## *Accelerated Publications*

# Multifunctional $Ca^{2+}$/Calmodulin-Dependent Protein Kinase Made $Ca^{2+}$ Independent for Functional Studies†

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ABSTRACT: Multifunctional $Ca^{2+}$/calmodulin-dependent protein kinase (CaM kinase) that is transiently expressed in COS-7 cells is essentially inactive when assayed without $Ca^{2+}$. Physiological activation of the kinase occurs by binding of $Ca^{2+}$/calmodulin near a putative autoinhibitory subdomain that contains the sequence $His^{282}$-Arg-Gln-Glu-$Thr^{286}$. We have markedly increased the $Ca^{2+}$-independent activity of CaM kinase by altering the charge of this sequence by site-directed mutagenesis. The mutant containing *$Asp^{282}$-Gly-Glu*-Glu-$Thr^{286}$ is 67% $Ca^{2+}$ independent. We also mimicked the effect of autophosphorylation at $Thr^{286}$ by the mutant containing $His^{282}$-Arg-Gln-Glu-*$Asp^{286}$*, which is 36% $Ca^{2+}$ independent. In addition to delineating the autoinhibitory domain by use of mutations that disable it, these constructs are of immediate practical value for simulating CaM kinase action in vivo without elevating $Ca^{2+}$. To this end, we show that nuclear microinjection of cDNA of a constitutive mutant, but not of the wild-type kinase, initiates maturation of *Xenopus* oocytes.

Multifunctional $Ca^{2+}$/calmodulin-dependent protein kinase (CaM kinase) is an important mediator of $Ca^{2+}$ action on diverse functions, including carbohydrate metabolism, neurotransmitter synthesis, and neurotransmitter release [reviewed in Schulman (1988), Schulman and Lou (1989), and Colbran et al. (1989)]. Purification and cloning have established that rat brain CaM kinase consists of homologous $\alpha$, $\beta$, $\beta'$, and $\gamma$ subunits in different isozymic ratios in a holoenzyme of 10–12 subunits. The N-terminal half of each subunit contains the catalytic domain. This is followed by subdomains that have autoinhibitory and calmodulin-binding functions (Figure 2) and by an association domain at the C-terminal end of the molecule (Lin et al., 1987). CaM kinase is kept inactive in its basal state by the putative autoinhibitory subdomain, whose function is disrupted by binding of $Ca^{2+}$/calmodulin leading to disinhibition of the kinase [reviewed in Schulman and Lou (1989) and Colbran et al. (1989)].

The study of CaM kinase function has been hampered by the lack of an active catalytic subunit that can be introduced into cells by transfection or microinjection, as has been done with cAMP kinase [e.g., Maurer (1989) and Castellucci et al. (1980)]. This has been particularly problematic, since, unlike cAMP which has few cellular targets, there are many $Ca^{2+}$-dependent proteins, thereby making it difficult to assign a given effect of $Ca^{2+}$ with a particular mediator of $Ca^{2+}$ action.

Biochemical analyses of CaM kinase suggest possible approaches for producing altered CaM kinase cDNA that would express $Ca^{2+}$-independent activity when introduced into mammalian cells. The sequence $His^{282}$-Arg-Gln-Glu-$Thr^{286}$ appears to be of central importance because (a) a synthetic peptide containing this sequence and extending on its C-terminal end inhibits kinase activity while a similar peptide lacking $His^{282}$ and $Arg^{283}$ is inactive (Kelly et al., 1988; Malinow et al., 1989); (b) autoinhibition can be disrupted by autophosphorylation of $Thr^{286}$, which converts the enzyme to a species that is largely $Ca^{2+}$ independent (Miller et al., 1988; Schworer et al., 1988; Thiel et al., 1988; Lou & Schulman, 1989); and (c) controlled proteolysis between the catalytic domain and $Arg^{283}$ generates a catalytic fragment while proteolysis that retains $Arg^{283}$ and $Thr^{286}$ produces an inactive fragment (LeVine & Sahyoun, 1987; Kwiatkowski & King, 1989). It may be possible to engineer a catalytic fragment by eliminating the cDNA coding for the autoinhibitory subdomain and expressing the truncated gene in situ. An alternative approach is to disable this subdomain by site-directed mutagenesis.

We now show that modifications aimed at disrupting the autoinhibitory domain produce a catalytically active enzyme with considerable $Ca^{2+}$-independent activity. However, cDNA constructs that truncate CaM kinase beyond its catalytic domain do not produce active enzyme when expressed in mammalian cells. Furthermore, we demonstrate that expression of a $Ca^{2+}$-independent construct in *Xenopus laevis* oocytes simulates a potential action of CaM kinase, the initiation of oocyte maturation.

## Experimental Procedures

*Materials.* Restriction enzymes and other DNA-modifying enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, MD) or New England Biolabs (Beverly, MA). The Bluescript KS cloning vector is from Stratagene (La Jolla, CA). Sequencing reagents and Sequenase enzyme were obtained from U.S. Biochemicals (Cleveland, OH). Bovine brain calmodulin and cytosolic rat brain CaM kinase were purified as described (Schulman & Greengard, 1978; Schulman, 1984). The immunoblotting kit was purchased from Vector Labs (Burlingame, CA). [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol) was purchased from ICN (Irvine, CA), and [$\alpha$-$^{35}$S]ATP was pur-

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chased from Amersham (Arlington Heights, IL). Dowex AG 1-X8 and electrophoresis reagents other than acrylamide were from Bio-Rad (Richmond, CA). All other chemicals were obtained from Sigma (St. Louis, MO). A synthetic peptide corresponding to amino acids 291–317 of α-CaM kinase, α-CaMK(291–317), was kindly provided by Dr. John Nestor (Syntex Research). Oligonucleotides were synthesized on a Biosearch DNA synthesizer and kindly provided by Dr. Jim Eberwine.

*Site-Directed Mutagenesis.* Oligonucleotide-directed mutagenesis was performed according to standard techniques (Zoller & Smith, 1983; Kunkel, 1985) with some modifications. Oligonucleotides were designed to introduce either stop codons or amino acid changes. In each oligonucleotide we introduced or eliminated a restriction site as an aid in screening for mutants. We will use the convention of referring to a construct that truncates the protein after amino acid 258 as α-CaM kinase-(1–258) and to constructs in which $Arg^{283}$ is replaced by Gly as $R^{283G}$. The sequences of the mutagenic primers used and the changes in restriction sites are as follows: α-CaM kinase-(1–258), 5′-GCCTCAGCGGCCGGAGCTCATTTGGACGGG-3′ (new *Sst*I site); α-CaM kinase-(1–267), 5′-GCGGTGCGAGATCCGAGCTCACTTGAGAGCCTCAGCG-3′ (new *Sst*I site); α-CaM kinase-(1–274), 5′-GCAGGAGGCCAGAGCTCAGCGGTGCG-3′ (new *Sst*I site); α-CaM kinase-(1–283), 5′-GGCAGTCCACGGGAGCTCATCTGTGCATGCAG-3′ (new *Sst*I site); $T^{286D}$, 5′-TCTTCAGGCAGTCGACGTCCTCCTGTCTGTGCATG-3′ (new *Aat*II site); $K^{300E}$, 5′-GGATGGCTCCCTCGAGCTTCCTCCTGGCATTG-3′ (new *Xho*I site); $H^{282D}R^{283G}Q^{284E}$ and $H^{282D}R^{283D}Q^{284E}$, 5′-GGCAGTCCACGGTCTCCTCG[C,T]CGTCCATGCAGGAGGC-3′ (eliminates *Sph*I site).

Mutagenic primers (5 pmol) were annealed to 1 pmol of uracil-containing single-stranded DNA in 10 μL of 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 50 mM NaCl for 5 min at 75 °C. The second-strand synthesis was accomplished in two steps. First, primer was extended in the presence of a limited amount of nucleoside triphosphates after addition of 4 μL of 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM DTT, 25 μM dNTP (each), and 3 units of Sequenase for 10 min at room temperature. Sequenase was inactivated by heating at 75 °C for 5 min, and second-strand synthesis was completed after addition of 16 μL of 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, 3 mM dNTPs (each), 0.5 unit of Klenow fragment of DNA polymerase I, and 6 units of T4 DNA ligase for 1–2 h at room temperature. DNA was isolated, and the mutants were identified from the introduced or removed restriction site. The mutants were verified by sequencing and transferred into the eukaryotic expression vector, SRα, as described (Hanson et al., 1989).

*Expression in COS Cells.* COS-7 cells were transfected with calcium phosphate coprecipitation (Chen & Okayama, 1987), harvested 68–75 h later, and lysed in 50 mM Pipes (pH 7.0), 1 mM EGTA, 10 μg/mL leupeptin, 1 mM PMSF, 1 μg/mL pepstatin A, 1 mM benzamidine, and 10% glycerol by sonication in a water cup sonicator (Heat Systems-Ultrasonics) at maximal power for 20 s at 0 °C. Cell extracts were prepared by centrifugation at 10000*g* for 10 min. Supernatants were used immediately or were frozen in liquid nitrogen and stored at −70 °C.

*Kinase Activity.* CaM kinase activity was assayed with either a synthetic peptide or synapsin I, as indicated. Phosphorylation of autocamtide-2 was performed as described (Hanson et al., 1989) with 250 μM [γ-$^{32}$P]ATP (60 Ci/mol) and an aliquot of COS cell extract containing 4–10 μg of protein in a final volume of 50 μL. Protein was determined by the method of Bradford (1976) using bovine γ-globulin as standard for cell extracts and BSA as standard for purified kinase. Reactions were initiated by addition of reaction mixture (45 μL) to cell extract (5 μL) and terminated after 60 s at 30 °C by addition of TCA to a final concentration of 2.5%. Precipitated protein was pelleted by centrifugation in a microfuge for 30 s. The sensitivity and reliability of the assay was greatly improved by reducing the level of [γ-$^{32}$P]ATP from the supernatant by use of spin columns of Dowex AG 1-X8. Aliquots of the supernatant (50 μL) were centrifuged through 100-μL spin columns for 10 s in a microfuge, and the columns were washed with 40 μL of $H_2O$. Combined eluates were spotted onto phosphocellulose paper (Whatman P81) as described (Roskoski, 1985). Radioactivity was quantified by Cerenkov radiation with a Beckman LS 3801 scintillation spectrophotometer. Phosphorylation of synapsin I was performed as above at pH 8.5 with 1 mg/mL synapsin I in 15-μL reaction mixtures. Reactions were terminated after 2 min at 30 °C by addition of 7 μL of SDS sample buffer. Labeled synapsin I was resolved on 9% SDS–polyacrylamide gels, the band containing synapsin I was excised, and $^{32}$P incorporation was quantified as above.

## Results and Discussion

Our preliminary studies indicated that some CaM kinase constructs that exhibit constitutive activity in bacterial expression systems are inactive when expressed in mammalian cells. Since one of our ultimate goals is to introduce cDNA coding for a constitutive kinase into eukaryotic cells, our strategy has been to examine the regulatory domain in a mammalian expression system. cDNA for wild-type or mutant kinase was transfected into COS-7 cells, and cellular proteins were resolved by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Expression level was compared by immunoblot (Figure 1A) and calmodulin overlay (Figure 1B), which demonstrate that the α subunit of CaM kinase was the most prominent stained band. As shown previously (Hanson et al., 1989), transfection using the SRα vector leads to high level of CaM kinase expression; in these experiments it was more than 14 times higher than the endogenous CaM kinase activity in mock-transfected cells.

We first established whether α-CaM kinase exhibits significant $Ca^{2+}$-independent activity due to either intrinsic basal activity or autophosphorylation in situ. Two key features of our assay were designed to make the assay of CaM kinase in cell extract containing other kinases both selective and sensitive. We utilized a highly selective synthetic substrate, autocamtide-2, which is poorly phosphorylated by cAMP kinase and kinase C (Hanson et al., 1989). In addition, we modified the protein kinase assay to greatly reduce the assay "blank" (see Experimental Procedures), thus permitting us to accurately determine even extremely low basal CaM kinase activity.

Basal activity of α-CaM kinase is only 0.33% of stimulated activity (0.083 nmol $min^{-1}$ $mg^{-1}$ with no $Ca^{2+}$ and 15 nmol $min^{-1}$ $mg^{-1}$ with $Ca^{2+}$). Stimulation by $Ca^{2+}$/calmodulin in numerous transfection experiments varied from 250-fold to 1000-fold, similar to what is found with the purified rat brain enzyme. The low background activity in the absence of $Ca^{2+}$ indicates the high specificity of the substrate used and the strict $Ca^{2+}$/calmodulin dependence of CaM kinase. This suggests that introducing modified forms of CaM kinase with even a small increase in its $Ca^{2+}$-independent activity could greatly increase CaM kinase activity in unstimulated cells.

Our first strategy for generating a constitutive enzyme was

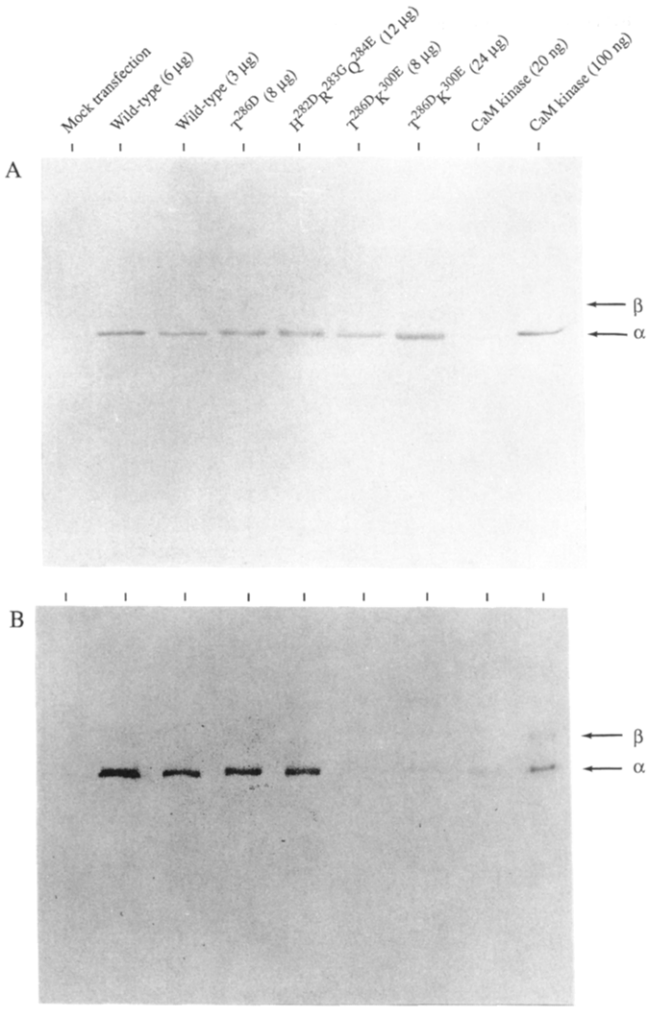


FIGURE 1: Immunoblot and calmodulin-binding overlay of wild-type and mutant CaM kinases. The labels above each lane indicate the amount of either purified kinase or total protein in extracts from COS cells that was resolved on SDS gels, transferred electrophoretically onto nitrocellulose, and analyzed by immunoblot (A) and by calmodulin overlay (B) as described (Hanson et al., 1989). Arrows indicate mobility of purified $\alpha$ and $\beta$ subunits of neuronal CaM kinase. The immunoblot was developed with monoclonal antibody CB-$\alpha$-1 that is selective for the $\alpha$ subunit (Scholz et al., 1988). Results similar to those shown were found with a polyclonal antibody kindly provided by Dr. E. Miyamoto (Fukunaga et al., 1988) (data not shown). Two concentrations of wild-type $\alpha$-CaM kinase and of purified rat brain CaM kinase were used to show the sensitivity of the method.

motivated by the demonstration that tryptic cleavage on the N-terminal side of $Thr^{286}$ produces an active monomeric fragment (LeVine & Sahyoun, 1987; Kwiatkowski & King, 1989). We therefore designed oligonucleotides that introduce stop codons at putative tryptic sites between $Thr^{286}$ and the end of the kinase catalytic domain [approximately at amino acid 258 or 259 (Hanks et al., 1988)]. We reasoned that at least one of the truncation mutants which end at $Lys^{258}$, $Lys^{267}$, $Arg^{274}$, and $Arg^{283}$ (Figure 2, arrowheads) would correspond to the tryptic fragment(s) generated biochemically.

Each of the four truncation mutants was expressed in COS cells as above, and CaM kinase activity was compared to that of wild type. Despite the fact that mutants contained the minimal sequence necessary to code for a catalytic domain, none increased $Ca^{2+}$-independent or $Ca^{2+}$-dependent CaM kinase activity in cell extracts. We would have detected increased activities as low as 1% of endogenous $Ca^{2+}$-stimulated activity or 0.05% of $Ca^{2+}$-stimulated $\alpha$-CaM kinase activity. The truncated kinases may be unstable, as described for catalytic fragments produced by proteolysis (Colbran et al., 1988). Alternatively, the catalytic domain of CaM kinase may not fold properly when expressed free of its regulatory and association domains in mammalian cells. a similar observation was made for a truncation mutant of protein kinase C, which showed negligible activity when assayed in vitro although it had some activity in situ (Muramatsu et al., 1989).

Our second strategy was to modify the autoinhibitory subdomain without changing the overall length of the protein. The autoinhibitory subdomain and overlapping calmodulin-binding subdomain of $\alpha$-CaM kinase are shown in Figure 2. These regions have been delineated on the basis of synthetic peptides which either inhibit catalysis (Kelly et al., 1988; Malinow et al., 1989) or inhibit activation by calmodulin (Hanley et al., 1988). $Arg^{283}$ is a critical cationic residue in the regulatory domain because it is essential for potency of autoinhibitory peptides (Kelly et al., 1988; Malinow et al., 1989). We therefore used oligonucleotide-mediated site-directed mutagenesis to alter this region by replacing $Arg^{283}$ with the neutral amino acid Gly. We use the convention of referring to such a construct as $R^{283G}$. We further modified the charge distribution in this region by substituting acidic residues for the two amino acids that flank it, $His^{282}$ and $Gln^{284}$, to generate mutant $H^{282D}R^{283G}Q^{284E}$.

We normalized kinase activity on the basis of CaM kinase protein assessed by immunoblot and calmodulin overlay (Figure 1) because the expression levels of constitutive constructs were consistently lower than that of the wild-type enzyme (Figure 3). We have made the assumption that the mutations have not altered these properties since the ratio of calmodulin binding and immunoreactivity with two independent antibodies was constant for the mutants analyzed. The modifications in mutant $H^{282D}R^{283G}Q^{284E}$ greatly disabled the autoinhibitory subdomain, raising $Ca^{2+}$-independent or constitutive activity to 67% of stimulated activity. Further increasing the anionic charge by producing mutant $H^{282D}R^{283D}Q^{284E}$ did not increase the constitutive activity (data not shown). $Ca^{2+}$-independent activity of these mutants is at least 300-fold higher than that in $\alpha$-CaM kinase. These

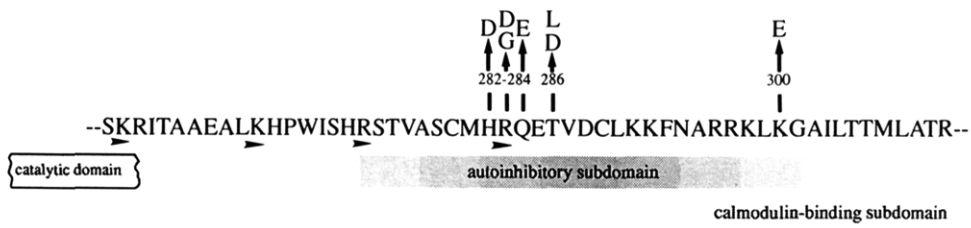


FIGURE 2: Mutagenesis of $\alpha$-CaM kinase regulatory domain. The regulatory region of $\alpha$-CaM kinase is shown in single-letter code with the autoinhibitory and calmodulin-binding domains delineated below. Position of site-directed mutations are indicated by arrows at amino acids numbered on the basis of the sequence of $\alpha$-CaM kinase (Lin et al., 1987). The C-terminal ends of the three truncation mutants are indicated by arrowheads below the sequence and represent, from left to right, $\alpha$-CaM kinase-(1–258), $\alpha$-CaM kinase-(1–267), $\alpha$-CaM kinase-(1–274), and $\alpha$-CaM kinase-(1–283).

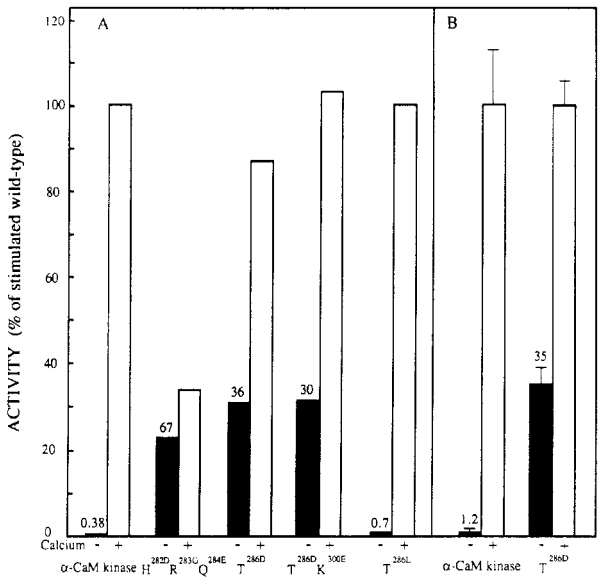


FIGURE 3: CaM kinase activity in extracts from transfected COS cells. The activity of wild-type and mutant CaM kinases in extracts of transfected COS cells was determined with autocamtide-2 or synapsin I as substrate, as described under Experimental Procedures. α-CaM kinase protein level was set at 1.0, and the others were normalized to this on the basis of comparison of immunoblot and calmodulin overlay (Figure 1), properties that are assumed not to vary among the constructs, after first subtracting basal activity of mock-transfected cells. Normalization factors were as follows: wild-type, 1.0; $T^{286D}$ and $T^{286D}K^{300E}$, 2.66; $H^{282D}R^{283G}Q^{284E}$, 4.0. Mutant $T^{286L}$ was not normalized since it was analyzed in a separate experiment; its $Ca^{2+}$-stimulated activity has previously been shown to be comparable to that of the wild-type enzyme and therefore set to 100% (Hanson et al., 1989). The constitutive activity, as a percent of the $Ca^{2+}$-stimulated activity, is indicated above the bar of $Ca^{2+}$-independent activity. (A) Autocamtide-2 phosphorylation was assayed in either the absence (−) or presence (+) of $Ca^{2+}$/calmodulin. Activities in pmol $min^{-1}$ (mg of extract)$^{-1}$ were as follows: α-CaM kinase, $1.50 \times 10^4$ ($+Ca^{2+}$) and 83 ($-Ca^{2+}$); mock transfected, $1.04 \times 10^3$ ($+Ca^{2+}$) and 26 ($-Ca^{2+}$). Activity of wild-type α-CaM kinase was set at 100%. Each determination was done in triplicate with standard deviation smaller than 10%. Similar results were obtained in five experiments with extracts from three transfections. (B) Constitutive synapsin I phosphorylation is enhanced in mutant $T^{286D}$. CaM kinase activity in extracts from COS cells transfected with α-CaM kinase or $T^{286D}$ was determined in duplicate with synapsin I as substrate, as described under Experimental Procedures. The specific activity of the wild-type kinase was $1.20 \times 10^4$ pmol $min^{-1}$ (mg of cell extract)$^{-1}$ and is defined as 100%.

mutations did reduce the specific activity of the kinase to approximately 40% of $Ca^{2+}$-stimulated wild-type kinase activity.

We also attempted to mimic the autophosphorylated state by substituting $Thr^{286}$ with Asp to generate mutant $T^{286D}$. The ability of acidic amino acids to mimic a phosphorylated amino acid has been previously shown for isocitrate dehydrogenase (Thorsness & Koshland, 1987), cAMP kinase (Kuret et al., 1988), and the eukaryotic translation initiation factor (Kaufman et al., 1989). We reasoned that the anionic aspartate moiety might simulate the anionic phosphothreonine, which is believed to debilitate the autoinhibitory subdomain and participate in fully activating the kinase. Colbran et al. (1988), for example, have shown that inhibition by a synthetic peptide whose sequence corresponds to the autoinhibitory and calmodulin-binding subdomains is decreased if the threonine corresponding to $Thr^{286}$ is phosphorylated. Indeed, the single amino acid substitution in mutant $T^{286D}$ was enough to relieve the normally stringent requirement for $Ca^{2+}$/calmodulin; the mutant enzyme is 36% constitutive (Figure 3). Full activity, comparable to that of α-CaM kinase, was attained in the presence of $Ca^{2+}$/calmodulin. The additional stimulation by $Ca^{2+}$/calmodulin was largely inhibited by addition of 2 μM α-CaMK(291–317), a synthetic peptide corresponding to the calmodulin-binding domain of α-CaM kinase (Lin et al., 1987). The constitutive activity was resistant to inhibition by this calmodulin antagonist (data not shown). The constitutive activity of $T^{286D}$ is therefore not likely to be due to tight binding of calmodulin present in the cell extracts.

Since $T^{286D}$ bound calmodulin normally (Figure 1B), we were able to exclude the possibility that the $Ca^{2+}$-independent activity was due to a protease-generated catalytic fragment. $T^{286D}$ should bind to a calmodulin–Sepharose affinity column whereas a catalytic fragment lacking the regulatory domain should not. In fact, no $Ca^{2+}$-independent activity was eluted from the column until all calmodulin-binding proteins were eluted with EGTA. CaM kinase activity in that eluate was approximately 40% $Ca^{2+}$ independent, as found for activity in the extract. This suggest that the constitutive activity was an intrinsic property of the mutant kinase and did not result from generation of an active proteolytic fragment.

We also examined a physiological substrate of CaM kinase, synapsin I (De Camilli & Greengard, 1986). Whereas the wild-type enzyme was highly $Ca^{2+}$ dependent, $T^{286D}$ was 35% $Ca^{2+}$ independent for synapsin I phosphorylation (Figure 3B). This is indistinguishable from the value of 36% obtained for autocamtide-2 phosphorylation. In the presence of $Ca^{2+}$, both wild type and mutant were equally effective in phosphorylating synapsin I. Thus, the mutation sufficiently altered the autoinhibitory subdomain to allow considerable $Ca^{2+}$-independent phosphorylation of a physiological substrate of CaM kinase.

The data suggest that a negatively charged amino acid at the position of $Thr^{286}$ can mimic the function of the phosphothreonine. Substituting a neutral amino acid, Leu, for $Thr^{286}$ to produce $T^{286L}$, for example, did not increase $Ca^{2+}$-independent activity whatsoever (Figure 3). These data support the model for generation of $Ca^{2+}$-independent or autonomous activity by autophosphorylation (Hanley et al., 1987; Lin et al., 1987; Schulman & Lou, 1989; Colbran et al., 1989). It is possible that $Arg^{283}$ and $Thr^{286}$ in the middle of the autoinhibitory domain are positioned near acidic residues on the catalytic domain so that introduction of a negative charge at or near either amino acid greatly reduces the function of the autoinhibitory subdomain.

We combined the $T^{286D}$ mutation with another modification at the C-terminal end of the putative autoinhibitory subdomain in the expectation that this would further disrupt this domain and increase $Ca^{2+}$-independent activity. We chose to replace $Lys^{300}$ with Asp since $Lys^{300}$ may be part of both the autoinhibitory and calmodulin-binding subdomains (Payne et al., 1988; Kelly et al., 1988; Malinow et al., 1989). Reduced calmodulin binding might be of advantage in functional studies since its overexpression is less likely to generally interfere with calmodulin-dependent processes. In fact, $T^{286D}K^{300D}$ had $Ca^{2+}$-independent activity, but it did not differ from $T^{286D}$; it was 30% $Ca^{2+}$ independent as compared to 36% $Ca^{2+}$-independent activity for $T^{286D}$ (Figure 3). $T^{286D}K^{300D}$ did exhibit reduced affinity for calmodulin when assayed at low calmodulin (Figure 1B). When assayed at excess calmodulin levels, as done in quantifying kinase activity (Figure 3), full activation could still be attained with this mutant. The reduction in calmodulin binding implicates $Lys^{300}$ as an important cationic component of the calmodulin-binding domain, consistent with conclusions from studies using synthetic peptides (Hanley et al., 1988). The inability of this second modification to increase $Ca^{2+}$-independent activity may suggest that the C-terminal

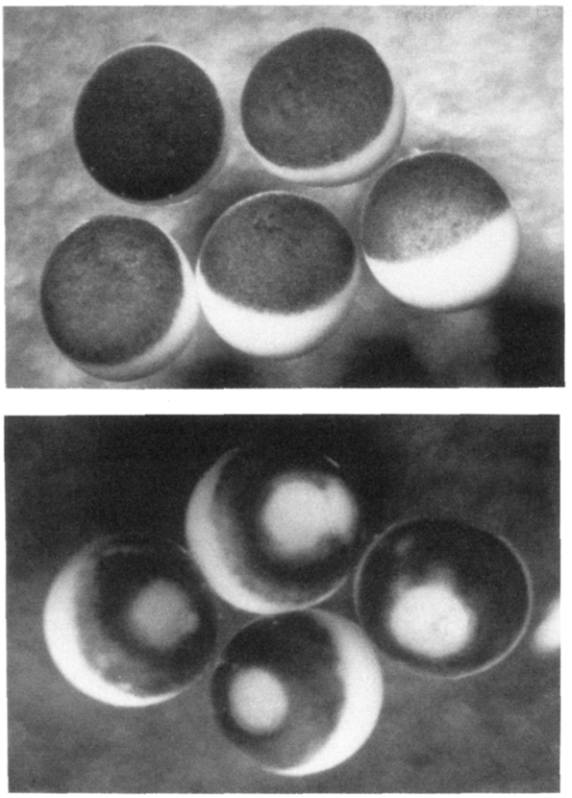


FIGURE 4: Effect of wild-type and constitutive CaM kinase expressed in *Xenopus* oocytes. Photograph of oocytes taken 27 h after nuclear microinjection of 6 ng of α-CaM kinase cDNA (A) and 6 ng of $T^{286D}$ cDNA (B). Prominent white spots in panel B indicate migration of the germinal vesicle to the animal pole.

end of the autoinhibitory subdomain does not extend as far as $Lys^{300}$.

The successful engineering of CaM kinase with constitutive activity in mammalian cells prompted us to examine whether they were capable of simulating CaM kinase action in vivo without elevating cellular $Ca^{2+}$. We chose *Xenopus* oocytes because protein kinases, including $Ca^{2+}$/calmodulin-dependent kinase(s), seem to play an important role in their maturation (Eckberg, 1988; Cicirelli et al., 1988) and because this system is amenable to nuclear microinjection. Our recent studies utilizing CaM kinase antibody and selective autoinhibitory peptides suggested that CaM kinase may be involved in mitosis in sea urchin eggs, a related biological system (Baitinger et al., 1989). We therefore tested whether expression of α-CaM kinase or $T^{286D}$ could induce maturation of *Xenopus* eggs, as recently shown with cDNA of constitutive protein kinase C constructs (Muramatsu et al., 1989).

Single stage VI oocytes from unprimed frogs were injected with 6 ng of the expression vector pSRα containing either α-CaM kinase, $T^{286L}$, or $T^{286D}$ cDNA as described (Muramatsu et al., 1989). Maturation is initiated by the movement of the nucleus (germinal vesicle), as judged by appearance of a white spot at the animal pole 27 h after injection. No effect on movement of the germinal vesicle was detected in 10 injections of α-CaM kinase or $T^{286L}$ cDNA, neither of which exhibits constitutive activity in vitro (Figure 3), or in 10 injections of buffer control. The inactive truncation mutant α-CaM kinase-(1–274) was also ineffective. By contrast, 8 of 10 oocytes injected with $T^{286D}$, which was 36% constitutive in vitro (Figure 3), showed migration of the germinal vesicle to the animal pole. This dramatic effect is shown in Figure 4 for oocytes injected with α-CaM kinase (panel A) or $T^{286D}$ (panel B). This morphological change is comparable to what is seen when maturation is induced by progesterone, with the exception that actual germinal vesicle breakdown did not occur with the constitutive construct.

We report here that some CaM kinase genes with a modified autoinhibitory subdomain exhibit kinase activity in vitro after expression in mammalian cells as well as activity in vivo without the normal requirement for $Ca^{2+}$. This study has both biochemical and practical implications. Modifications introduced in the autoinhibitory and calmodulin-binding subdomains directly demonstrate the importance of certain amino acids in these two functions. Since pseudosubstrate or autoinhibitory subdomains have been shown to involve an interaction with the cationic residue that would be part of a substrate consensus sequence (Hardie, 1988), we reasoned that placement of anionic charge in this region would disrupt the autoinhibitory function. Indeed, mutant $H^{282D}R^{283G}Q^{284E}$ harboring such changes is 67% constitutive. This provides an additional and more direct demonstration that this region is important for maintaining the kinase almost completely inactive in the absence of $Ca^{2+}$.

We had previously used site-directed mutagenesis to show that phosphorylation of $Thr^{286}$ was necessary for conversion of the kinase to a $Ca^{2+}$-independent species (Hanson et al., 1989). A similar conclusion has recently been reported by Fong et al. (1989). They used in vitro translation to screen and characterize mutant constructs and show that $T^{286D}$ has a 4-fold higher level of $Ca^{2+}$-independent activity than the wild type. Our study demonstrates, by mammalian expression, that substitution of aspartic acid, but not of leucine, for the threonine at this position of the autoinhibitory subdomain (mutant $T^{286D}$) is sufficient for producing a $Ca^{2+}$-independent or constitutive enzyme. Since neither aspartic acid nor leucine has the shape of phosphothreonine, but only the aspartic acid substitution increased $Ca^{2+}$-independent activity of the enzyme, it is likely that the anionic charge of the phosphate moiety is responsible for producing the $Ca^{2+}$-independent activity during autophosphorylation of the enzyme.

Overexpression of a constitutive construct ($T^{286D}$) activated a putative CaM kinase pathway and elicited a biological response in oocytes. We do not know whether the endogenous *Xenopus* CaM kinase is normally involved in germinal vesicle movement, although it has been implicated in other forms of motility because of its ability to phosphorylate myosin light chains. Additional experiments, such as microinjection of selective CaM kinase inhibitory peptides, will be needed before the biological implication of the oocyte experiment can be appreciated. Nonetheless, our aim was to determine whether constitutive CaM kinase can be functionally expressed in a biological system, and this has been successfully accomplished. Expression of the mutant constructs, such as $H^{282D}R^{283G}Q^{284E}$ which is 67% $Ca^{2+}$ independent or $T^{286D}K^{300E}$ which has reduced calmodulin binding, should be useful, for example, in studying neurotransmitter release and synaptic plasticity where CaM kinase has been implicated (Llinas et al., 1985; Malinow et al., 1989).

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# Mechanistic Studies of Peptidyl Prolyl Cis–Trans Isomerase: Evidence for Catalysis by Distortion

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ABSTRACT: Cyclophilin, the cytosolic binding protein for the immunosuppressive drug cyclosporin A, has recently been shown to be identical with peptidyl prolyl cis–trans isomerase [Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989) *Nature 337*, 476; Takahashi, N., Hayano, T., & Suzuki, M. (1989) *Nature 337*, 473]. To provide a mechanistic framework for studies of the interaction of cyclophilin with cyclosporin, we investigated the mechanism of the PPI-catalyzed cis to trans isomerization of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA (Xaa = Ala, Gly). Our mechanistic studies of peptidyl prolyl cis–trans isomerase include the determination of steady-state kinetic parameters, pH and temperature dependencies, and solvent and secondary deuterium isotope effects. The results of these experiments support a mechanism involving catalysis by distortion in which the enzyme uses free energy released from favorable, noncovalent interactions with the substrate to stabilize a transition state that is characterized by partial rotation about the C–N amide bond.

The recent finding (Fischer et al., 1989a; Takahashi et al., 1989) that cyclophilin and peptidyl prolyl cis–trans isomerase (PPI)[1] are identical and that cyclosporin A potently inhibits PPI raises the possibility of designing immunosupressive agents

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