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Regulation of intrasteric inhibition of the multifunctional calcium/calmodulin-dependent protein kinase

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ABSTRACT **A regulatory region involved in both autoinhibition and calmodulin (CaM) binding has previously been identified in the multifunctional Ca^{2+}/CaM-dependent protein kinase (CaM kinase II). We have tested the role of various segments of the regulatory region in autoinhibition by the analysis of a series of truncation, substitution, and deletion mutants of the CaM kinase II α subunit (CaM kinase IIα). Unexpectedly, the sequence Lys-Lys-Phe-Asn at positions 291–294, adjacent to the CaM binding domain, was found to be sufficient to maintain an inhibited state in a truncated form of the kinase. However, these residues are not essential in the context of the full-length protein, indicating the importance of additional residues from the overlapping CaM binding domain. We propose here a molecular model for CaM kinase IIα based on the three-dimensional structure of the cAPK–PKI-(5–24) (protein kinase inhibitor fragment) complex. It is predicted from this model that autoinhibition is of the pseudosubstrate variety and that autophosphorylation of Thr-286 could occur by an intersubunit reaction in the holoenzyme complex.**

Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaM kinase II) is one of several serine/threonine protein kinases known to be maintained in an inactive state by the interaction of the catalytic region with an autoinhibitory domain located on the same polypeptide (1–4). These enzymes can be activated by limited proteolysis, which removes the autoinhibitory domain. In the cAMP-dependent protein kinase (cAPK) (5), the inactive state of the holoenzyme is maintained by an inhibitory amino acid sequence located on the regulatory subunit. Upon dissociation of the regulatory subunit, the enzyme can also be inhibited by interaction with the thermostable protein kinase inhibitor (PKI). Synthetic peptides derived from PKI have provided evidence that inhibition occurs by the interaction of a pseudosubstrate sequence on the PKI with the active site. This pseudosubstrate type of inhibition is also the mechanism involved in the intrasteric regulation of MLCK and PKC and has now been proposed for CaM kinase II (1–4, 6).

CaM kinase II, the most abundant protein kinase in brain tissue (7), is a multimer of 10–12 catalytic subunits. All subunit types described to date (α, β, β', γ, δ) have an NH_2-terminal catalytic domain containing the conserved blocks of homology found in serine/threonine kinases (8), a central regulatory domain, and a COOH-terminal domain responsible for the association of the subunits into a multimer. Upon activation by Ca^{2+}/CaM, CaM kinase II undergoes rapid autophosphorylation. In the α subunit, the predominant type in forebrain, this occurs by phosphorylation of Thr-286 (9). This event is responsible for the development of an autonomous catalytic activity that is maintained even after removal of Ca^{2+}/CaM from the enzyme.

Efforts have been made to map the regulatory region of CaM kinase II. Our studies showed that residues 291–294 were required for a synthetic peptide analog to inhibit the Ca^{2+}/CaM-independent form of the kinase (10). Other groups revealed that inhibitory potency could be enhanced by using longer peptides containing Thr-286 (11–13). Site-directed mutagenesis studies have predominantly been restricted to attempts to mimic the effect of autophosphorylation by replacement of Thr-286 and its neighboring residues with negatively charged amino acids (14–16). Since none of these mutations resulted in complete autonomous activity, other residues within the autoinhibitory domain must be involved in establishment of the autoinhibitory state.

For this study of residues of the regulatory region responsible for autoinhibition we generated a series of truncation, substitution, and deletion mutants of the α subunit of CaM kinase II and measured their Ca^{2+}/CaM-independent activity. We show that residues 291–294 are sufficient to impart autoinhibition to a truncated form of the kinase and that, in the context of the full-length enzyme, amino acids involved in binding CaM also may participate in autoinhibition. In addition, a molecular model is proposed for the three-dimensional structure of a truncated form of CaM kinase IIα based on similarities to the structure of the cAPK complexed to its inhibitor peptide.

METHODS

Site-Directed Mutagenesis and Expression of Mutants. A cDNA encoding rat brain CaM kinase IIα (17) was introduced into either pET3a (18) or pT7-7 (19) after generation of a *Nde* I site at the translation initiation codon by site-directed mutagenesis (20). All mutants were made by the same method. The sequence of oligonucleotides used for generation of truncation ("stop"), deletion (del.), and substitution (subst.) mutants are available upon request. The resulting constructs, pETCKα and pT7-CaMk, allowed the expression of proteins in *Escherichia coli* by induction of T7 RNA polymerase or by *in vitro* transcription/translation. Purified DNA from clone pETCKα and each one of the mutants was transcribed as described (22). For *in vitro* translation reactions, 1–3 μg of CaM kinase IIα transcript was used in a 50-μl reaction containing 35 μl of rabbit reticulocyte lysate (Promega) and 25 μCi (925 kBq) of [^{35}S]methionine (22). Expression of translated mutant proteins relative to wild type (wt) was compared by the intensity of radiolabeled products on SDS/PAGE. For measurement of kinase activity, identi-

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Abbreviations: CaM, calmodulin; CaM kinase II, Ca^{2+}/CaM-dependent protein kinase; cAPK, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; MLCK, myosin light chain kinase; wt, wild type.

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cal reactions were carried out with unlabeled methionine. RNA was omitted from control reactions.

CaM Overlays, Immunoblots, and Kinase Assays. To visualize expressed proteins, 5 μl of labeled translation reactions were loaded on 10% polyacrylamide gels, autoradiographed, and quantified by laser scanning. For Western blots, *E. coli* cells carrying T7 polymerase-driven expression constructs (Fig. 1) of each mutant were induced to express CaM kinase IIα by infection with λ phage carrying T7 polymerase. Western immunoblots (22) using a polyclonal antibody specific for the α subunit and ^{125}I-labeled CaM overlays of crude bacterial lysates were carried out as described (23). Kinase activity was measured as described (22, 24) by phosphorylation of a synthetic peptide derived from glycogen synthase (Pro-Leu-Arg-Arg-Thr-Leu-Val-Ala-Ala) (25) in 50 μl of reaction mixture (50 mM Hepes, pH 7.5/50 mM $Mg(OAc)_2$/1 μM CaM/50 μM ATP/0.2 mM dithiothreitol/50 μM peptide substrate/0.2 μg of cAPK inhibitor per ml/20 μCi of [γ-^{32}P]ATP) per reaction. Aliquots (10 μl) were applied directly to a P81 phosphocellulose filter and washed with 75 mM phosphoric acid. After drying, the filters were counted in scintillation fluid. Reactions were done in duplicate. Typically, a 4- to 5-fold increase in kinase activity was observed in a translation reaction containing CaM kinase IIα mRNA compared with no RNA.

Molecular Modeling of CaM Kinase IIα. The coordinates of the cAPK–PKI-(5–24) complex crystal structure were kindly provided by Susan Taylor (University of California at San Diego). Modeling was carried out with the Sybyl 5.4 package (Tripos Associates, St. Louis) on an Evans–Sutherland system. Replacement of side chains and construction of additional chains were carried out with the BIOPOLYMER program. Alignment of cAPK and CaM kinase IIα amino acid sequences was carried out with the BESTFIT program (GCG package).

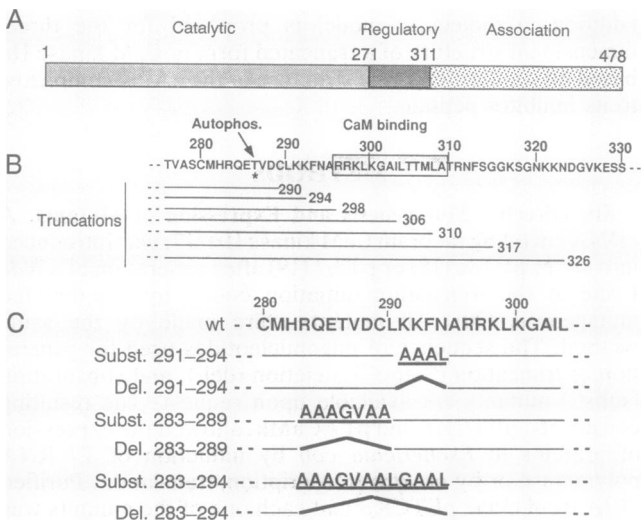

FIG. 1. Organization of the CaM kinase IIα and mutants. CaM kinase IIα (17) is composed of three major regions: a catalytic region (1–270) that contains the 11 blocks of homology found in protein kinases (8), a central regulatory region responsible for CaM binding and autoinhibition (271–311) (26), and a COOH-terminal region involved in association of subunits. (312–474) (27). COOH-terminal truncations were made that maintained the catalytic region intact. Substitutions were made in the context of the full-length kinase with new residues underlined. The amino acids used for substitutions were chosen to avoid the introduction of charged or bulky side chains. In some cases, glycine residues were used to impart flexibility to the structure. Deletions are shown as angled lines. Autophos., autophosphorylation (site indicated by asterisk).

RESULTS AND DISCUSSION

To study its regulatory region, a series of truncation, substitution, and deletion mutants of the rat brain CaM kinase IIα were created (Fig. 1), and each one of the mutants was tested for its ability to bind Ca^{2+}/CaM as well as both Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent kinase activity. For expression of mutated proteins, we used expression vectors driven by T7 polymerase which allowed production of the wild-type (wt) and mutant proteins either in *E. coli* or by *in vitro* transcription/translation. However, since bacterial expression of CaM kinase IIα invariably yields also a fragment of about 33 kDa (11, 28), we preferred the *in vitro* translation system as a source of enzyme for our kinase assays and used bacterial expressed proteins only in CaM-overlay assays.

As predicted from previous studies of synthetic peptides suggesting that residues 296–309 are important for Ca^{2+}/CaM binding (6), truncation mutants ending at positions 306, 298, 294, and 290 did not bind Ca^{2+}/CaM, while longer versions of the enzyme terminating at 317 and 326 or the full-length polypeptide bound Ca^{2+}/CaM (Fig. 2*B*). No high-affinity binding was detected in the mutant ending at residue 310, suggesting that Arg-311 corresponds to an arginine shown to be essential for CaM binding in other enzymes (23). As shown on Fig. 2*C*, the holoenzyme or mutants truncated at residues 326 or 317 were activated by binding of Ca^{2+}/CaM, whereas the mutant ending at 310 was only weakly activated (22%) at saturating CaM concentrations. In contrast, truncation mutants ending at 306, 298, and 294 that did not bind CaM exhibited no enzymatic activity in either the presence or absence of Ca^{2+}/CaM. Surprisingly, removal of the sequence Lys-Lys-Phe-Asn at positions 291–294 created

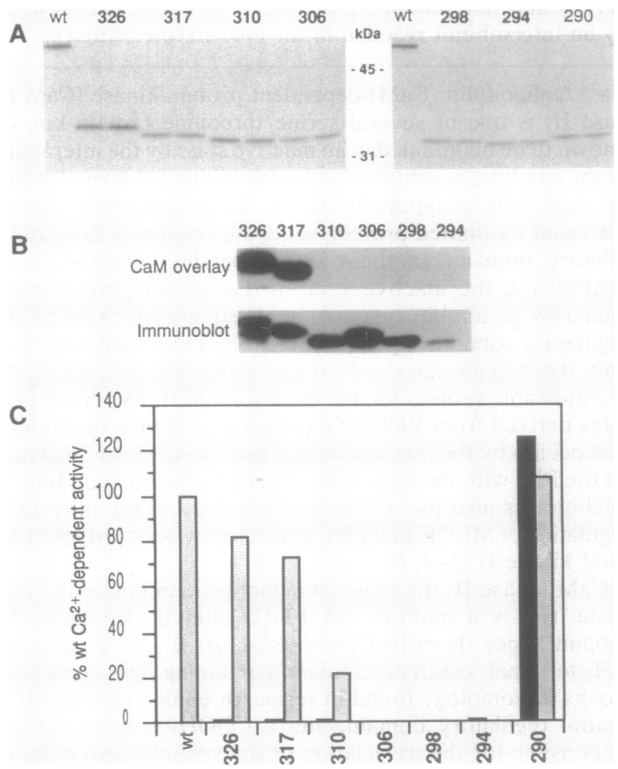

FIG. 2. Expression and analysis of truncation mutants. (*A*) SDS/PAGE of ^{35}S-labeled *in vitro* translated mutants. Duplicate translation reactions are shown. (*B*) ^{125}I-labeled CaM overlay and immunoblot analysis using crude lysates of *E. coli* expressing the CaM kinase IIα polypeptides. (*C*) Phosphorylation of GS-10 peptide substrate in the presence of CaM and Ca^{2+} (gray bars) or EGTA (black bars) as measured with *in vitro* translated proteins as the source of kinases.

a mutant that, while not capable of binding Ca^{2+}/CaM, was constitutively active at levels similar to that of the activated wt enzyme.

Results obtained with the truncation mutants indicate that the COOH-terminal boundary of a minimal autoinhibitory domain lies between residues 291 and 294, NH_2-terminal to the CaM-binding sequence, but COOH-terminal to Thr-286, the autophosphorylation site that confers Ca^{2+}/CaM-independence (9). These data (Fig. 2) concur with the observations that constitutively active fragments can be generated by truncation of the enzyme at residue 282 by site-directed mutagenesis (28) whereas proteolytic digestion can yield an inactive fragment ending at 293 (29), suggesting that, while residues 283–290 are not sufficient to inhibit CaM kinase IIα, the addition of residues 291–293 (or 294) inhibits kinase activity. Alternatively, the lack of activity observed in the mutant truncated at 294 could be explained by improper folding caused by the addition of residues 291–294 to the mutant truncated at 290. However, this is unlikely since the longer truncated versions (Fig. 2) have activities near wt levels. It is noteworthy that a similar constitutively active truncation mutant has been constructed by using the mouse CaM kinase IIα (30) containing the additional sequence Lys-Arg-Pro-Ala-Gly-Met-Gln-Ala at the COOH terminus after Leu-290. The constitutive activity of this mutant suggests that the inhibition by residues 291–294 described here is specific. Finally, the activity of the mutant ending at 290 could not be attributed to phosphorylation of Thr-286 in this protein since mutation of Thr-286 to alanine did not alter the constitutive activity of the enzyme truncated at Leu-290 (not shown).

Having shown that extension of the constitutively active kinase by amino acids 291–294 was sufficient to inhibit catalytic activity, it was of interest to test the requirement for these residues when they were adjacent to the normal CaM binding domain. Therefore, we substituted Ala-Ala-Ala-Leu for the sequence Lys-Arg-Arg-Asn at positions 291–294 in the context of the full-length protein (Fig. 1*C*). Like the enzyme isolated from mammalian brain, the full-length α subunit expressed in *E. coli* formed a multimer, as assessed by gel filtration (not shown). Multimer formation has been shown in other *in vitro* systems in which the α subunit has been expressed such as CHO cells (31) and baculovirus (32). The mutant carrying the substitution at 291–294 was predominantly Ca^{2+}/CaM-dependent with a low level (7%) of independent activity (Table 1). However, disruption of the domain by deletion of these residues resulted in higher constitutive activity (38%). In addition, because of the higher inhibitory potency of synthetic peptides that include residues 283–289 (13, 33), these amino acids were also substituted or deleted. As with the replacement of residues 291–294, mutation of 283–289 resulted in only a slight increase in independent activity, whereas deletion had a larger effect. More drastic changes in enzymatic properties were introduced by replacement or deletion of the entire sequence 283–294. In both cases the resulting mutants showed 95% independent activity, suggesting a nearly complete disruption of intrasteric regulation.

Table 1. Activity of CaM kinase IIα substitution and deletion mutants

Mutant	Phosphorylation activity		
	With Ca^{2+}	With EGTA	% Ca^{2+}-indep.
wt	87.4	0.2	0
Subst. 291–294	77.4	5.41	7
Del. 291–294	301.5	113.6	37.7
Subst. 283–289	197.5	25.5	13
Del. 283–289	65.1	23.1	35.5
Subst. 283–294	26.1	24.9	95
Del. 283–294	64.6	60.3	95

Phosphorylation is expressed as pmol of phosphorylated peptide substrate per min per μg of lysate. Activities were normalized to equal amounts of CaM kinase IIα polypeptide based on the relative expression of these mutants in reticulocyte lysates. Kinase activity endogenous to the lysate (i.e., no CaM kinase IIα RNA added) was subtracted in each case. Percentage of Ca^{2+}-independent activity was calculated from the ratio of activities in the presence of EGTA vs. Ca^{2+}. Subst., substitution; Del., deletion; indep., independent.

That the truncation mutant ending at Leu-290 was unregulated with a total activity similar to that of activated full-length enzyme, while residues 291–294 are sufficient to confer autoinhibition of the truncated kinase but were not essential in the context of the full-length enzyme, suggests that an extended domain beyond Leu-290 could be involved in autoinhibition. The ability of sequences other than residues 291–294 to provide autoinhibition would be consistent with the observation that deletions result in larger increases in independent activity, whereas short substitutions with noncharged residues have milder effects. Thus, residues NH_2-terminal to position 291 would be unlikely to play a direct role in the intrasteric inhibition but would ensure the correct positioning of the autoinhibitory domain. The extended autoinhibitory domain would then encompass Phe-293, followed by separate blocks of basic and hydrophobic residues. This pattern of side chains is reminiscent of that of the peptide inhibitor of cAPK, PKI-(5–24) (Fig. 3). In this peptide, phenylalanine at position 11 [P(−11)] upstream from phosphorylation site P(0) [Phe-(−11); corresponds to Phe-293 of CaM kinase IIα] is critical for high-affinity binding to the catalytic subunit, and substitution of this amino acid decreases affinity by a factor of 100 (34). The importance of Phe-293 and the similar pattern of side chains suggested that the intrasteric autoinhibition of CaM kinase II could have functional and structural similarities to the inhibition of cAPK by the inhibitor peptide. Thus, we prepared a model for CaM kinase IIα based on the three-dimensional structure of the catalytic subunit of cAPK and modeled the autoinhibitory domain according to the coordinates of PKI-(5–24) (35, 36). A similar approach has been used recently to elaborate a model for smooth muscle MLCK (37). Fig. 4 compares the structures of the cAPK–PKI-(5–24) complex with our model for CaM kinase IIα. In Fig. 4*A*, the NH_2-terminal end of cAPK (in magenta) contains a long α-helix (helix A) absent in CaM kinase IIα. Residues 31–296 of cAPK (shown in cyan) represent the core of the enzyme homologous to the catalytic region of CaM kinase IIα (residues 1–270). The last residue conserved in this region is Trp-296 (Trp-270 in CaM kinase IIα) and is shown in yellow. The sequence following Trp-296 is a random coil structure (magenta) with no homology to CaM kinase IIα. The catalytic core is composed of two lobes. The upper lobe, mainly formed by β-strands, is involved in binding ATP (Lys-72 of the phosphate anchor is displayed in blue). The lower lobe, rich in α-helices, is responsible for interacting with the peptide. Within this lobe, residues Glu-

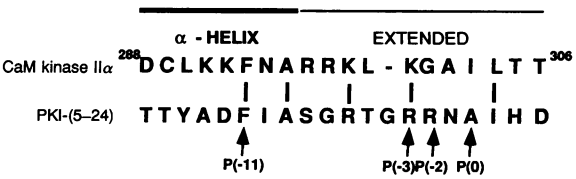

FIG. 3. Comparison of the CaM kinase IIα autoinhibitory domain to PKI-(5–24). Amino acids 288–306 of CaM kinase IIα are aligned with PKI-(5–24). P(0) indicates the position of the alanine residue that occupies the site of phosphorylation in a cAPK substrate. Arginine residues at P(−2) and P(−3) are recognized by acidic residues in the peptide binding site of the kinase.

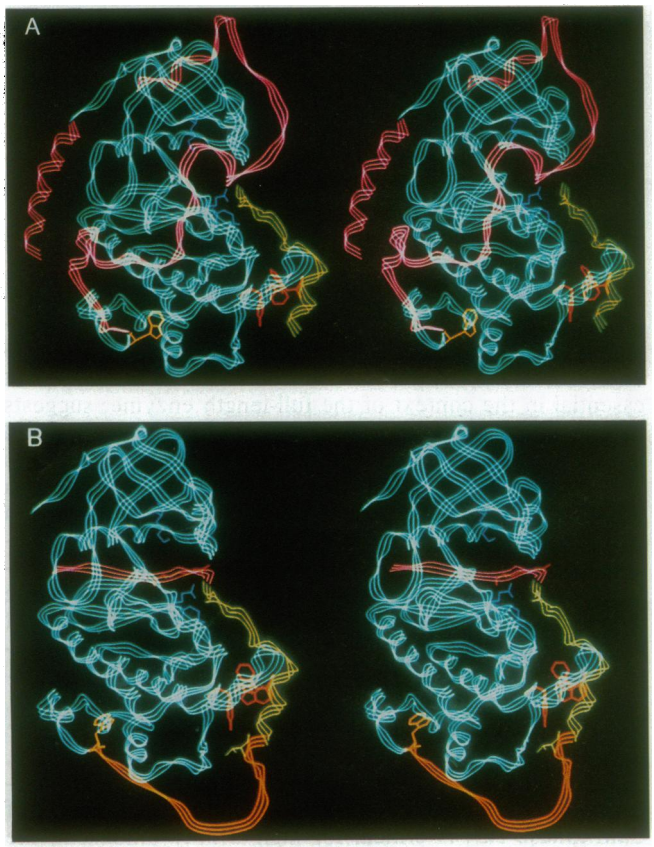

FIG. 4. Molecular modeling of CaM kinase IIα. (*A*) Structure of the cAPK–PKI-(5–24) complex. Colors: Cyan, homologous core catalytic region (residues 35–270); magenta, nonhomologous regions of cAPK; green, PKI-(5–24). (*B*) Molecular model for monomeric CaM kinase IIα (amino acids 1–317). Colors: Cyan, core catalytic domain (1–270); green, Asp-288 to Thr-306 [aligned with PKI-(5–24)]; orange, Ile-271 to Val-287 (connecting peptide in CaM kinase IIα); magenta, residues 306–317 (COOH end of the CaM binding domain). Side chains of several important residues are shown colored: yellow, Trp-270, the last residue of the core catalytic domain; green, Thr-286, autophosphorylation site (facing the inner side of the enzyme); blue, residues forming the ATP binding loop (Lys-72 and -42) and the catalytic loop (Glu-127 and -96, Glu-170 and -139) in cAPK and CaM kinase IIα. The coordinates for this model are available upon request.

127 and -170 are involved in recognizing the basic residues found in the consensus cAPK substrates [positions P(−3) and P(−2), respectively]. The inhibitor peptide (in green) is composed of an NH_2-terminal five-residue α-helix containing Phe-(−11) (displayed in orange), followed by an extended structure containing the pseudosubstrate sequence. Phe-(−11) interacts with a hydrophobic pocket between helices F and G of the kinase, formed by Tyr-210 and Phe-214.

To build our model, we aligned the catalytic subunit (residues 1–350) with residues 1–317 of CaM kinase IIα. This alignment showed that residues 1–270 of CaM kinase IIα contained all of the blocks of homology typical of serine/threonine protein kinases. Furthermore, all α-helices and β-strands would be predicted to have the same length as in cAPK, except for helix β (on the small lobe), which is one residue shorter. Other inserts or deletions in the CaM kinase IIα would be predicted to occur in loops. Several residues within α-helices and β-strands as well as within loops involved in binding the peptide inhibitor are remarkably conserved. In particular, (*i*) Glu-96 is situated at a position equivalent to Glu-127 in cAPK and could be involved in recognizing the basic residue required at position P(−3) in most CaM kinase II substrates; (*ii*) the hydrophobic loop involved in interacting with a nonpolar residue at the P(+1) position in both cAPK and CaM kinase IIα is conserved; (*iii*) unlike cAPK, CaM kinase IIα does not require a second basic residue at position P(−2), as the latter enzyme does not contain a residue equivalent of Glu-230; and finally (*iv*) conservation of the hydrophobic pocket between helices F and G (Gly-Tyr-Pro-Pro-Phe-Phe-Ala-Asp at 234–241 in cAPK, and Gly-Tyr-Pro-Pro-Phe-Trp-Asp-Glu at 209–216 in CaM kinase IIα), which is the site of the high-affinity interaction with Phe-(−11) on the inhibitor.

When optimally aligned, amino acids 1–270 of CaM kinase IIα are equivalent to residues 31–296 of cAPK. In the model, each of the side chains of cAPK in this region was replaced with the side chains of the equivalent residue in CaM kinase IIα on the α-carbon backbone of the cAPK structure. After all the side chains had been replaced, the entire modeled structure was energy-minimized to ensure the best possible fit. This replacement did not affect the overall distribution of charged residues preferentially exposed to the solvent or hydrophobic residues predominantly directed inwards. Subsequently, the structure of residues from Asp-288 to Met-306 (regulatory region) was modeled according to the coordinates of PKI-(5–24) by aligning Phe-293 of CaM kinase IIα and Phe-(−11) of PKI-(5–24) as shown in Fig. 3. The possibility that the autoinhibitory domain conforms to the PKI-(5–24) structure is also suggested by the fact that circular dichroism measurements of the peptide from Met-281 to Ala-302 have been shown to have 57% α-helical character (36). To connect the aligned regulatory region to the core of the kinase, residues 271–283 were modeled to form a random coil followed by one turn of the α-helix at 283–287, joining the helix at the NH_2-terminal end of the PKI alignment. No secondary structure has been modeled for this segment of the polypeptide to show that, regardless of the type of secondary structure, the number of residues between Trp-271 and Val-287 (orange) (64 Å) allows sufficient distance to accommodate all of the intervening amino acids [in the PKI-(5–24)–cAPK structure, the distance between Trp^{296} and the NH_2-terminal end of PKI P(−16), is 30 Å].

In our model shown in Fig. 4*B*, the following features can be observed. (*i*) Alignment of Phe-(−11) with Phe-293 of CaM kinase IIα positions the latter residue at the hydrophobic pocket formed by Tyr-210 and Trp-214 of CaM kinase IIα at the same location as Tyr-235 and Phe-239 of cAPK. This interaction is responsible for the high affinity of PKI-(5–24) for cAPK (32) and could explain the tight inhibition of the kinase in the absence of Ca^{2+}/CaM. (*ii*) Some of the residues involved in CaM binding (Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala-Thr-Arg at 296–311) at the COOH-terminal end of the regulatory region could also be involved in a direct interaction with the catalytic site (either the ATP binding loop or the residues involved in recognizing the substrate). The α-helical character of PKI-(5–24) is limited to the first eight residues, whereas the COOH-terminal 12 residues adopt a relaxed conformation that in CaM kinase II could be more extended, allowing the positioning of Lys-298 at position P(−6), Lys-300 at P(−3), Ile-303 at P(0), and Leu-304 at P(+1). (*iii*) Thr-286, the autophosphorylation site responsible for autonomous activity, is located far from the catalytic site at a position more likely to be available for intersubunit phosphorylation upon CaM binding. It is also useful to consider whether Thr-286 could become phosphorylated in an intrasubunit reaction. This would require sliding this residue into the active site in response to a conformational change due to CaM binding. To accomplish this, residues 271–285 would need to be fully extended across the surface of the kinase, and such a structure would not be energetically likely. Thus, we favor intersubunit phosphorylation of Thr-286. Experimental evidence for intersubunit phosphorylation after CaM binding

has been provided by anisotropy measurements of a monomeric recombinant CaM kinase (38). These experiments showed that CaM trapping, which depends on autophosphorylation, is not possible when this mutant is diluted. In addition, coexpression of mutated forms of CaM kinase IIα in COS cells has recently demonstrated that autophosphorylation at Thr-286 is an intersubunit event (H. Schulman, personal communication).

The results and the model presented here define a minimal autoinhibitory domain composed of residues 291–294 interacting with the catalytic domain of the same subunit. We cannot rule out the possibility that in the multimer, the autoinhibitory domain of one subunit interacts with the catalytic domain of an adjacent subunit such that residues 271–285 extend as a tether between subunits. However, two facts argue against this possibility: (*i*) phosphorylation of Thr-286 occurs only upon CaM binding, suggesting that this residue is not accessible to another subunit before activation; and (*ii*) proteolysis at sites located between residues 271 and 287, which leads to constitutive activity, occurs only upon CaM binding, suggesting that the autoinhibitory domain is protected in the inactive state. Consistent with the results of our mutational studies, truncation at Leu-290 would remove the key inhibitory interactions, including the contributions of Phe-293. Extending the sequence by only four residues (291–294) may occlude substrate binding sufficiently to inactivate the enzyme, without necessarily interacting with the phosphate anchor or the catalytic loop. This model indicates that replacement of residues 291–294 with Ala-Ala-Ala-Leu would result in only a small effect on the level of CaM-independent activity because of the remainder of the COOH-terminal end of the autoregulatory region, which extends through the active-site groove. This region includes residues Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala at 301–309 containing Thr-305 and -306. These two residues are now known to be involved in an inhibitory autophosphorylation reaction and proposed to be situated close to the active site (39).

Based on the model and data, we speculate that activation of CaM kinase II may be initiated by binding of CaM at the COOH-terminal end of the autoinhibitory domain, which includes Arg-311 as a critical residue. This interaction would precipitate major conformational changes in CaM as well as throughout the entire length of the CaM binding region of the enzyme. Based on the NMR and three-dimensional structures of CaM/MLCK–peptide complexes (21, 40), we predict that the CaM-binding region will form a long, rigid α-helix. Such a conformational alteration would separate the remainder of the autoinhibitory domain including Phe-293 from the core of the kinase, exposing Thr-286 for intersubunit phosphorylation and allowing phosphorylation of other substrates. The phosphate group on Thr-286 could prevent reassociation of the autoinhibitory domain with the body of the kinase by either stabilizing the helix and/or by electrostatic repulsion. Obviously, solution of the crystal structure of CaM kinase IIα will be required to confirm or deny our suggestions. However, until such structure is available, the model presented here can serve as a basis for the design of additional mutations that could lead to the identification of other important elements in this kinase.

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1. Kemp, B. E. & Pearson, R. (1991) *Biochim. Biophys. Acta* **1094**, 67–76.
2. House, C. & Kemp, B. E. (1987) *Science* **238**, 1726–1728.
3. Bagchi, I. C., Kemp, B. E. & Means, A. R. (1992) *Mol. Endocrinol.* **6**, 621–626.
4. Kennelly, P. J., Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *J. Biol. Chem.* **262**, 11958–11963.
5. Taylor, S. S. (1989) *J. Biol. Chem.* **264**, 8443–8446.
6. Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R., Kemp, B. E., Soderling, T. R. & Means, A. R. (1988) *J. Biol. Chem.* **263**, 7190–7195.
7. Schulman, H. (1988) *Adv. Second Messenger Phosphoprotein Res.* **22**, 39–111.
8. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
9. Miller, S. G., Patton, B. L. & Kennedy, M. B. (1988) *Neuron* **1**, 593–604.
10. Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R. & Means, A. R. (1988) *J. Biol. Chem.* **263**, 7190–7195.
11. Waxham, M. N., Aronowski, J. & Kelly, P. T. (1989) *J. Biol. Chem.* **264**, 7477–7482.
12. Colbran, R. J., Fong, Y.-L., Schworer, C. M. & Soderling, T. R. (1988) *J. Biol. Chem.* **263**, 18145–18151.
13. Colbran, R. J., Smith, M. K., Schworer, C. M., Fong, L.-Y. & Soderling, T. R. (1989) *J. Biol. Chem.* **264**, 4800–4804.
14. Fong, Y.-L., Taylor, W. L., Means, A. R. & Soderling, T. R. (1989) *J. Biol. Chem.* **264**, 16759–16763.
15. Waxham, M. N., Aronowski, J., Westgate, S. A. & Kelly, P. T. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1273–1277.
16. Waldman, R., Hanson, P. I. & Schulman, H. (1990) *Biochemistry* **29**, 1679–1684.
17. Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H. & Rosenfeld, M. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5962–5966.
18. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
19. Tabor, S. & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1074–1078.
20. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
21. Meador, W. E., Means, A. R. & Quiocho, F. A. (1992) *Science* **256**, 1199–1202.
22. Means, A. R., Cruzalegui, F., LeMagueresse, B., Needleman, D., Slaughter, G. & Ono, T. (1991) *Mol. Cell. Biol.* **11**, 3960–3971.
23. Bagchi, I. C., Huang, Q. & Means, A. R. (1992) *J. Biol. Chem.* **267**, 3024–3029.
24. Fong, Y.-L. & Soderling, T. R. (1990) *J. Biol. Chem.* **265**, 11091–11097.
25. Pearson, R. B., Woodgett, J. R., Cohen, P. & Kemp, B. E. (1985) *J. Biol. Chem.* **260**, 14471–14476.
26. Hanley, R. H., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N. & Kelly, P. T. (1987) *Science* **237**, 293–297.
27. Schulman, H. & Lou, L. L. (1989) *Trends Biochem. Sci.* **14**, 62–66.
28. Hagiwara, T., Ohsako, S. & Yamauchi, T. (1991) *J. Biol. Chem.* **266**, 16401–16408.
29. Yamagata, Y., Czernik, A. J. & Greengard, P. (1991) *J. Biol. Chem.* **266**, 15391–15397.
30. Planas-Silva, M. D. & Means, A. R. (1992) *EMBO J.* **11**, 507–517.
31. Yamauchi, T., Ohsako, S. & Deguchi, T. (1989) *J. Biol. Chem.* **264**, 19108–19116.
32. Brickey, D. A., Colbran, R. J., Fong, Y.-L. & Soderling, T. R. (1990) *Biochem. Biophys. Res. Commun.* **173**, 578–584.
33. Kelly, P. T., Weinberger, R. P. & Waxham, M. N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4991–4995.
34. Glass, D. B., Cheng, H.-C., Mende-Mueller, L., Reed, J. & Walsh, D. A. (1989) *J. Biol. Chem.* **264**, 8802–8810.
35. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S. & Sowadski, J. M. (1991) *Science* **253**, 407–414.
36. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N.-h., Taylor, S. S. & Sowadski, J. M. (1991) *Science* **253**, 414–420.
37. Knighton, D. R., Pearson, R. B., Sowadski, J. M., Means, A. R., Ten Eyck, L. F., Taylor, S. S. & Kemp, B. E. (1992) *Science* **257**, 130–135.
38. Meyer, T., Hanson, P. I., Sryer, L. & Schulman, H. (1992) *Science* **256**, 1199–1202.
39. Hanson, P. & Schulman, H. (1992) *J. Biol. Chem.* **267**, 17216–17224.
40. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B. & Bax, A. (1992) *Science* **256**, 632–638.