Mechanisms for Regulation of Calmodulin Kinase IIα by Ca^{2+}/Calmodulin and Autophosphorylation of Threonine 286[†]

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ABSTRACT: A mechanism that relates calmodulin (CaM) binding to enzyme activation remains to be established within the context of full-length calmodulin kinase IIα (CaM KIIα). Previous studies using peptides and/or truncated enzymes have shown that L299 of CaM KIIα represents an "anchor" for Ca^{2+}/CaM binding and that F293 is required for autoinhibition. We have substituted each of these residues with a W in full-length CaM KIIα and measured the W fluorescence to evaluate the location of these side chains in the absence and presence of Ca^{2+}/CaM. Fluorescence emission of the L299W mutant indicates that L299 is solvent accessible in the absence of Ca^{2+}/CaM but becomes internalized in the presence of Ca^{2+}/CaM. On the other hand, examination of F293W indicates that Ca^{2+}/CaM binding promotes enzyme activation by transferring F293 from an internal location in the inactive enzyme to a more solvent accessible position in the active enzyme. In addition, F293 interacts with Ca^{2+}/CaM as a consequence of autophosphorylation at T286, thus providing a mechanism for CaM trapping. Whereas in the absence of autophosphorylation the exposure of F293 is reversed by dissociation of CaM leading to enzyme autoinhibition, after autophosphorylation of T286, F293 is retained in an exposed position due to dissociation of CaM, consistent with the retention of autonomous activity. Proline mutants were introduced at positions between T286 and F293 to explore the basis of CaM-independent, autonomous activity. The observation that an L290P mutant displayed a high level of activity independent of Ca^{2+}/CaM or phosphorylation of T286 indicates that a change in the conformation of the polypeptide main chain at L290 might contribute to the mechanism for generating autophosphorylation-dependent autonomous activity.

The stimulation of many cells by extracellular agonists induces transient and localized changes in the amplitude and/or frequency of oscillations in intracellular free calcium (Ca^{2+}) (*1*). These Ca^{2+} signals encode information that can be detected by Ca^{2+} sensors, such as calmodulin (CaM),[1] a ubiquitous protein that regulates a number of effector proteins in a Ca^{2+}-dependent manner (*2*). Indeed, the complex of CaM with one effector, the CaM-dependent protein kinase II (CaM KII), transduces different frequencies of Ca^{2+} oscillations into changes in enzyme activity (*3*). CaM KII is also ubiquitously distributed, but is abundantly expressed in neurons, where it is important for learning and memory (*4*). Several isoforms of the enzyme (α, *β*, *γ*, and *δ*) can combine in different ratios to form a ringed, bilayered, multimeric (between 8 and 12 monomers) holoenzyme (*5*). Each subunit of CaM KIIα consists of an N-terminal catalytic domain (residues 1−270) and a C-terminal association domain (residues 311−478) that is responsible for oligomerization and subcellular localization (Figure 1). A central autoregulatory sequence (residues 276−310) autoinhibits kinase activity and binds to Ca^{2+}/CaM with residues 293−310 (Figure 1).

The activation of CaM KIIα by Ca^{2+}/CaM promotes autophosphorylation of T286 (Figure 1). This event acts as a "molecular memory" by increasing the affinity of CaM KIIα for Ca^{2+}/CaM by ∼1000-fold, termed "CaM trapping" (*6*), and maintains the enzyme in a partially activated, or "autonomous", state even after dissociation of CaM. The properties of CaM trapping and autonomous activity are maintained in monomeric fragments of the enzyme lacking the C-terminal association domain (*6*, *7*). Autophosphorylation has important physiological consequences as mice in which wild-type CaM KIIα is replaced with a T286A point mutant are impaired in spatial learning and long-term potentiation (LTP) in their hippocampal neurons (*8*).

Prior studies have primarily addressed mechanisms that are responsible for maintaining the autoinhibited state of CaM KII by introducing substitutions into the autoinhibitory sequence. Some of these mutations generate constitutive enzymatic activity in a manner that does not require the addition of CaM. This constitutive activity has been termed "CaM-independent" activity (*9*−*14*). However, although it is possible to introduce mutations into CaM KIIα that result in CaM-independent activity, the mechanisms underlying how the binding of Ca^{2+}/CaM mediates the transition from the autoinhibited to the activated state of the enzyme still

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[1] Abbreviations: CaM, calmodulin; CaM KIIα, Ca^{2+}/calmodulin-dependent protein kinase IIα; CaM KI, Ca^{2+}/calmodulin-dependent protein kinase I; PCR, polymerase chain reaction; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; smMLCK, smooth muscle myosin light chain kinase.

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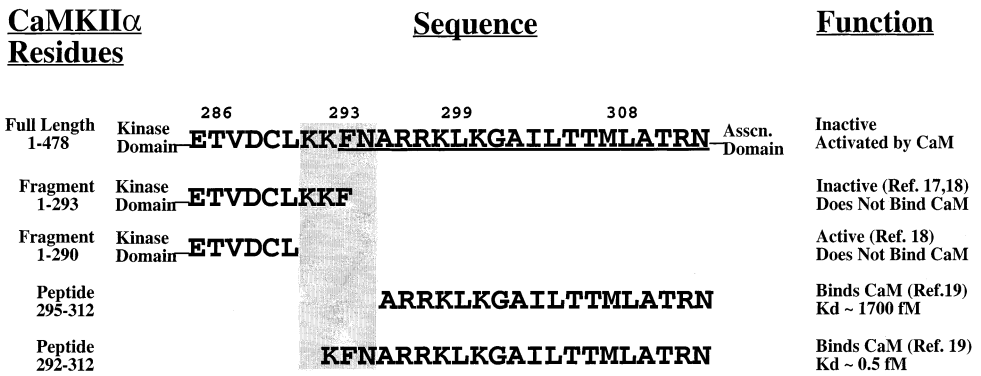

FIGURE 1: Autoregulatory domain of calmodulin kinase IIα. The amino acid sequence from the autoregulatory domain of CaM kinases IIα is shown above with the CaM binding domain underlined and important positions indicated. Enzyme fragments and peptides derived from CaM kinases IIα are shown below. The functionally important KKFN residues are shaded. Functions of the various proteins and/or peptides are presented with references.

remain to be elucidated in biochemically precise terms. Insights into the CaM binding process are provided by the crystal structure of Ca^{2+}/CaM in complex with a CaM binding peptide from CaM KIIα (residues 290−314). This structure reveals that CaM engulfs the helical peptide, and two leucines from the peptide, equivalent to L299 and L308 in the enzyme (Figure 1), were concluded to be important for "anchoring" it to Ca^{2+}/CaM (*15*). Significantly, L299 is homologous to W303 from the closely related enzyme, CaM kinase I (CaM KI), and in the crystallographic structure of autoinhibited CaM KI, W303 is situated in a solvent accessible loop that may promote the recognition of that enzyme by Ca^{2+}/CaM (*16*).

The previous structural studies (*15*, *16*) imply that sequestration of the CaM binding domain of CaM KIIα by Ca^{2+}/CaM is an important step in enzyme activation. However, C-terminal deletion of the entire CaM binding domain, including a "pseudosubstrate" sequence (residues 296−300), believed to be important for autoinhibition, fails to activate the remaining catalytic fragment consisting of residues 1−293 (*17*, *18*) (Figure 1). Importantly, the further removal of only the adjacent residues (K, K, and F at positions 291−293, respectively) is sufficient to generate an activated enzyme fragment (residues 1−290) in the absence of Ca^{2+}/CaM (*18*) (Figure 1). The proximity of this "core" autoinhibitory sequence (KKF), located between the CaM binding domain (residues 294−310) and T286 (Figure 1), suggested that the removal of the KKF residues may be linked to the generation of both Ca^{2+}/CaM-dependent and T286-dependent autonomous activity in the full-length enzyme. Furthermore, as the attachment of residues 292−294 (KFN) to a CaM binding peptide of CaM KIIα increases its affinity for Ca^{2+}/CaM by ~3400-fold (*19*) (Figure 1), it seemed to be plausible that the KFN sequence might have the additional feature of participating in the trapping of Ca^{2+}/CaM by the CaM KIIα enzyme.

In this study, we have investigated mechanisms underlying the regulation of CaM KIIα by Ca^{2+}/CaM and autophosphorylation at T286, by introducing mutations at strategic residues within the autoregulatory domain of the full-length enzyme. Functional analyses of the mutant proteins, together with the results obtained from examining the conformations of W introduced at positions 293 and 299 by fluorescence spectroscopy, reveal a novel connection between CaM KIIα autoinhibition, activation by Ca^{2+}/CaM, Ca^{2+}/CaM trapping, and autonomous activity that involves the relocation of F293.

MATERIALS AND METHODS

Restriction enzymes, T4 ligase, ATP, AMP-PNP, and CIP were purchased from Roche. Antibodies to CaM KII were purchased from Promega. The pRK-5CaMKIIα plasmid was provided by A. Chantry (*20*). GS-10 peptide prepared by solid-phase synthesis was purified by HPLC and confirmed by mass spectral analysis. Radiolabeled [γ-^{32}P]ATP was purhased from Amersham. CaM from an affinity resin was isolated from bull testes, whereas chicken CaM used in enzyme assays was expressed and isolated from bacteria (*21*). Reagents used in bacterial and tissue cultures were of the highest available quality.

CaM KIIα Expression Plasmids. Individual CaM KIIα mutant plasmids were created from the wild-type rat CaM KIIα gene cloned into the mammalian expression plasmid pRK-5. PCR mutagenesis (*20*) using the pRK-5CaMKIIα plasmid as a template and digestion of the product with unique *Bcl*I and *Bsp*EI restriction sites at coding positions 740 and 940 resulted in an ~200 bp cassette containing the mutation(s) of choice. An appropriate vector was prepared by digesting the wild-type pRK-5CaMKIIα plasmid (isolated from an *Escherichia coli* dam− strain) to remove the corresponding *Bcl*I−*Bsp*EI fragment, followed by treatment with CIP. The cassette containing the mutation was then ligated into this vector with T4 ligase and transformed into DH5α bacteria to allow the isolation of DNA from individual colonies. The identity of the mutated plasmids was verified by sequencing the entire insert, including the unique insertion sites.

Expression and Purification of CaM KIIα. Wild-type and mutant CaM KIIα were expressed in human 293 cells (American Type Culture Collection number CRL-1573) using modifications of a previously described procedure (*22*). Freshly trypsinized 293 cells ($5-7 \times 10^6$) were placed in 150 mm diameter Falcon plates with 20 mL of DMEM/10% FBS medium and left for 7−8 h to incubate in a 5% CO_2 atmosphere and 37 °C. Cells were transiently transfected using a Ca_2PO_4 precipitation procedure (*22*) by adding 60 μg of the pRK-5CaMKIIα plasmid in 2 mL of Bes-buffered saline (BBS). The cells were then incubated in a 3% CO_2

atmosphere at 35 °C for 16−20 h, followed by washing and a change to fresh medium. Cells were harvested after incubation for an additional 24 h in a 5% CO_2 atmosphere and 37 °C. Cells were lysed at 4 °C in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 1 mM EGTA, 10% glycerol, and 1% Triton X-100 in the presence of the protease inhibitors leupeptin, Pefabloc, aprotinin, PMSF, and pepstatin. The supernatant was collected after centrifugation at 13000*g* for 20 min at 4 °C.

Enzymes that were expressed in sufficient quantities were isolated by batch affinity chromatography on CaM-Sepharose, resulting in a highly purified fraction of CaM KIIα. The CaM affinity resin was extensively washed as previously described (*22*), followed by washing in a buffer of 50 mM Hepes, 150 mM NaCl, 1 mM DTT, 10% glycerol, and 0.1 mM $CaCl_2$, before being eluted in the same buffer but containing 5 mM EDTA and 40% glycerol and stored at −70 °C. Where a highly purified protein was required, cell extracts were first fractionated by gel filtration chromatography on a Sephadex G-200 column (25 cm × 120 cm), equilibrated, and eluted in 50 mM Hepes (pH 7.5), 1 mM DTT, 1 mM EDTA, and 10% glycerol. All mutant proteins exhibited a similar elution volume like the wild-type protein, just after the column void volume, indicating that they were not monomeric but had retained their oligomeric structures. This procedure was followed by anion exchange chromatography by FPLC on a Mono-Q (FF) column (2 mL) eluted with a 0 to 0.8 M NaCl gradient. CaM-Sepharose chromatography was then used to concentrate the protein. Concentrations of purified proteins were determined by the method of Bradford (*23*) using BSA as a standard. Average yields of protein ranged from 10 to 30 $\mu g/10^6$ cells. In cases where enzymes were assayed in cell extracts or in a partially purified state, protein concentrations were estimated by Western blot analysis using previously purified CaM KIIα as a standard.

SDS−PAGE and Western Immunoblot Analysis. Proteins were examined for purity by 7.5% SDS−PAGE analysis followed by staining with Coomassie blue. For immunoblots, proteins were transferred in a buffer of 25 mM Tris-base (pH 8.3), 192 mM glycine, 0.02% SDS, and 20% methanol to PVDF membranes at 4 °C for 2 h at 80 V. Membranes were blocked in a buffer of 25 mM Tris-base (pH 7.4), 140 mM NaCl, 3 mM KCl, and 0.1% Tween 20 (TBST) with 5% powdered milk and 0.5% fish skin gelatin, followed by overnight incubation at 4 °C with a 1/1000 dilution of the rabbit anti-CaM KII antibody in the same buffer. After three or four washes with TBST and incubation with an anti-rabbit peroxidase secondary antibody for 1 h, the membrane was washed three or four times with TBST before being developed with an ECL solution and exposed to film.

Assays of CaM KIIα Enzymatic Activity. Protein kinase assays were performed in a solution of 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 80, 10% glycerol, 0.2 mg/mL BSA, and 0.2 mM ATP (0.2 μCi of [γ-^{32}P]ATP) with 200 μM peptide GS-10 as a substrate. $CaCl_2$ (1 mM) and CaM (various concentrations) were added to determine K_{CaM}s with Kaleidagraph as previously described (*24*). Otherwise, a fixed CaM concentration of 5 μM was added to determine CaM-dependent activity, whereas CaM-independent activity was determined in the presence of 2 mM EGTA. Assays were performed in triplicate, initiated by the addition of enzyme to a final concentration of 2 nM/subunit, and allowed to proceed for 3 min at 30 °C. Samples were loaded onto Whatman P-81 filters and washed in 75 mM phosphoric acid. Dried filters were counted on a Beckman LS 6000 scintillation counter. Autonomous activity was measured after preincubating CaM KII in 50 mM Hepes (pH 7), 5 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 80, 10% glycerol, 0.2 mg/mL BSA, and 0.5 mM ATP at 4 °C for 1 min.

Fluorescence Analysis of CaM KIIα. Fluorescence emission scans of protein samples in a 1 mL cell (Hellma) were performed in either a SLM Aminco Bowman Series 2 or a Photon Technology International (PTI) luminescence spectrometer at 30 °C. Samples were excited at 297 nm, and fluoresecence emission spectra were recorded between 310 and 380 nm at a scan speed of 100 nm/min. Samples consisted of equal concentrations of wild-type (WT) and mutant CaM KIIα (0.8 μM each) in a solution of 25 mM Hepes (pH 7.4), 0.16 M NaCl, 0.5 mM DTT, and 50 μM EGTA. Experiments for studying the effects of Ca^{2+}/CaM included 0.2 mM $CaCl_2$ and 2 μM CaM. Experiments for studying the effects of autophosphorylation included 10 μM ATP or AMP-PNP and 1 mM $MgCl_2$ with Ca^{2+}/CaM. Dissociation of CaM was achieved by addition of EGTA to a final concentration of 5 mM. The data from single scans are presented for one set of experiments and are representative of two to three sets of experiments.

RESULTS

Fluorescence Emission of Tryptophan 299 in CaM KIIα. To explore the role of the L299 anchor in Ca^{2+}/CaM binding, we replaced L299 with W, a fluorophore that reports on the environment of this side chain. A comparison of the fluorescent emission spectrum of the nine W residues from the L299W mutant protein with that of the eight tryptophans in the WT enzyme reveals that both proteins exhibit a wavelength of maximum emission (λ_{max}) of 327 nm (Figure 2A). Unlike other CaM kinases, the autoregulatory sequence of CaM KIIα contains no W, so its introduction to this domain is unique. We took advantage of this characteristic of CaM KIIα to define the environment of residue 299. In contrast to the spectrum of the whole proteins, the difference spectrum (L299W − WT), which provides information about the location of the W299 side chain only (Figure 2B), exhibited a λ_{max} of 350 nm, comparable to that of the solvent accessible, free amino acid L-tryptophan (L-Trp) which exhibits a similar λ_{max} of 352 nM (Table 1). In this series of experiments, the λ_{max} parameter was chosen for determining the relative exposure to solvent of the additional W side chain over fluorescence intensity (*F*), which is not as easily interpreted due to its wide variation in different environments (*25*). Consequently, a λ_{max} close to that of the reference L-Trp, is characteristic of a solvent accessible environment, whereas a lower λ_{max} is consistent with a more internalized location. Therefore, the observed λ_{max} for W299 of 350 nm provides the first indication that this side chain is located in a solvent accessible environment prior to the binding of Ca^{2+}/CaM.

As the accessibility of W299 might enable the full-length CaM KIIα to interact with Ca^{2+}/CaM, the fluorescence emission spectra were recorded for both L299W and WT in the presence of Ca^{2+}/CaM. Importantly, the emission spec-

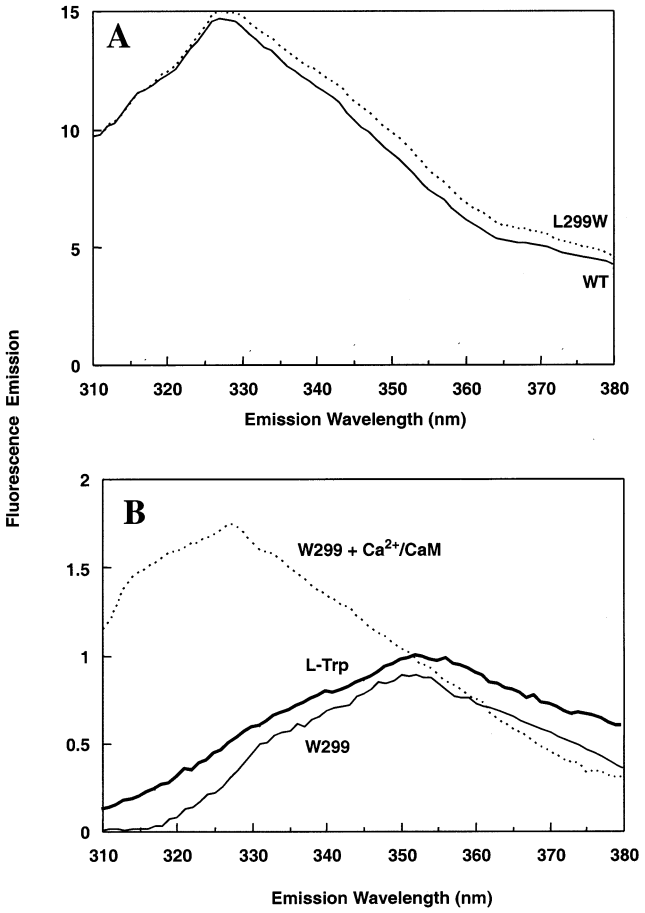

FIGURE 2: Fluorescence emission spectra of tryptophans from wild-type CaM KIIα and the L299W mutant. Tryptophan residues from 0.8 μM wild-type or L299W mutant CaM KIIα were excited at 297 nm and their fluorescence emission spectra monitored from 310 to 380 nm. (A) Emission spectra of WT CaM KIIα (—) and L299W (•••) in the absence of Ca^{2+}/CaM. (B) Emission spectra of W299 derived from the difference spectrum (L299W − WT) in the absence (—) and presence (•••) of 0.2 mM Ca^{2+} and 2 μM CaM compared with 1 μM L-Trp.

Table 1: Wavelength (nm) of Maximum Fluorescence Emission from Difference Spectra of Calmodulin Kinase IIα

		CaM KIIα residue	
treatment	L-Trp	W299	W293
none	352	350	327
Ca^{2+}/CaM		327	344
Ca^{2+}/CaM with EGTA		349	328
Ca^{2+}/CaM with Mg^{2+}/ATP			327
Ca^{2+}/CaM with Mg^{2+}/ATP and EGTA			342

trum of Ca^{2+}/CaM, which contains no W, is indistinguishable from that of a buffer solution (data not shown), indicating that the resulting spectra are solely due to W in CaM KIIα. The resulting difference spectrum for W299 in the presence of Ca^{2+}/CaM exhibits a significant "blue shift", with a λ_{max} of 327 nm (Figure 2B and Table 1), indicating that W299 now resides in a nonpolar environment. This result provides the first demonstration that the observed interactions between a hydrophobic crevice of Ca^{2+}/CaM and L299 from the CaM KIIα peptide (*15*) are applicable to the intact enzyme. Dissociation of CaM, induced by the addition of the Ca^{2+} chelator EGTA, returned the λ_{max} of W299 to 349 nm (Table 1).

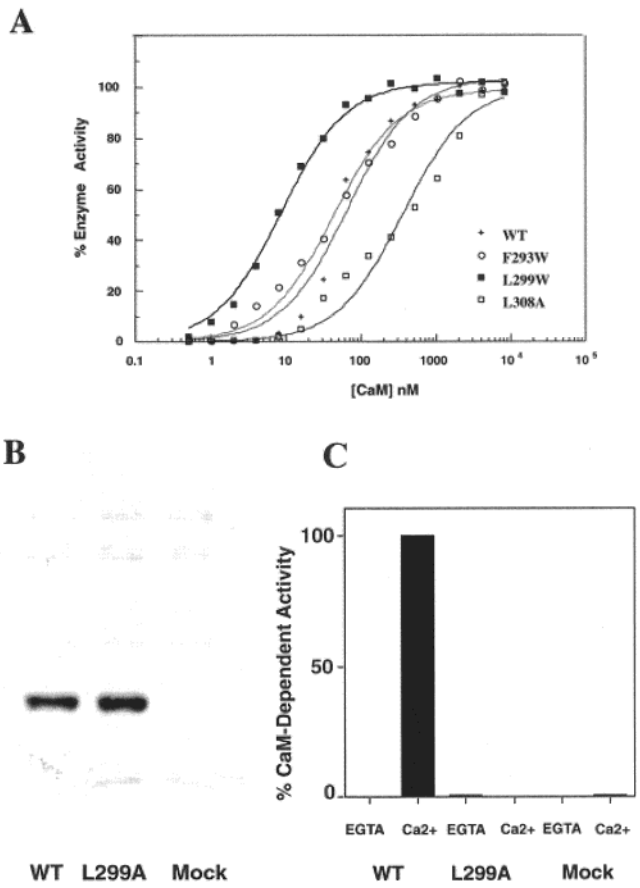

FIGURE 3: Calmodulin-dependent activation of CaM KIIα. (A) Enzymatic activities of wild-type CaM KIIα (WT) and the mutants F293W, L299W, and L308A at increasing concentrations of CaM. The activities of the mutants are expressed as a percentage of the maximal activity of WT at 5 μM CaM (specific activity = 4.4 μmol min^{-1} mg^{-1}). (B) Immunoblot from supernatants of cell extracts containing wild-type (WT)-, L299W-, or mock-transfected cells. (C) Enzymatic activity from mock-, wild-type CaM KIIα (WT)-, and L299A-transfected cell extracts. The activity of the mutant is expressed as a percentage of the activity of the WT at 5 μM CaM.

Contribution of Anchors to the Regulation of CaM KIIα by Ca^{2+}/CaM. As seen in Figure 3A, the L299W mutant was activated to a maximal specific activity similar to that of the WT, providing evidence that the extra W299 does not alter the catalytic function of CaM KIIα. Indeed, L299W required a 4-fold lower concentration of CaM than the WT (10 vs 45 nM) (Figure 3A) to achieve 50% maximal enzyme activity (K_{CaM}). To determine the effect of a smaller hydrophobic anchor in CaM KIIα, an L299A mutant (Figure 3B) was tested for its ability to bind to CaM-Sepharose (*26*). Unlike the WT, the L299A mutant did not bind to CaM-Sepharose (data not shown), indicating a large loss in affinity for Ca^{2+}/CaM due to the mutation. This result was confirmed by enzyme assays of cell extracts containing L299A that were inactive in the presence of up to 5 μM CaM (Figure 3C). In contrast to L299A, a mutant protein of the other CaM binding anchor L308A readily bound to CaM-Sepharose (data not shown). Additionally, L308A exhibited a K_{CaM} of 400 nM which was ~10-fold higher than that of the WT (Figure 3A).

Fluorescence Emission of Tryptophan 293 in CaM KIIα. As previous experiments indicate that residues 291−294 (KKFN) are important for the separate functions of enzyme autoinhibition, CaM-dependent activation, CaM trapping, and CaM-independent activation of full-length CaM KIIα (*17*−

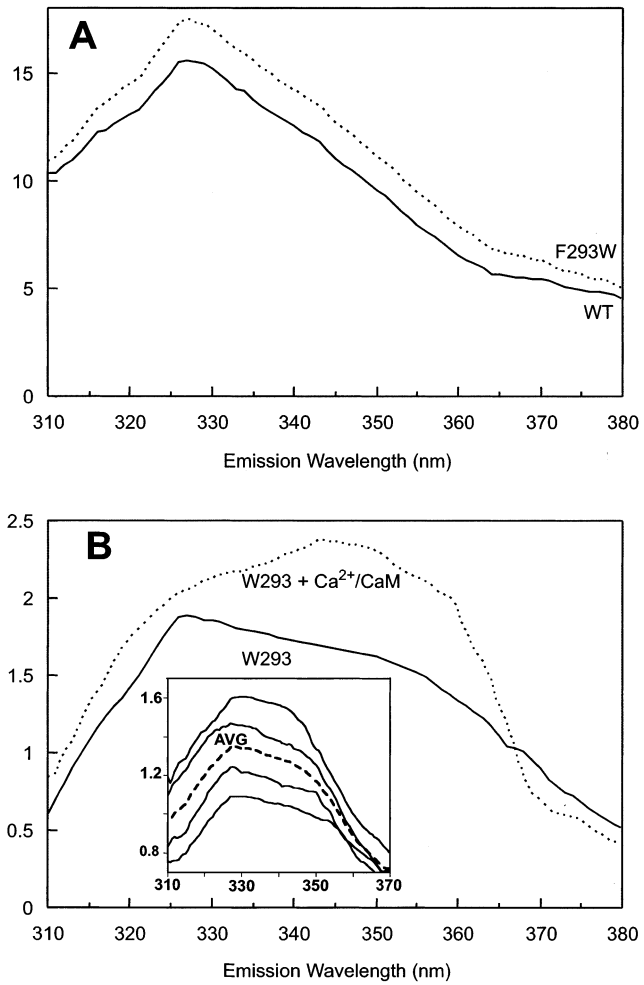

FIGURE 4: Tryptophan fluorescence emission of wild-type CaM KIIα and F293W. Tryptophan residues from 0.8 μM wild-type or F293W mutant CaM KIIα were excited at 297 nm and their fluorescence emission spectra monitored from 310 to 380 nm. Emission spectra of WT CaM KIIα (—) and F293W (•••) in the absence of Ca^{2+}/CaM. Emission spectra of W293 derived from the difference spectrum (F293W − WT) in the absence (—) and presence (•••) of 0.2 mM Ca^{2+} and 2 μM CaM. The inset shows the difference spectra of W293 (F293W − WT) in the absence of CaM derived at concentrations of F293W 1-, 1.03-, 1.07-, and 1.1-fold that of the WT. The averaged difference spectrum (- - -) is also shown.

19), we analyzed a F293W mutant of CaM KIIα. Catalytic activity measurements indicated that the F293W mutant behaved like the WT enzyme as it was inactive in the absence of Ca^{2+}/CaM and exhibited a similar K_{CaM} (Figure 3A). Similarly, the fluorescence emission spectrum of the F293W protein exhibited a λ_{max} identical to that of the WT (Figure 4A), demonstrating that the introduction of W293 had little effect on the general conformation of CaM KIIα. The difference spectrum (F293W − WT) contrasted with that of L-Trp, however, with a lower λ_{max} of 327 nm for W293 (Figure 4B and Table 1). Indeed, in experiments where up to 10% more mutant L293W protein was present than WT, the identical λ_{max} of 327 nm was maintained even though the fluorescence intensity (*F*) varied (inset of Figure 4B). These results provide evidence of an interaction between F293 and the kinase domain that is involved in autoinhibition of the full-length enzyme.

We next compared the W fluorescence spectra of WT and the F293W mutant in the presence of Ca^{2+}/CaM. The resulting difference spectrum exhibited a shift in the λ_{max} of W293 to 344 nm. However, W293 did not achieve the λ_{max} of 352 nm observed for L-Trp, indicating that the Ca^{2+}/CaM-induced position of W293 is not fully solvent accessible (Figure 4B and Table 1). Indeed, a careful inspection of the spectrum also reveals the presence of a shoulder at 327 nM, providing evidence for another conformation of W293 that does not respond to CaM binding. Regardless, these results provide the first experimental evidence that the binding of Ca^{2+}/CaM to the CaM binding domain is coupled to the displacement of the adjacent core autoinhibitory F293. As in the case of W299, the dissociation of CaM by addition of EGTA reversed the effect of Ca^{2+}/CaM on λ_{max} (Table 1) by allowing F293 to revert to its original position.

Fluorescence Emission of Tryptophan 293 in Phosphorylated CaM KIIα. Western blot analysis with an antibody specific for phospho-T286 reveals that Ca^{2+}/CaM and Mg^{2+}/ATP promote autophosphorylation of T286 (Figure 5A) that leads to autonomous activity in both the WT and the F293W mutant, but not in the T286V mutant (Figure 5B). We examined the effects of T286 phosphorylation on W293 by analyzing the difference spectrum of F293W and WT derived in the presence of Ca^{2+}/CaM and Mg^{2+}/ATP. Under these conditions, the λ_{max} of W293 changed from 344 nm in the absence of Mg^{2+}/ATP to 327 nm after autophosphorylation (Figure 5C and Table 1). In contrast, the addition of the nonhydrolyzable ATP analogue, AMP-PNP, instead of ATP did not induce a similar change in the λ_{max} of W293 (inset of Figure 5C). These results provide evidence that autophosphorylation of T286 induced the transfer of W293 to a more hydrophobic environment possibly due to an intersubunit interaction within the holoenzyme, or as a consequence of an interaction between W293 and CaM.

We then evaluated the W fluorescence emission spectra for WT and F293W in the autonomously active state. After the release of CaM from both the autophosphorylated WT and F293W enzymes, due to the addition of EGTA, the resulting difference spectrum for W293 exhibited a λ_{max} of 344 nm (Figure 5C and Table 1), indicating the exposure of W293 in the autonomously active state. Additional scrutiny of the spectra of W293 in either the CaM-trapped or autonomous state (Figure 5C) reveals the existence of a shoulder at 352 nm which might indicate the presence of a another conformation for W293 that is even more solvent accessible. However, the presence of the λ_{max} at 344 nm demonstrates that dissociation of CaM from the autonomously active enzymes returns W293 to a solvent accessible position that is more similar to that induced by Ca^{2+}/CaM-dependent binding (Table 1). Furthermore, the externalization of W293 prompted by the release of CaM supports the idea that W293 interacts with CaM in the prior "trapped" state.

Investigation of Mechanisms that Are Responsible for Autonomous Activity. Although phosphorylation T286 might exert effects on autonomous activity at least in part by repulsing hydrophobic groups on its own kinase domain (*14*), an additional mechanism for generating autonomous activity might involve a change in the conformation of the polypeptide backbone between T286 and F293 that would prevent autoinhibitory interactions with the kinase domain. The most recent molecular model of autoinhibited CaM KIIα shows residues 283−293 in a helical conformation (*14*), where T286 and F293 interact with a complementary surface from the

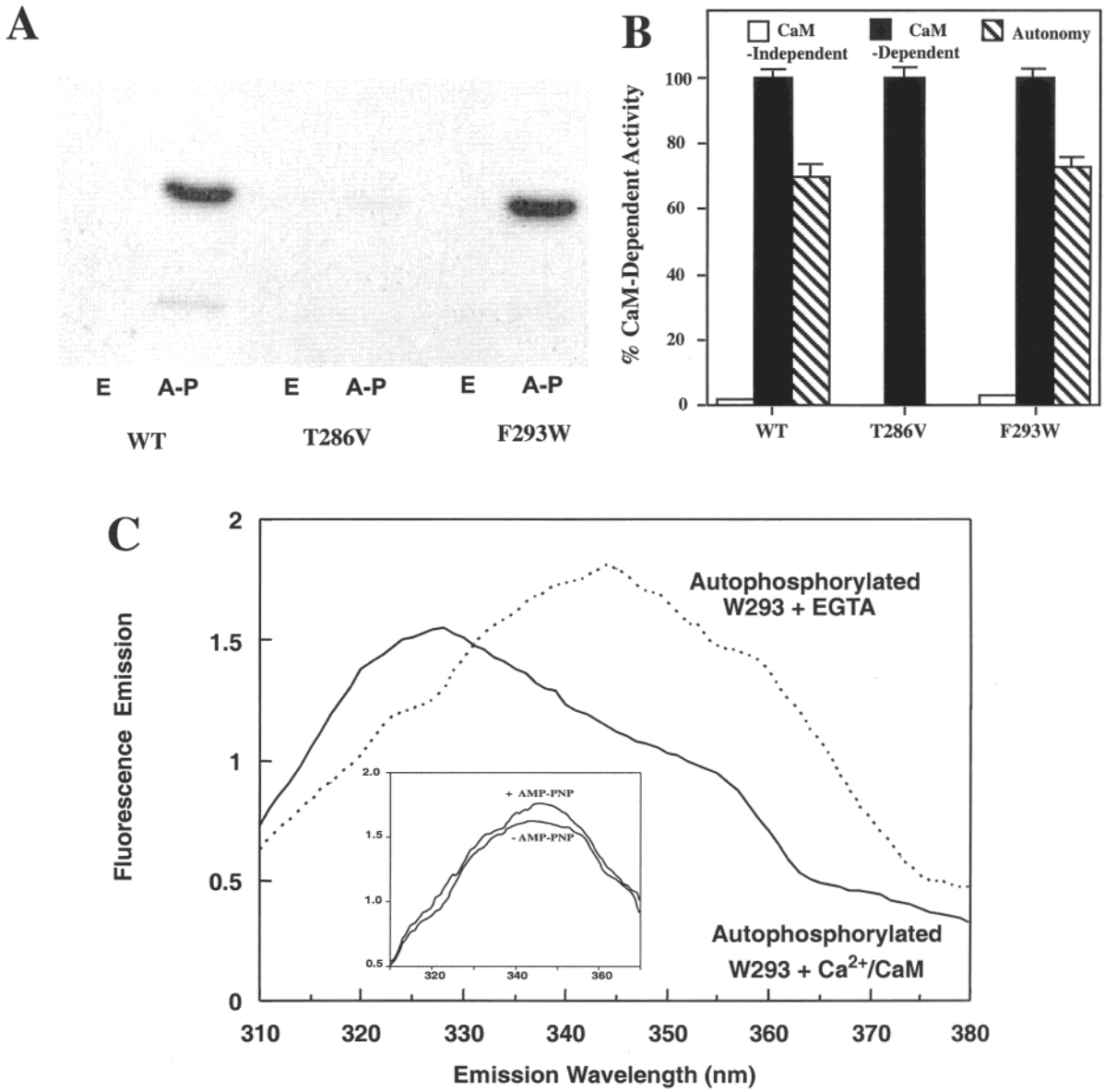

FIGURE 5: Autophosphorylation and EGTA regulate the conformation of W293. (A) Immunoblot of phospho-T286 from WT, T286V, and F293W CaM KIIα, in the presence of EGTA (E) and after autophosphorylation of T286 (A-P) due to the addition of Ca^{2+}/CaM and Mg^{2+}/ATP. (B) CaM-independent and autonomous enzymatic activities of WT, T286V, and F293W CaM KIIα. The activities of the mutants are expressed as a percentage of WT at 5 μM CaM. (C) Difference emission spectra (F293W − WT) of W293 from autophosphorylated WT and F293W CaM KIIα in the presence of 0.2 mM Ca^{2+} and 2 μM CaM with 1 mM Mg^{2+} and 10 μM ATP (—) and after dissociation of CaM by the addition of 5 mM EGTA (•••). The inset shows difference emission spectra (F293W − WT) of W293 from CaM KIIα in the presence of 0.2 mM Ca^{2+} and 2 μM CaM (-AMP-PNP) and after the addition of 1 mM Mg^{2+} and 10 μM AMP-PNP (+AMP-PNP).

kinase domain. This is represented in an α-helical wheel diagram of residues 286−294 that place T286 and F293 on the same helical surface (Figure 6A). The diagram also reveals that the peptide bond nitrogen of L290 is located on the same helical surface as T286 and F293.

Model α-helices of residues 286−294 suggest that the L290P (trans) substitution mutant bends the helical axis perpendicular to (away from) the surface of T286, L290, and F293 (Figure 6A). Such a conformational change could potentially disrupt interactions between autoinhibitory F293 and the kinase domain to produce high levels of CaM-independent activity. In contrast, the peptide bonds of residues C289 and K291 are predicted to be rotated 100° to either side of the helix, relative to L290 (Figure 6A), such that a P at these positions would bend the helical axis in a plane parallel to the facing catalytic domain and, therefore, does not disrupt autoinhibitory interactions to the same extent as L290P.

To test the hypothesis that a change in the conformation of the helical backbone near L290 might induce CaM-independent activity in CaM KIIα, residue 289, 290, or 291 was individually changed to P. The C289P mutant has been previously characterized (*11*) but is presented here for purposes of comparison. As the L290P protein was expressed to a much lower extent (<5% total WT enzyme activity in cell extracts) in abnormally appearing cells and was unable to be isolated in sufficient quantities, all three mutant proteins were compared to the WT after partial purification by gel filtration and anion exchange chromatography. Enzymatic activity measurements show that the three P mutants were regulated by Ca^{2+}/CaM (Figure 6B), but in contrast to the WT, the mutant proteins exhibited varying degrees of Ca^{2+}/

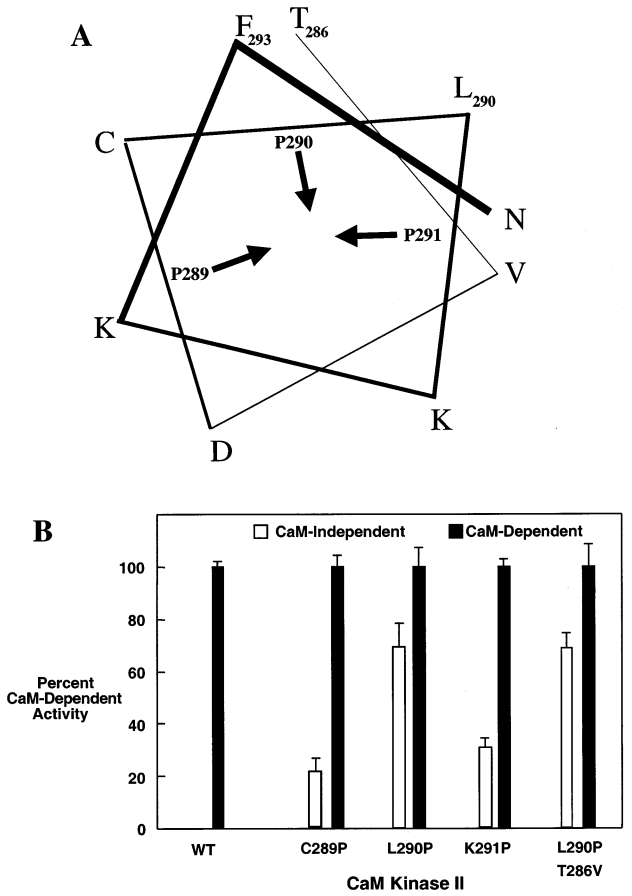

FIGURE 6: CaM-independent activities of CaM KIIα with individual proline mutations. (A) α-Helical wheel representation of residues 286−294 (TVDCLKKFN). The top of the helix comprised of the T286 and F293 side chains faces the catalytic domain of CaM KIIα. Arrows show the directions in which prolines at positions 289−291 change the helical axis. (B) CaM-independent enzymatic activities in 2 mM EGTA of partially purified CaM KIIα with proline mutations at C289, K291, or L290 with or without T286V, expressed as a percentage of their CaM-dependent activity at 1 mM Ca^{2+} and 5 μM CaM.

CaM-independent activity. Furthermore, whereas the CaM-independent activities of P289 and P291 were 23−35% of that observed in the presence of Ca^{2+}/CaM, the L290P mutant exhibited a higher level of CaM-independent activity (65%). To determine whether L290P required phosphorylation of T286 for CaM-independent activity, a T286V mutation was combined with L290P. Cells transfected with this double mutant exhibited an abnormal appearance similar to that of those expressing L290P, and in addition, the partially purified T286V/L290P protein showed a high level of CaM-independent activity comparable to that of the L290P mutant (Figure 6B). These results demonstrate that a change in the conformation of the main chain at L290 is sufficient for recapitulation of autonomous activity.

DISCUSSION

Although hydrophobic interactions between CaM and its partners are believed to be important for transducing Ca^{2+} signals, there have been few attempts to investigate the roles of specific hydrophobic groups from the CaM binding domain of an effector protein, such as CaM KIIα, in its regulation by CaM. X-ray crystallographic and NMR structural analyses reveal that CaM responds to Ca^{2+} signals by exposing hydrophobic groups in its two domains (*27*). We have previously shown that several of these hydrophobic residues in CaM are important for binding and activation of CaM-regulated enzymes such as CaM KIIα (*21*). In this study, we provide evidence that Ca^{2+}/CaM interacts with, and alters the conformation of functionally important hydrophobic residues from, CaM KIIα. We took advantage of the lack of W in both the autoregulatory domain of CaM KIIα and CaM to follow changes in the environment of individual W mutant side chains by analyzing the W fluorescence emission difference spectra derived from WT and mutant forms of CaM KIIα. As we report, the solvent accessibility and hydrophobicity of the N-terminal anchor L299 is particularly important for targeting CaM KIIα to Ca^{2+}/CaM. The fact that the second anchor, L308, exerts comparatively less influence emphasizes the functional differences between these two anchor residues as defined by the crystal structure of the Ca^{2+}/CaM−peptide complex (*15*).

Two related mechanisms are involved in the Ca^{2+}/CaM-dependent activation of CaM KIIα. One involves the conventional form of regulation at low subunit occupancy by Ca^{2+}/CaM whereby the enzyme is activated or autoinhibited due to the binding or release of CaM. The second or autonomous form of activation occurs when two subunits of CaM KIIα are simultaneously occupied by Ca^{2+}/CaM to allow the phosphorylation of one CaM-bound "substrate" subunit at T286 by a spatially adjacent CaM-bound "kinase" subunit followed by the release of CaM from the substrate subunit (*3*). Recent results from chemical quench flow studies combined with dynamic light scattering experiments provide evidence for such an intersubunit interaction (*28*). Our experiments showing the Ca^{2+}/CaM-induced exposure of the internal W293 suggest that the binding of Ca^{2+}/CaM to the CaM binding domain of CaM KIIα is directly linked to the displacement of the adjacent core autoinhibitory residue F293. This conventional mechanism of activation by Ca^{2+}/CaM might also apply to other CaM kinases which contain a core autoinhibitor similar to that of CaM KIIα (*29*). Furthermore, the exposure of W293 in the autonomously active enzyme is consistent with the observation that the removal of F293 is common to both Ca^{2+}/CaM-dependent and CaM-independent activation of CaM KIIα.

The differing effects of EGTA on the CaM-bound enzyme provide insight into the mechanisms underlying these two ways of activating CaM KIIα. The reversal of W293 in the absence of phosphorylation agrees with the situation when a potential substrate subunit is not phosphorylated, due to the low occupancy of adjacent Ca^{2+}/CaM-activated kinase subunits, and is reversibly inactivated by the release of CaM. Conversely, the exposure of W293 from the T286 autophosphorylated enzyme after release of CaM agrees with the generation of autonomous activity in a substrate subunit that has a high Ca^{2+}/CaM occupancy of adjacent kinase subunits. These results provide additional support for the hypothesis that displacement of F293 from the kinase domain is an important feature of CaM-independent activation of CaM KIIα.

The exposure of W293 in the autonomously active enzyme indicates that phosphorylation of T286 affects residues on the autoregulatory domain, such as F293, that are critical for autoinhibition and CaM-independent activity. The effect

of the L290P mutant on CaM-independent activity identifies a regulatory site on the polypeptide backbone that provides a potential link between T286 phosphorylation and the retention of autonomous activity. In addition, a class of electrostatic interactions between backbone amide groups at the N-termini of helices and a phosphate group, sometimes called a helix dipole−phosphate interaction, have been observed in several proteins (*30*). It is tempting to suggest that this type of electrostatic interaction may represent a mechanism for maintaining autonomous activity in CaM KIIα whereby autophosphorylation of T286, due to CaM binding, places a negatively charged phosphate group in the proximity of main chain amides, such as at L290, to allow an interaction with the N-terminal (positive) dipole of the helical CaM binding domain. Such an interaction might sufficiently alter the conformation of the autoinhibitory domain to maintain F293 in an exposed position and generate autonomous activity even after dissociation of CaM.

Whereas autonomous activity is defined by the release of CaM from CaM KIIα, the immediate effect of autophosphorylation at T286 is an ~1000-fold higher affinity or trapping of Ca^{2+}/CaM (*6*). Our results provide a mechanism for Ca^{2+}/CaM trapping by indicating that autophosphorylation induces the internalization of W293 that was previously exposed to solvent due to the prior Ca^{2+}/CaM binding event. Evidence in favor of an interaction between W293 and Ca^{2+}/CaM in the trapped state is provided by the subsequent exposure of W293 due to the release of CaM. Such an interaction in the trapped state would further promote activation of CaM KIIα by providing an alternative binding site for W293 with Ca^{2+}/CaM instead of interacting with its own kinase domain. This additional hydrophobic interaction with Ca^{2+}/CaM would contribute to the binding energy for trapping.

The internalization of W293 after autophosphorylation also implies that disruption of the interactions between CaM and F293 should impair trapping. Indeed, mutations of either F293 in full-length CaM KIIα or the complementary binding surface on CaM, which includes E120 and M124, interfere with Ca^{2+}/CaM trapping (*31*). M124 of CaM is particularly noteworthy as it simultaneously interacts with the critical L299 anchor residue in the CaM binding domain, and previous experiments with CaM have implicated M124 in the binding and activation of CaM KIIα (*21*). The convergence of L299 and F293 from CaM KIIα with M124 from CaM identifies a potential "hot spot" on the Ca^{2+}/CaM−CaM KIIα interface (*15*) that is responsible for stabilizing both the low- and high-affinity complexes. This interpretation implies that the crystal structure of the Ca^{2+}/CaM−CaM KIIα peptide complex represents the trapped form rather than the lower-affinity state of the complex.

The suggestion that phosphorylation of T286 might regulate both autonomous activity and trapping, at least in part by changing the conformation of W293, implies that the same phosphate−dipole interaction might also promote the observed trapping interaction of W293 with Ca^{2+}/CaM. Although autonomous activation of CaM KIIα and CaM trapping could be regulated by distinct mechanisms, a common mechanism has the additional advantage of explaining how autonomous activity and CaM trapping are observed in both full-length CaM KIIα and fragments of the enzyme lacking the C-terminal association domain (*6*, *7*). However, in contrast to enzyme fragments, the mutants of the full-length proteins that interfere, to varying degrees, with CaM function still retain their respective C-terminal domains that are responsible for oligomerization and subcellular localization. Our study also suggests that these proteins that exhibit significantly elevated levels of CaM-independent activity might have profound consequences on cellular function, as indicated by the abnormal appearance of 293 cells expressing CaM KIIα bearing the L290P mutation.

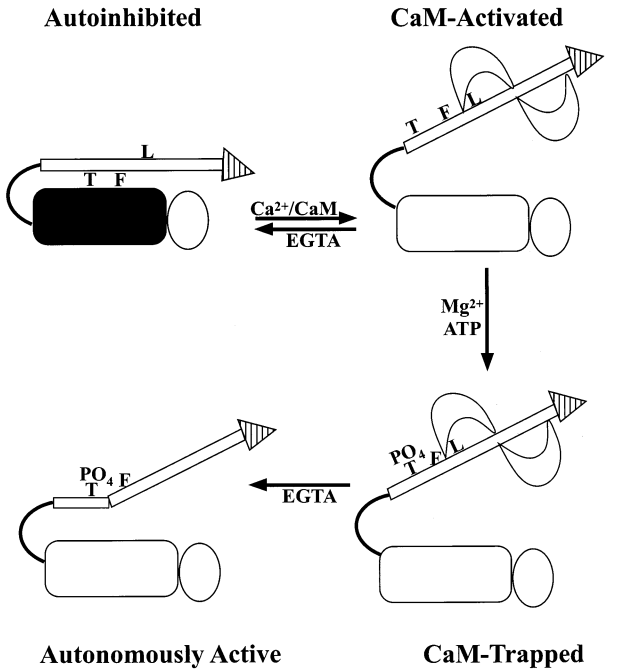

FIGURE 7: Mechanisms for activation of CaM KIIα. A CaM KIIα subunit is depicted as being comprised of a bilobal kinase domain, an autoregulatory domain represented by a rectangular bar, and an association domain shown as a striped triangle. In the Autoinhibited panel, the kinase domain is inactive (black) due to its interaction with the autoinhibitory domain. The relative positions of T286 (T), F293 (F), and L299 (L) are indicated. In the CaM-Activated panel, the C-domain of Ca^{2+}/CaM, represented by the two crescent-shaped domains, binds to L299. This activates the kinase (white) by exposing the inhibitory F293 and the phosphorylation site at T286. Dissociation of CaM reverses this process. In the CaM-Trapped panel, phosphorylation of T286 promotes the interaction of F293 with Ca^{2+}/CaM. In the Autonomously Active panel, dissociation of CaM from phosphorylated CaM KIIα exposes F293 to maintain activity in the kinase.

In conclusion, activation of full-length CaM KIIα involves a series of closely linked steps (Figure 7). Formation of a complex between Ca^{2+}/CaM and CaM KIIα is promoted by the internalization of complementary, solvent accessible, hydrophobic groups such as the L299 anchor with the C-terminal domain of Ca^{2+}/CaM. This induces a sequestration of the helical CaM binding domain of CaM KIIα by Ca^{2+}/CaM, which promotes a conformational change that disrupts the interaction between F293 in the core autoinhibitory sequence and the kinase domain. As in the deletion experiments (*17*, *18*), the removal of both the CaM binding and core autoinhibitory sequences is sufficient to activate the enzyme. In the absence of autophosphorylation at T286, the resulting exposure of the kinase domain still activates the enzyme, by a process which is reversed by the dissociation of CaM. However, upon phosphorylation of T286, the activated form of the enzyme is favored by inducing a trapping interaction of F293 with Ca^{2+}/CaM. The control of

F293 by the phosphate at T286 has a dual role. It not only prevents the autoinhibitory interaction of F293 with the kinase domain but also promotes autonomous activity by retaining F293 in an exposed position after release of CaM. Thus, the theme of internalization and exposure of hydrophobic groups in response to the binding of Ca^{2+} by CaM recurs in the regulation of CaM KIIα by Ca^{2+}/CaM and autophosphorylation of T286.

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REFERENCES

1. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) *Nat. Rev. Mol. Cell Biol. 1*, 11−20.
2. Chin, D., and Means, A. R. (2000) *Trends Cell Biol. 10*, 322−328.
3. De Koninck, P., and Schulman, H. (1998) *Science 279*, 227−230.
4. Malenka, R. C., and Nicoll, R. A. (1999) *Science 285*, 1870−1874.
5. Kolodziej, S. J., Hudmon, A., Waxham, M. N., and Stoops, J. K. (2000) *J. Biol. Chem. 275*, 14354−14359.
6. Meyer, T., Hanson, P. I., Stryer, L., and Schulman, H. (1992) *Science 256*, 1199−1202.
7. Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) *Neuron 12*, 943−956.
8. Giese, K. P., Fedorov, N. B., Filipkowski, R. K., and Silva, A. J. (1998) *Science 279*, 870−873.
9. Waldmann, R., Hanson, P. I., and Schulman, H. (1990) *Biochemistry 29*, 1679−1684.
10. Smith, M. K., Colbran, R. J., Brickey, D. A., and Soderling, T. R. (1992) *J. Biol. Chem. 267*, 1761−1768.
11. Mukherji, S., Brickey, D. A., and Soderling, T. R. (1994) *J. Biol. Chem. 269*, 20733−20738.
12. Brickey, D. A., Bann, J. G., Fong, Y.-L., Perrino, L., Brennan, R. G., and Soderling, T. R. (1994) *J. Biol. Chem. 269*, 29047−29054.
13. Mukherji, S., and Soderling, T. R. (1995) *J. Biol. Chem. 270*, 14062−14067.
14. Yang, E., and Schulman, H. (1999) *J. Biol. Chem. 274*, 26199−26208.
15. Meador, W. E., Means, A. R., and Quiocho, F. A. (1993) *Science 262*, 1718−1721.
16. Goldberg, J., Nairn, A. C., and Kuriyan, J. (1996) *Cell 84*, 875−887.
17. Yamagata, Y., Czernik, A. J., and Greengard, P. (1991) *J. Biol. Chem. 266*, 15391−15397.
18. 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.
19. Waxham, M. N., Tsai, A.-l., and Putkey, J. A. (1998) *J. Biol. Chem. 273*, 17579−17584.
20. Feinmesser, R. L., Wicks, S. J., Taverner, C. J., and Chantry, A. (1999) *J. Biol. Chem. 274*, 16168−16173.
21. Chin, D., and Means, A. R. (1996) *J. Biol. Chem. 271*, 30465−30471.
22. Feinmesser, R. L., Gray, K., Means, A. R., and Chantry, A. (1996) *Oncogene 12*, 2725−2730.
23. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
24. Chin, D., Schreiber, J. L., and Means, A. R. (1999) *Biochemistry 38*, 15061−15069.
25. Callis, P. R. (1997) *Methods Enzymol. 278*, 113−115.
26. Yokokura, H., Piccioto, M. R., Nairn, A., and Hidaka, H. (1995) *J. Biol. Chem. 270*, 23851−23859.
27. Zhang, M., Tanaka, T., and Ikura, M. (1995) *Nat. Struct. Biol. 2*, 758−767.
28. Bradshaw, J. M., Hudmon, A., and Schulman, H. (2002) *J. Biol. Chem. 277*, 20991−20998.
29. Chin, D. (2000) Calcium Signaling. *CRC Methods in Signal Transduction*. (Putney, J., Jr., Ed.) pp 297−319, CRC Press, Boca Raton, FL.
30. Hol, W. G. (1985) *Prog. Biophys. Mol. Biol. 45*, 149−195.
31. Singla, S. I., Hudmon, A., Goldberg, J. M., Smith, J. L., and Schulman, H. (2001) *J. Biol. Chem. 276*, 29353−29360.

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