in bacteria as previously described (*11*). Myosin light chain peptide and GS-10 peptides were provided as a gift courtesy of B. E. Kemp from the Holt Laboratory of St. Vincent's Institute (Melbourne, Australia). Other reagents were of the highest available quality.

Methods

Construction of Kinase Expression Plasmids. (1) Wild-Type smMLCK. A *Bam*HI restriction site was inserted 15 bp upstream of the ATG start codon for chicken smMLCK in pGEM (*5*) by USE mutagenesis (*13*). The cDNA containing the entire coding region of smMLCK was then excised by *Bam*HI digestion and inserted in the correct orientation into the unique *Bam*HI site of the baculovirus expression plasmid pVL1393.

(2) His-Tagged PVL1393. To facilitate the expression of His-tagged proteins, the hexyl histidine tag from pTrcHisB (Invitrogen) was isolated by PCR and inserted between the unique *Bam*HI site and a newly created *Spe*I site of pVL1393. This new expression vector was called pVLHis.

PCR mutagenesis was used to generate all chimeric protein kinases. Reactions were performed in a Perkin-Elmer Cetus DNA Thermo Cycler in a total volume of 0.1 mL. All reaction mixtures contained Taq polymerase, Taq buffer, 0.2 mM dNTPs, 0.1 μg of forward and reverse oligonucleotide primers, and 0.5 μM template DNA. In most reactions, each cycle consisted of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, which was then repeated 33 times.

(3) MK. PCR was used to introduce a *Bam*HI site at the nucleotides between E471 and S472 of smMLCK in pGEM and also to introduce a *Pst*I site 3′ to the coding sequence. The resulting 1.5 kb *Bam*HI−*Pst*I fragment of smMLCK was inserted between the *Bam*HI and *Pst*I sites of pVLHis and the resulting construct designated MK.

(4) MK(CK281−478). PCR was used to insert an *Eco*RI site immediately 5′ to M281 of CaMKIIα and simultaneously add a *Pst*I site immediately 3′ to the stop codon. The resulting 0.6 kb *Eco*RI−*Pst*I fragment of CaMKIIα was attached to an *Eco*RI site previously inserted at M751 of the wild-type smMLCK. The resulting 1.5 kb *Bam*HI−*Pst*I fragment, containing residues 1−751 of smMLCK fused with CaMKIIα residues 281−478, was inserted between the *Bam*HI and *Pst*I sites of pVLHis.

(5) MK(CK281−315). PCR was used to introduce a *Bsp*EI site immediately 5′ to the DNA encoding smMLCK A817 and to simultaneously introduce a *Pst*I site 3′ to the protein coding sequence. The resulting 0.5 kb *Bsp*EI−*Pst*I fragment of smMLCK was inserted between the *Bsp*EI and *Pst*I sites of MK(CK281−478) to generate a new construct, MK-(CK281−315).

(6) MK(CK301−315). PCR was used to create a silent mutation *Nar*I site (with an *Eco*RI site on its 5′ flank) within CaM KIIα residues 901−906 of the MK(CK281−315) construct. A *Pst*I site was also simultaneously created at the 3′ end of the terminal stop codon for MK(CK281−305). The resulting *Eco*RI−*Pst*I fragment was inserted into a shuttle vector. PCR was also used to create a *Bam*HI site prior to and including the sequence encoding wild-type smMLCK S472 as well as to introduce a complementary *Eco*RI, *Nar*I site at the 3′ end. The resulting 1 kb *Bam*HI−*Eco*RI fragment was cloned next to the 5′ end of the previous *Eco*RI−*Pst*I fragment in the shuttle vector. The intervening *Nar*I fragment was then removed by digestion and religation to create the construct MK(CK301−315).

(7) MK EF. PCR was used to insert an *Eco*RI site immediately 5′ to E782 of smMLCK and also to create a *Pst*I site 3′ to the stop codon. The resulting 0.6 kb *Eco*RI−*Pst*I fragment of smMLCK was inserted between the *Eco*RI and *Pst*I sites of MK(CK281−315), leading to a MK construct with E and F inserted between M781 and E782, designated MK EF.

To confirm the correct identity of the chimeras, DNA sequencing was performed on the protein coding sequences of all constructs starting from both the amino and carboxyl termini, as well as spanning their entire autoregulatory regions.

Expression and Purification of Protein Kinases. Baculovirus stocks were generated from plasmids by transfecting Sf9 cells with reagents provided from the Baculogold Transfection Kit (Pharmingen) using previously established protocols (*11*). This typically resulted in viral stocks with a concentration of 10^8−10^9 pfu/mL. Proteins were then expressed in Sf9 cells as previously described (*11*). The His-tagged protein kinases were purified by affinity chromatography with a nickel−NTA resin. The infected Sf9 cells were first harvested by centrifugation and lysed by sonication. The collected supernatant was first mixed with 1 mL of Ni−NTA resin at 4 °C for 2 h. The resin was next washed with 5 volumes of buffer A [50 mM Hepes (pH 7.5), 0.5 M NaCl, and 10% glycerol] followed by 12.5 volumes of buffer B [50 mM K-MES (pH 6.3), 0.5 M NaCl, and 10% glycerol]. The resin was then washed with 12.5 volumes of buffer B, containing 50 mM imidazole, before finally eluting the proteins with 3−5 volumes of buffer B, containing 200 mM imidazole. The concentration of purified proteins was determined by the method of Bradford (*14*) using γ-globulin protein standards, and the purity of all proteins was checked by SDS−PAGE analysis. The proteins were finally stored for later use at −70 °C in 20% glycerol.

Calmodulin Overlays. Protein samples were transferred overnight at 4 °C from a SDS−PAGE gel to an Immobilon-P membrane (Millipore). The membrane was incubated with 100 mM Imidazole (pH 7.0) for 10 min, followed by incubation in buffer C [20 mM imidazole (pH 7.0), 200 mM KCl, 0.1% BSA, 0.02% NaN_3, and 1 mM $CaCl_2$] for 2 h.

The membrane was then incubated for 2 h in buffer C, containing [^{125}I]CaM (0.5×10^6 cpm/mL), followed by three washes of 20 min each in buffer C only, before drying and exposing to film.

Calmodulin Equilibrium Binding Assay. CaM was biotinylated with *N*-hydroxysuccinimide-LC biotin by combining 5 mol of biotin reagent/mol of CaM in 50 mM $NaHCO_3$ (pH 8.5) for 2 h on ice. The biotinylated CaM was next immobilized by passing it over a BIAcore chip fixed with streptavidin that was part of a BIAcore system (Pharmacia Biosensor). The affixed CaM was equilibrated with a running buffer of 10 mM Hepes (pH 7.5), 150 mM NaCl, 0.5 mM $CaCl_2$, and 0.005% Tween 20. All enzyme constructs were dialyzed extensively against running buffer prior to application on the BIAcore system. Eight different concentrations of each enzyme construct ranging from 0.5 nM to 2 μM enzyme were passed over the CaM chip, with each concentration repeated to ensure the accuracy of the results. Between each concentration, the apparatus was washed with running buffer containing EGTA instead of calcium, followed by extensive equilibration with the original running buffer.

Sensorgrams showing association and dissociation curves obtained from the BIAcore system were analyzed using the BIAevaluation 2.0 software to determine K_d values for binding to CaM based on a single-site interaction model where $K_d = k_{off}/k_{on}$.

k_s was derived from a nonlinear least-squares fit of the association curves to the equation

$$R = r_0/k_s \times [1 - e^{-k_s(t-t_0)}]$$

where R is the instrument signal, r_0 is the baseline signal, t is time (seconds), t_0 is time at addition of enzyme, and $k_s = k_{on}C + k_{off}$, where C is the enzyme concentration. The value of k_{on} was determined from linear regression analysis of a secondary plot of k_s versus C.

k_{off} was derived from a nonlinear least-squares fit of the disocciation curves to the equation

$$R = R_0 e^{-k_{off}(t-t_0)}$$

where R_0 is the instrument signal at the start of the disocciation and t_0 is the time at the start of the dissociation.

Gel Permeation Chromatography. Gel permeation chromatography was performed on a Pharmacia FPLC system using a 24 mL Superdex 200 HR analytical gel filtration column (10 mm $\times$ 300 mm) equilibrated and eluted with buffer at a flow rate of 0.4 mL/min.

Limited Proteolysis. Protein kinases (0.2 mg/mL) were proteolyzed by the addition of trypsin (0.08 mg/mL) in a solution of 30 mM Tris-HCl (pH 7.5), 50 mM KCl, and 1 mM EGTA at 0 °C for times varying from 0 to 60 min. At different time intervals, 20 μL aliquots were taken and added to solutions of trypsin inhibitor at 4 °C to give a final 10:1 ratio of inhibitor to trypsin. These samples were then assayed for protein kinase activity.

Protein Kinase Assays. Protein kinase assays were performed as previously described (*11*) in a solution of 50 mM Hepes (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 0.1% Tween 80, 0.5 mg/mL BSA, 100 μM ATP (0.2 mCi), and 0.5 mM $CaCl_2$ (or 1 mM EGTA) in the presence of either 200 μM peptide MLC(11−23) or the 20 kDa MLC as protein substrates (25 μM), in a final volume of 50 μL. Assays were initiated by the addition of enzymes to final concentration of 1 nM and allowed to proceed for 10 min at 30 °C. Under these conditions, less than 5% of the substrates were consumed in the reactions. Forty microliters of each reaction mixture was then spotted on either Whatman P-81 filter paper [MLC(11−13) peptide] or Whatman 3MM filter paper (20 kDa MLC). The P-81 filters were extensively washed in 75 mM phosphoric acid, whereas the 3MM filters were washed in a solution of 10% TCA and 2% sodium pyrophosphate, prior to quantitation on a Beckman LS 6000 scintillation counter.

K_{CaM} values were determined by direct nonlinear least-squares fitting of calmodulin concentration curves to the equation

$$v - v_0 = V_m - v_0[\text{CaM}]/(K_{CaM} + [\text{CaM}])$$

using Kaleidagraph version 3.0.2 (Abelbeck Software), where v is the rate at subsaturating concentrations of CaM, v_0 is the rate in the absence of CaM, V_m is the maximal rate achieved at saturating CaM concentrations (1 μM), and K_{CaM} is the concentration of CaM required to elicit 50% of the CaM-dependent activity of the enzyme.

RESULTS

PCR mutagenesis was employed to create hexyl histidine-tagged, truncated forms of smMLCK in which different segments of the autoregulatory domain of CaM KII were exchanged for the corresponding region of smMLCK (Figure 1A). The N-termini of all the truncated enzymes started at residue 472 of smMLCK, approximately 40 residues before the beginning of the kinase domain of the enzyme. All chimeras extended through the entire kinase domain of smMLCK (residues 513−774) and, with one exception, terminated at C-terminal residue 972 of the wild-type smMLCK. The exception was the chimera MK(CK281−478) which spliced C-terminal residue 781 of smMLCK with N-terminal residue 281 of CaM KII and terminated at residue 478 of CaM KII. The chimeric protein MK(CK281−315) replaced smMLCK residues 781−817 with CaM KII residues 281−315. In the cases of MK(CK281−478) and MK-(CK281−315), two additional residues, E and F, were inserted between smMLCK residue 781 and CaM KII residue 281 as a consequence of creating a new DNA restriction site. We therefore created a control enzyme, MK EF, in which the two identical residues (EF) were inserted between residues 781 and 782 of smMLCK. The final chimeric construct, MK(CK301−315), replaced smMLCK residues 801−817 with CaM KII residues 310−315, but without including the EF insert. The exact amino acid sequences of the various enzymes, highlighting the CaM KII chimeric residues, are presented in Figure 1B.

The truncated, His-tagged smMLCK chimeric proteins were expressed in Sf9 insect cells with the baculovirus system and purified in a single step by the identical method of nickel column affinity chromatography. Typical yields of the purified enzymes from 120×10^6 Sf9 cells were several milligrams of protein. Figure 2A shows a Coomasie blue-stained SDS−PAGE gel of equal amounts of the various purified proteins. All the His-tagged proteins migrated in the expected M_r range (between 55 and 65 kDa), although MK-

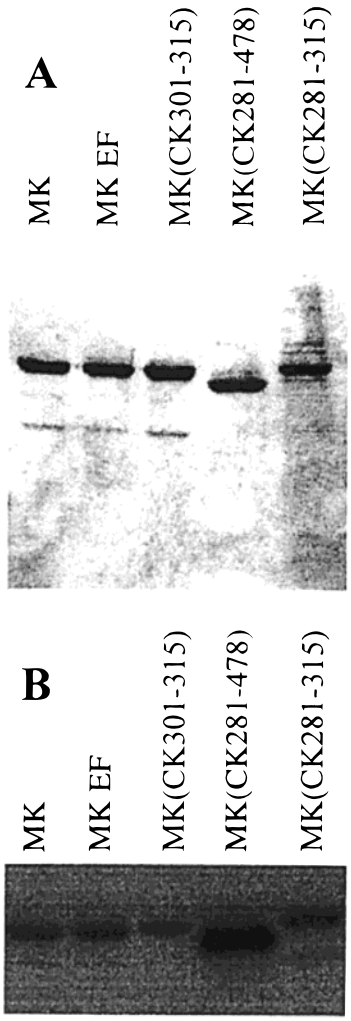

FIGURE 2: Analysis of proteins by SDS−polyacrylamide gel electrophoresis. (A) Coomassie blue stain of equal amounts of purified proteins. The identities of the proteins are presented above each lane. (B) [^{125}I]CaM gel overlay of purified proteins.

(CK281−478) had a slightly faster migration rate than expected. To qualitatively determine whether the inserted CaM KII chimeric sequences inhibited the ability of the enzymes to interact with CaM, an [^{125}I]CaM overlay was performed on equal amounts of the purified proteins. The results (Figure 2B) demonstrate that all the expressed proteins were capable of binding CaM in the presence of calcium, with MK(CK281−478) appearing to exhibit a higher affinity for CaM than the other proteins. To quantitate the relative abilities of these proteins to bind to CaM, different concentrations of each protein were passed over biotinylated CaM that had been immobilized on a BIAcore chip, and the results are shown in Figure 3. The CaM binding constants (K_d) of the enzymes are consistent with the results from the CaM overlay assay (Table 1). Three of the constructs, MK EF, MK(CK281−478), and MK(CK281−315), were very similar to the wild-type MK enzyme, whereas the fourth, MK-(CK301−315), had an approximately 10-fold lower affinity for CaM.

In contrast with the other chimeras, MK(CK281−478) also harbored the C-terminal domain of CaM KII (residues 320−478), the portion of the enzyme responsible for its oligomerization properties. To ascertain whether the oligomerization function of CaM KII was transferred to MK(CK281−478), analytical gel filtration chromatography was performed on this chimera and the peak of enzymatic activity compared with those from wild-type MK, another chimeric protein MK-(CK281−315), and wild-type CaM KII. The results from gel filtration on a Superdex 200 column (Figure 4) demonstrate that oligomerized wild-type CaM KII ($M_r \sim 600$ kDa) elutes at a volume close to the void volume (V_o), whereas chimeras MK and MK(CK281−315) elute at a position similar to that of a protein standard, BSA (~65 kDa). In contrast, the activity of the chimera MK(CK281−478) eluted at a position very close to that of wild-type CaM KII (600 kDa), confirming that the presence of the oligomerization domain of CaM KII led to a multimerization of the smMLCK domain.

Although the CaM gel overlay and BIAcore assays indicated that all the chimeric proteins were capable of binding CaM to some extent, it is also known that the initial binding of CaM by smMLCK can be separated from a later step of enzyme activation (*12*, *15*, *16*). We therefore compared the enzymatic activity of the various purified proteins in the absence of CaM with their activity in the presence of increasing amounts of CaM (Figure 5). The results show that in the absence of CaM, the wild-type enzyme MK has virtually no kinase activity, whereas it is activated by CaM with an activation constant (K_{CaM}) of 9 nM (Table 1). In contrast, chimeras MK(CK281−315) and MK(CK281−478) had 23 and 37% of the maximal kinase activity toward the 20 kDa substrate protein MLC in the absence of CaM, respectively. When tested with a considerably smaller peptide substrate, MLC(11−23), this CaM-independent activity increased even further, to 30% for MK(CK281−315) and 54% for MK(CK281−478) (Table 1). Interestingly, although these two chimeras were active in the absence of CaM, they were still capable of additional activation by exogenously added CaM, with K_{CaM} values of 7.5 and 22 nM, respectively (Table 1). Indeed, at high CaM concentrations, the chimeras exhibited enzymatic activities comparable to that of the wild-type MK enzyme. Thus, the chimeric CaM KII sequences (residues 281−315 and 281−478) had different effects on autoinhibition and CaM-dependent activation. On one hand, they were unable to completely inhibit the activity of the enzymes in the absence of CaM. On the other hand, the chimeric CaM KII sequences behaved like the original smMLCK sequence with respect to CaM binding and activation.

Since the two-amino acid insert (EF) could, in part, account for some of the CaM-independent activity of the chimeras, we tested the activity of a control construct (MK EF), where the same two amino acids (EF) were inserted in the identical position of MK, between residues 781 and 782. In the presence of CaM, MK EF behaved very much like the wild-type enzyme MK, with an almost identical specific activity and a K_{CaM} of 6 nM (Figure 5 and Table 1). However, the results in the absence of CaM demonstrate that MK EF, unlike chimeras MK(CK281−315) and MK(CK281−478), exhibited little CaM-independent activity. The two-residue EF insertion is thus unlikely to account for the CaM-independent activity of the chimeric proteins.

A region within the CaM KII chimeric sequences might also be responsible for the CaM-independent activities of chimeras MK(CK281−315) and MK(CK281−478). It has been previously demonstrated that phosphorylation of T286 in CaM KIIα leads to an autonomous enzyme, i.e., one which

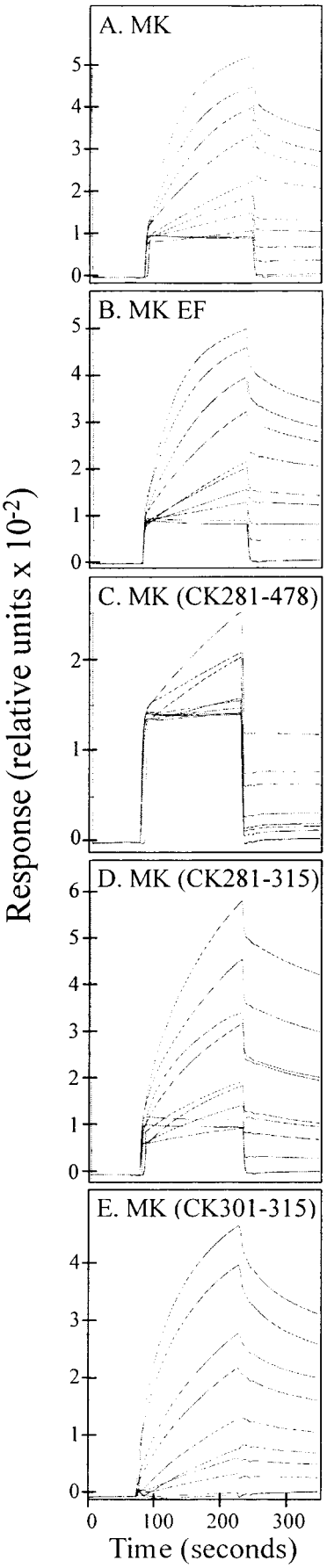

FIGURE 3: Sensorgrams showing the phases of association and dissociation of proteins with CaM. The proteins are (A) MK, (B) MK EF, (C) MK(CK281−478), (D) MK(CK281−315), and (E) MK(CK310−315).

Table 1: Calmodulin-Dependent and -Independent Properties of Wild-Type and Chimeric Enzymes

protein	K_d (nM)	K_{CaM} (nM)	% Ca^{2+}-independent activity: peptides	% Ca^{2+}-independent activity: light chains
MK	10.3 ± 2.0	9.0 ± 1.3	0	0
MK EF	8.6 ± 0.6	6.6 ± 1.1	0	0
MK(CK281−478)	9.0 ± 1.6	21.6 ± 3.8	54	37
MK(CK281−315)	5.6 ± 2.2	7.5 ± 1.3	30	23
MK(CK301−315)	116.5 ± 59.2	LA[a]	0	0

[a] Low activity.

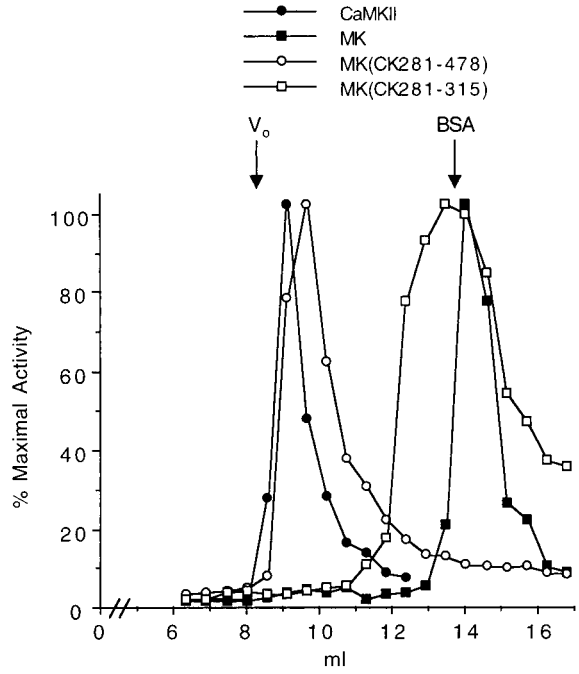

FIGURE 4: Gel filtration analysis of wild-type and chimeric proteins. Individual samples of proteins were loaded on a Superdex 200 FPLC column, eluted, and fractions were collected. Enzymatic activities were assayed as described in Methods and expressed as a percentage of the peak fraction. The void volume (V_o) was calibrated by using dextran blue (M_r = 600 kDa). BSA (M_r = 66 kDa) was also used as a molecular mass standard.

is active in the absence of Ca^{2+}/CaM (*17*). Since T286 is present in both chimeras MK(CK281−315) and MK(CK281−478), it is possible that the unusual presence of this potential regulatory site in smMLCK might contribute to the CaM-independent activity exhibited by the two chimeric enzymes. To test whether T286 contributed to the observed CaM-independent activities, two additional constructs were created by site-directed mutagenesis in which the corresponding T286 of chimeras MK(CK281−315) and MK(CK281−478) was individually changed to a structurally similar residue, V, which was incapable of being phosphorylated. The activities in the absence and presence of CaM of the T to V point mutants, MK(CK281−315)TV and MK(CK281−478)TV, were then compared to their parental chimeras, MK(CK281−315) and MK(CK281−478), respectively. The results demonstrate that the CaM-independent and CaM-dependent activities of the mutant MK(CK281−315)TV were almost identical to those of the parental MK(CK281−315) (Figure 6). However, the CaM-independent activity of the T to V mutant MK(CK281−478)TV was almost twice that of the parental chimera MK(CK281−478) (65 vs 35%), a result which indicates that the substitution

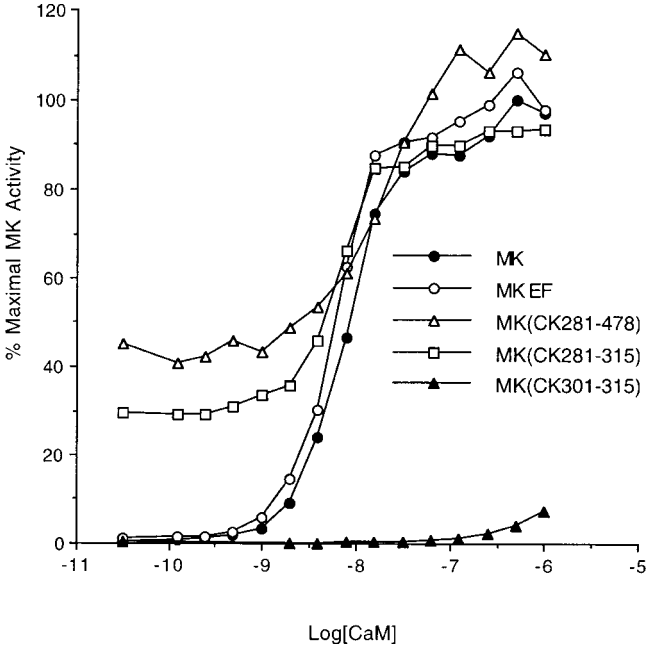

FIGURE 5: Activation of wild-type and chimeric enzymes by calmodulin. The specific activity of the individual enzymes was measured at increasing CaM concentrations and expressed as a percentage of the activity of the wild-type MK protein determined at 1 μM CaM. The concentration of the enzymes was 1 nM.

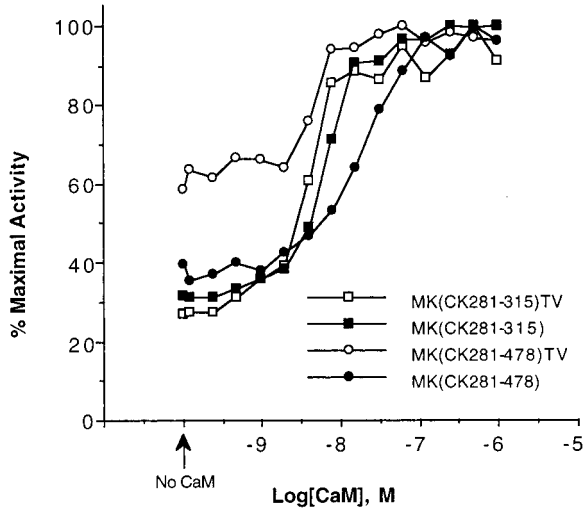

FIGURE 6: Activity of chimeric and threonine 286 to valine mutant enzymes in the absence and presence of calmodulin. The enzymatic activities of the chimeric proteins was measured either in the absence of CaM or at increasing CaM concentrations and expressed as a percentage of the activity of wild-type MK determined at 1 μM CaM.

of V for T286 had not abolished, but had instead increased, the activity of this enzyme in the absence of CaM. Taken together, the results with these mutations demonstrate that the presence of T286 has little effect on generating CaM-independent activity in the chimeric enzymes, and may in fact contribute to maintaining the enzyme in an inactive state when not phosphorylated.

Like the wild-type MK enzyme, the chimera MK(CK301−315) did not exhibit CaM-independent activity (Figure 5 and Table 1), indicating that CaM KII residues 301−315 did not interfere with the normal autoinhibitory function of the enzyme. In contrast to MK, however, MK(CK301−315) was resistant to activation by CaM, exhibiting <5% of the control

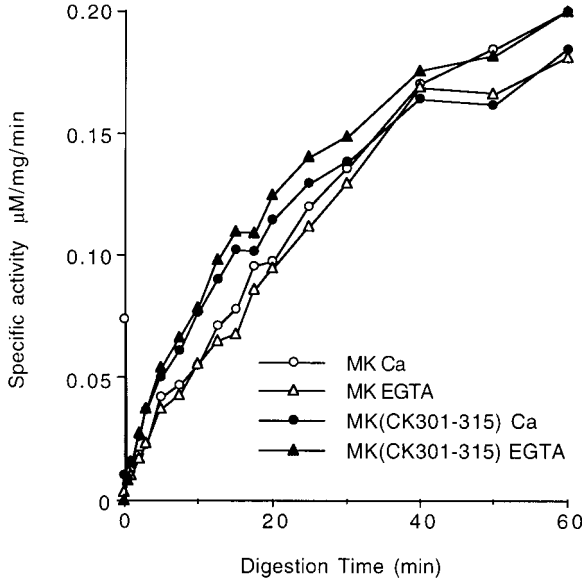

FIGURE 7: Activation of wild-type and chimeric enzymes by limited proteolysis. Wild-type MK and chimeric enzymes were treated with trypsin, and their specific activities were measured over time. Experiments were performed in the presence of Ca^{2+} or EGTA.

MK activity, even at concentrations of CaM approaching 1 μM (Figure 5). When combined with the results from the CaM gel overlay (Figure 2) and BIAcore assays (Table 1) demonstrating that MK(CK301−315) binds CaM, this result indicates that MK(CK301−315) interacts with CaM but is activated with great difficulty. One possible explanation for the inability of MK(CK301−315) to be activated by CaM might be a misfolding of the kinase domain of this particular enzyme as a result of the unusual changes in its autoregulatory sequence. To check for the presence of a correctly folded kinase domain, we compared the effects of limited trypsinolysis on the activity of MK(CK301−315) with that of the wild-type MK enzyme, to determine whether cleavage of their respective autoinhibitory domains would result in constitutively active enzymes. The results of this study demonstrate that both MK and MK(CK301−315) are activated at almost identical initial rates due to the cleavage of the autoinhibitory domains by trypsin (Figure 7). Both enzymes were activated to a similar specific activity by treatment with trypsin for up to 1 h, and this activity was independent of the presence of CaM. The kinase domains of both enzymes are therefore capable of CaM-independent enzymatic activities, and are thus unlikely to be responsible for the inability of MK(CK301−315) to be activated by CaM.

DISCUSSION

The enzyme smMLCK is inactive in the absence of CaM, with its activity strictly controlled by an autoregulatory domain that performs the dual functions of autoinhibition and CaM-dependent activation. The CaM-independent activities observed in the chimeric enzymes MK(CK281−315) and MK(CK281−478) suggest that the specific sequence of the smMLCK autoinhibitory domain is necessary for complete inhibition of enzymatic activity (Table 1). These results indicate that the CaM KII autoregulatory sequence is able to partially compensate for the autoinhibitory function of the original smMLCK autoregulatory domain but was unable to

fully inhibit the enzymatic activity of the smMLCK catalytic domain in an intrasteric manner. A similar inability to completely autoinhibit enzyme activity has also been observed in mutants of the related CaM-dependent protein kinases, CaM KII and CaM KI, where substitutions were introduced into the autoinhibitory sequences of these two enzymes (*18*, *19*). Together, these results indicate that at least three CaM-regulated protein kinases are partially activated by mutations within their respective autoinhibitory sequences.

Recently, a strategy similar to the one used herein on smMLCK has been employed in replacing the autoregulatory sequence of rabbit skMLCK with that of either rabbit smMLCK, CaM KII, or neuronal nitric oxide synthase. The resulting skMLCK chimeras did not exhibit CaM-independent activity as was observed in the smMLCK chimeras described here but were instead completely inactive in the absence of CaM (*20*). The differences between the primary structures and domain organizations of skMLCK and smMLCK, as well as the high substrate specificity of smMLCK, indicate a tighter degree of regulation of smMLCK. These differences might account for the inability of two of the CaMKII chimeras to maintain smMLCK in an inactive state, whereas the skMLCK enzyme can tolerate these changes.

In contrast to MK(CK281−315), which exhibited significant CaM-independent activity, the lack of activity of the chimera MK(CK301−315), in the absence of CaM (Figure 5 and Table 1), indicates that this chimera contains sequences important for the effective autoinhibition of the smMLCK catalytic domain. One clear difference between these two proteins is the presence of smMLCK residues 782−801 in the chimera MK(CK301−315) instead of the homologous autoregulatory residues of CaM KII found in MK(CK281−315) (Figure 1B). This suggests that smMLCK residues 782−801 are responsible for the observed autoinhibition of enzymatic activity and conversely that the chimeric autoregulatory residues of CaM KII were unable to make the same specific intramolecular contacts with the smMLCK catalytic domain. Indeed, the part of the autoregulatory domain that resembles the natural substrate, smooth muscle myosin light chain amino acids 1−22, designated the "pseudosubstrate" inhibitor sequence, also resides between smMLCK residues 787 and 808 (*21*, *22*).

Whereas in the absence of CaM chimeras MK(CK281−475) and MK(CK281−315) were unable to completely autoinhibit the catalytic domain of smMLCK, the same enzymes behaved like the wild-type MK enzyme in the presence of CaM. These results demonstrate that although they may have dissimilar primary structures, the CaM binding domain of CaM KII can functionally replace the CaM binding domain of smMLCK. Similar results have been obtained when the entire CaM binding domain of CaM KII was substituted for its counterpart in smMLCK (*23*), and when smMLCK or CaM KII autoregulatory domains were substituted in skMLCK (*20*). The CaM binding properties of these chimeras in which the entire CaM binding domains are swapped can be readily explained by the similarities between the crystallographic structures of CaM in complex with peptides corresponding to the CaM binding domains of either smMLCK (*10*) or CaM KII (*24*). Although the two CaM binding domains have different amino acid sequences, their similar interactions with CaM may explain the ability of the intact CaM binding domain of CaM KII to functionally substitute for the original smMLCK CaM binding domain within the context of the smMLCK enzyme.

The ability of MK(CK301−315) to bind to CaM but remain inactive is the most intriguing result of this study. It has been previously demonstrated that the conversion of W800 in the N-terminal half of the CaM binding domain to a variety of residues with smaller side chains severely diminishes and in some cases abolishes CaM binding to smMLCK (*6*, *7*). On the basis of these results, the ability of MK(CK301−315) to bind CaM resides in its retention of the N-terminal half of the CaM binding domain of smMLCK, which includes W800 (Figure 1B). However, the inability of MK(CK301−315) to be activated by micromolar concentrations of CaM indicates that the chimeric CaM KII sequences of MK(CK301−315) do not allow CaM-dependent activation. It has been previously observed that autophosphorylation of T305/306 in CaM KIIα can prevent CaM-dependent activation of CaM KII (*25*, *26*). These residues are also present in the MK(CK301−315) chimera. However, the phosphorylation of these residues is an unlikely explanation for the inability of the MK(CK301−315) chimera to be activated by CaM for three reasons. First, phosphorylation of T305/306 prevents CaM binding to CaM KII, whereas the MK(CK301−315) chimera is still capable of binding CaM in gel overlays as well as in solution. Second, the same two T residues are also present in chimeras MK(CK281−315) and MK(CK281−478) (Figure 1), both of which were activated in a CaM-dependent manner (Figure 5). Third, the treatment of MK(CK301−315) with protein phosphatases did not result in an enzyme capable of being activated by Ca^{2+}/CaM (data not shown).

The relative spacing of hydrophobic residues in the CaM KII sequence of MK(CK301−315) may be responsible for the inability of this chimera to be activated by CaM. The crystal structures of Ca^{2+}/CaM in complex with the CaM binding peptides of smMLCK or CaMKII show that the N-terminal domain of CaM makes a prominent hydrophobic interaction with the residue corresponding to L813 of the smMLCK peptide or L308 in the case of the CaM KII peptide (*10*, *24*). The chimera MK(CK301−315) retains W800 in the N-terminal half of the CaM binding domain of smMLCK but substitutes the C-terminal half of the CaM KII sequence. This changes the relative position of smMLCK residue L813 which is normally separated by 12 residues from W800, and instead introduces L308 of CaM KII which is now separated from W800 by only 8 residues. The misalignment of the C-terminal halves of these two different CaM binding domains might account for the failure of MK(CK301−315) to be activated by CaM since the change in spacing from 12 to 8 residues relative to W800 may not allow the N-terminal domain of CaM to properly interact with the newly introduced CaM KII chimeric sequence.

Residues other than L813 or the critical spacing between two important hydrophobic residues may also be responsible for the inability of MK(CK301−315) to be activated by CaM. An earlier mutagenesis study of the kinase domain of smMLCK demonstrated that several positions within the CaM binding domain of smMLCK had substantial effects on its regulation by CaM (*6*). Specifically, individual A substitutions at K802, R808, I810, and L813 of smMLCK resulted in enzymes that had a reduced affinity for CaM as well as a lowered enzymatic activity in the presence of CaM.

The same residues, as well as several others, are changed in the MK(CK301−315) chimera (Figure 1B), which in comparison produced similar effects. For example, I810 of smMLCK is an A in MK(CK301−315) (Figure 1B). The I810A mutant of smMLCK has been shown to have a reduced affinity for CaM as well as reduced enzymatic activity (*6*). Therefore, it is possible that the individual A point mutants of smMLCK are behaving like MK(CK301−315) but exhibit lesser effects due the smaller number of changes they incorporate into the enzyme.

Finally, it is worth noting that the results from the smMLCK−CaM KII chimeras are complementary with those from mutants of CaM (*11*, *12*). Since the CaM binding domain of smMLCK overlaps its autoinhibitory domain, it is likely that CaM binding disrupts intramolecular contacts between the autoinhibitory sequence and the kinase domain of the enzyme. This removes the entire autoinhibitory domain from the active site, resulting in the generation of enzymatic activity. Recent small-angle X-ray scattering studies on the homologous CaM-dependent enzyme, skMLCK, provide evidence for just such a dramatic conformational change on binding CaM (*27*). The relevance of this mechanism to the activation of other CaM-dependent kinases remains to be investigated.

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SUPPORTING INFORMATION AVAILABLE

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