Substrate-directed Function of Calmodulin in Autophosphorylation of Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II*  

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Autophosphorylation of Thr\(^{286}\) in Ca\(^{2+}\)/calmodulin-dependent protein kinase II occurs within each holoenzyme by an intersubunit reaction and is essential for kinase function in vivo. In addition to a kinase-directed function of calmodulin to activate the kinase, a second calmodulin is required for the autophosphorylation of each Thr\(^{286}\) (Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) Neuron 12, 943–956). We have engineered heteromeric holoenzymes comprising distinct “kinase” and “substrate” subunits to test for kinase- and substrate-directed functions of calmodulin. The obligate kinase subunits have aspartate residues substituted for threonine at positions 286, 305, and 306 (the autophosphorylation and calmodulin-binding sites), making it constitutively active but unable to bind calmodulin. Obligate substrate subunits are catalytically inactive (K42M mutation) but are able to bind calmodulin. Phosphorylation of substrate subunits occurs specifically at Thr\(^{286}\) and is completely dependent upon the presence of calmodulin. Blocking the ability of the substrate subunit to bind calmodulin, either with inhibitor KN-93 or by mutagenesis of the calmodulin-binding domain of the substrate subunit, prevents its phosphorylation, consistent with a substrate-directed function of calmodulin that requires its direct binding to the subunit being phosphorylated.

Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) regulates a wide variety of neuronal processes, including neurotransmitter synthesis and secretion, carbohydrate metabolism, receptor and ion channel function, and gene expression by phosphorylation of critical enzymes and proteins (reviewed in Ref. 1). It does so in response to increases in intracellular Ca\(^{2+}\) levels initiated by diverse signal transduction pathways. Accordingly, the kinase is both highly abundant in brain and distributed in diverse subcellular compartments (reviewed in Ref. 2). Four genes (\(a, b, \gamma, \) and \(\delta\)) give rise to many related CaM kinase II isoforms, with the \(a\) and \(b\) isoforms predomniating in brain (1). Each isoform has an amino-terminal catalytic domain, a central regulatory domain containing autoinhibitory and calmodulin-binding segments, and an association domain at its carboxyl terminus. Interactions between association domains of individual subunits allow 6–12 subunits to form a multimeric holoenzyme. Electron microscopy suggests that the catalytic and regulatory domains radiate out from a central core of assembled association domains in a flower-petal-like arrangement (3).

CaM kinase II can undergo distinct steps of autophosphorylation at Thr\(^{286}\) within the autoinhibitory domain and at Thr\(^{305}\) and Thr\(^{306}\) in the calmodulin-binding site. Activation of CaM kinase II in the presence of Ca\(^{2+}\)/calmodulin results in rapid autophosphorylation at Thr\(^{286}\) (4–6). Subsequent dissociation of calmodulin exposes Thr\(^{305}\) and Thr\(^{306}\) for autophosphorylation, which prevents rebinding of Ca\(^{2+}\)/calmodulin (7–10). Autophosphorylation of Thr\(^{286}\) has significant consequences for some neuronal functions in vivo, as demonstrated with mice in which Thr\(^{286}\) of the endogenous kinase is mutated to Ala, preventing autophosphorylation at this position without disturbing kinase expression or its catalytic site (11, 12). The mutant mice are impaired in spatial learning tasks requiring the hippocampus and long-term potentiation in hippocampal slices cannot be induced (12). Conversion of CaM kinase II to its autonomous form is mimicked in mutant T286D-CaM kinase II (13, 14). Consequently, long-term potentiation and spatial learning are altered in transgenic mice expressing T286D mutant kinase (15–18).

There are several possible biochemical consequences of Thr\(^{286}\) phosphorylation. Phospho-Thr\(^{286}\)-bearing subunits have a markedly reduced calmodulin off-rate and thereby “trap” calmodulin, perhaps providing a mechanism for CaM kinase II to sequester calmodulin from other enzymes and proteins while prolonging its activated state (19). Even after calmodulin dissociates, the presence of phospho-Thr\(^{286}\) disables the autoinhibitory domain, leaving the kinase 50–70% Ca\(^{2+}\)-independent or autonomous (20–23). Finally, autophosphorylation makes activation of the kinase sensitive to the frequency of Ca\(^{2+}\) oscillations (24). During brief Ca\(^{2+}\) transients and with submaximal calmodulin levels, significant occupancy of calmodulin-binding sites and high activation only occur as the frequency of stimulation exceeds a threshold that achieves autophosphorylation (24).

Calmodulin trapping, autonomous activity, and frequency-dependent activation all require the autophosphorylation of Thr\(^{286}\). Given both its mechanistic and functional consequences, it is crucial to understand the mechanism underlying this phenomenon.

Autophosphorylation of Thr\(^{286}\) is associated with the multimeric structure of CaM kinase II, yet activation by bound calmodulin is an inherent property of individual subunits, with each subunit binding one molecule of calmodulin (19, 25). Autophosphorylation of its critical autonomy site is an intraholoenzyme reaction (20, 26), which occurs via an intersubunit mechanism (27, 28). Calmodulin exhibits cooperativity in calmodulin trapping and autophosphorylation (24, 27, 29), sug-
gesting that calmodulin serves a second function in addition to activation. This is consistent with the finding that Thr286 is not constitutively autophosphorylated in holoenzymes in which some or all of the subunits are mutated to be constitutively active (27, 30). The simplest mechanism to explain these findings is for one molecule of calmodulin to bind for activation of a “kinase” subunit (a kinase-directed action) coincident with a second mole of calmodulin that must be bound to the “substrate” subunit (a substrate-directed function; Ref. 31), e.g. to expose Thr286 for autophosphorylation (27). However, evidence supporting such a requirement for direct binding of calmodulin to the substrate subunit during autophosphorylation of CaM kinase II holoenzymes has not been demonstrated.

In this report, we present such evidence using CaM kinase II heteromultimers in which subunits are mutated to assume obligatory roles, either as a kinase or a substrate subunit. Occupation by calmodulin on each of the two subunits participating in CaM kinase II autophosphorylation explains the cooperative effect of calmodulin (25, 27), and may underlie the high sensitivity of CaM kinase II to changes in Ca2+/calmodulin concentrations and frequencies of Ca2+ oscillations (24).

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Porcine brain calmodulin was purchased from Ocean Biologicals (Edmonds, WA). Both KN-93 and immobilized calmodulin were obtained from Life Technologies, Inc. Restriction enzymes and other DNA-modifying enzymes were purchased through New England Biolabs or Life Technologies, Inc. The supplier for [γ-32P]ATP (6000 Ci/mol) was NEN Life Science Products.

Construction of Expression Vectors—The plasmid used for expression of wild type and mutant α-CaM kinase II constructs was the pCD derivative SRe (32). Plasmids encoding wild type, K42M mutant, K42M/T286A double mutant, and tag-CaM kinase II (in which an 18-amino acid sequence from the influenza hemagglutinin HA1 protein was inserted by site-directed mutagenesis into CaM kinase II between Thr89 and Ile94 to generate a “tagged” form of the enzyme) were those as described previously (27).

Three additional constructs in SRe, used as parent plasmids for subcloning to create mutant CaM kinase II constructs described herein, were T286/305/306D-α-CaM kinase II, T305/306D-α-CaM kinase II, and K42M/C289P-α-CaM kinase II, which were engineered by and obtained from M. Srinivasan and H. Schulman. These α isoforms have a 11-amino acid insert between Lys328 and Glu329 containing a high sensitivity of CaM kinase II to changes in Ca2+/calmodulin while being active, i.e. that it be constitutively active. These features are those of wild type CaM kinase II that is autophosphorylated at Thr286 (which makes it autonomous of Ca2+/calmodulin) and at Thr305 and Thr306 (which disables calmodulin binding; Refs. 8 and 27), which can be mimicked by engineering aspartate in place of the three threonine residues. To distinguish these subunits immunologically and on SDS-polyacrylamide gels, we epitope-tagged the kinase subunits near the amino terminus (see “Experimental Procedures”) to increase their size by 2 kDa without affecting its activity, as previously reported (see Fig. 1 in Ref. 27). This kinase subunit is termed tag-T286/305/306D mutant, the PstI/EcoRI fragment from SRe-tag-CaM kinase II was subcloned into SRe-T286/305/306D-CaM kinase II.

Expression and Purification of CaM Kinase II—The calcium phosphate precipitation method of Chen and Okayama (34) was used to transiently transfet or cotransfect COS cells, equivalent total kinase activity in COS lysates was achieved by diluting lysates expressing higher levels of kinase activity with mock-transfected COS lysate. Routinely, 5 μl of COS lysate was used in reactions. Dilution buffer for purified kinases consisted of 2 mM PIPES (pH 7.0) with 1.0 mg/ml BSA, and these dilutions were adjusted to ensure comparable total activity between wild type and mutant CaM kinase II in phosphorylation reactions.

Detection of CaM Kinase II Subunits on Blots—Proteins were separated by electrophoresis on 9% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). CaM kinase II subunits on membranes were visualized by using calmodulin overlay assay, autoradiography (for 32P-labeled samples), or immunodetection. The calmodulin overlay assay was performed with biotinylated calmodulin and enhanced chemiluminescence as described (39). Immunodetection specific for α-CaM kinase II was conducted with anti-CaM kinase II monoclonal CB-α2 antibody as described (40).

For purified kinases, autophosphorylated as described above except that nonradioactive ATP was used in place of [γ-32P]ATP, immunodetection was also performed with anti-phospho-Thr286 CaM kinase II rabbit polyclonal antiserum generously supplied from D. D. Ginty. This antiserum selectively recognizes CaM kinase II phosphorylated at Thr286. Immunoblots were prepared by blocking membranes in buffer A (20 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20) plus 5% (w/v) BSA for 1 h at room temperature, and then incubating in primary antibody diluted 1:500 in buffer A plus 1% BSA for 2.5 h. Bound antibodies were detected by incubation for 1 h with donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:2500 in buffer A plus 1% BSA and visualized using the Enhanced Chemiluminescence detection kit and film from Amersham Pharmacia Biotech following the manufacturer’s directions.

RESULTS

We engineered heteromultimers of CaM kinase II composed of subunits that can function as either kinase or substrate subunits in order to test for obligatory binding of calmodulin to the substrate subunit during autophosphorylation. The requisite feature of the kinase subunit is that it be unable to bind calmodulin while being active, i.e. that it be constitutively active. These features are those of wild type CaM kinase II that is autophosphorylated at Thr286 (which makes it autonomous of Ca2+/calmodulin) and at Thr305 and Thr306 (which disables calmodulin binding; Refs. 8 and 27), which can be mimicked by engineering aspartate in place of the three threonine residues. To distinguish these subunits immunologically and on SDS-polyacrylamide gels, we epitope-tagged the kinase subunits near the amino terminus (see “Experimental Procedures”) to increase their size by 2 kDa without affecting its activity, as previously reported (see Fig. 1 in Ref. 27). This kinase subunit is termed tag-T286/305/306D mutant, the PstI/EcoRI fragment from SRe-tag-CaM kinase II was subcloned into SRe-T286/305/306D-CaM kinase II.

Wild type and mutant CaM kinases II subunits were expressed in COS-7 cells by transfection with corresponding DNAs, and expression levels examined by immunoblot with α-CaM kinase II specific monoclonal antibody CB-α2 and for the ability to bind calmodulin by calmodulin overlay (Fig. 1). Homomultimers composed of either kinase (tag-T286/305/306D), apparent molecular mass of 56 kDa) or substrate (K42M, apparent molecular mass of 54 kDa) subunits or het-

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eromultimers composed of both subunits achieved by cotransfection a mixture of corresponding DNAs were well expressed and distinguishable by mobility on SDS-polyacrylamide gels (Fig. 1A). Calmodulin overlay assay of identical lysates reveal bands corresponding to K42M mutant (Fig. 1B, lanes 1, 3, and 4) and to wild type kinase (Fig. 1B, lane 5) but not to the tag-T(286/305/306)D mutant (Fig. 1B, lanes 2, 3, and 4). Thus, replacement of Thr305 and Thr306 with aspartate has the intended effect of mimicking autophosphorylation of these sites by similarly blocking binding of calmodulin (7–10).

The activities of expressed kinases were found to be appropriate for the substrate and kinase subunits needed for our studies (Fig. 2C). Lysates with expressed homomultimers of K42M mutant (Fig. 2C, lanes 1 and 2) exhibited little or no Ca$^{2+}$/calmodulin-stimulated activity, similar to that found in mock-transfected cells (data not shown). By contrast, lysates of cells expressing homomultimers of tag-T(286/305/306)D exhibited high activity in either the presence or absence of added Ca$^{2+}$/calmodulin (Fig. 2C, lanes 7 and 8). Comparison of expressed kinase subunit migrating at 56 kDa and substrate subunit at 54 kDa (Fig. 2B) with activities of comparable samples (Fig. 2C) indicates that kinase activities in lysates expressing both types of subunits are largely due to the presence of tag-T(286/305/306)D, the kinase subunit. The tag-T(286/305/306)D mutant is therefore an appropriate obligate kinase subunit for our studies, with high Ca$^{2+}$-independent activity and no calmodulin binding (Fig. 1).

Previous studies showed that Thr$^{286}$ autophosphorylation in CaM kinase II occurs via an intraholoenzyme reaction (20, 26) and involves an intersubunit mechanism (27, 28). We therefore reasoned that the phosphorylation of K42M by tag-T(286/305/306)D should be a likely indicator of whether the two subunits were coassembled into heteromultimers. Indeed, Fig. 2A illustrates that phosphorylation of the K42M subunit only occurred in COS lysates in which the tag-T(286/305/306)D mutant was coexpressed (and in the presence of Ca$^{2+}$/calmodulin; see lane 6). No subunit $^{32}$P incorporation was seen in cell lysates expressing homomultimers of either subunit (lanes 1 and 2 for K42M, and lanes 7 and 8 for tag-T(286/305/306)D), nor when these lysates were mixed together (lanes 3 and 4). Phosphorylation of K42M does not occur even though the amount of kinase protein and activity in the two preparations (mixed versus coexpressed) is similar. These data suggest that the tag-T(286/305/306)D and K42M subunits coassemble to form heteromeric holoenzymes. This conclusion is also supported by the observation that tag-T(286/305/306)D is copurified with K42M using a procedure (see "Experimental Procedures") involving immobilized calmodulin chromatography (lanes 1–4 in Fig. 3B). In addition to mutant K42M, all other substrate subunits used in
CaM Kinase II Autophosphorylation

**Fig. 3.** Calmodulin must bind to a substrate subunit within a holoenzyme for autophosphorylation of Thr\(^{286}\). Identical samples were examined for phosphorylation of Thr\(^{286}\), substrate expression, and activity. A, purified heteromultimers (1.2 μg) containing K42M and tag-T286/305/306D mutant subunits (lanes 1–4) or purified wild type CaM kinase II (90 ng, lanes 5–8) were included in autophosphorylation reactions either in the absence (−) or presence (+) of Ca\(^{2+}\)/calmodulin and 5 μM KN-93. Proteins were resolved by SDS-polyacrylamide gel and transferred to nitrocellulose. Phosphorylation of Thr\(^{286}\) was assessed by immunoblot developed with anti-phospho-Thr\(^{286}\) rabbit polyclonal antisemur, which selectively recognizes the autophosphorylated form of CaM kinase II. The positions of the kinase subunit tag-T286/305/306D (lanes 1) and the substrate subunit K42M are indicated by the upper and lower arrows, respectively. B, expression of kinase subunits was assessed by immunoblot developed with anti-α-CaM kinase monoclonal antibody CB-2α to show the presence of all CaM kinase II subunits independent of their phosphorylation state. C, kinase activity was determined under conditions indicated in A using autocamtide-3 as exogenous substrate.

The experiments below formed heteromeric holoenzymes when coexpressed with tagged kinase subunits, as judged by co-immunoprecipitation using an antibody specific to the hemagglutinin epitope tag (data not shown).

We purified heteromeric CaM kinase II composed of obligate kinase (tag-T286/305/306D) and substrate (K42M) subunits in order to test the possible requirement for calmodulin binding to the substrate subunit with Thr\(^{286}\). Heteromeric (lanes 1–4) or wild type (lanes 5–8) CaM kinase II was incubated under autophosphorylation conditions in either the absence (−) or presence (+) of Ca\(^{2+}\)/calmodulin (Fig. 3). In this experiment, phosphorylation of Thr\(^{286}\) was detected by immunoblot with polyclonal antibody that selectively detects phospho-Thr\(^{286}\) CaM kinase II kindly provided by D. Ginty. Although the heteromeric kinase expressed both kinase and substrate subunits (Fig. 3B, lanes 1 and 2) and was constitutively active when presented with an exogenous substrate with no difference in activity in the absence or presence of Ca\(^{2+}\)/calmodulin (Fig. 3C, lanes 1 and 2), it was unable to phosphorylate itself, *i.e.* its substrate subunit, unless Ca\(^{2+}\)/calmodulin was present (Fig. 3A, compare lanes 1 and 2). Thus, autophosphorylation, but not phosphorylation of exogenous substrates, by the constitutively active heteromultimer is conditional on the presence of Ca\(^{2+}\)/calmodulin. By contrast, Ca\(^{2+}\)/calmodulin was required for both substrate phosphorylation and autophosphorylation by wild type CaM kinase II (Fig. 3C, lanes 5 and 6; Fig. 3A, lanes 5 and 6). We took advantage of KN-93, an inhibitor that blocks binding of calmodulin to the kinase without interfering with the Ca\(^{2+}\)/calmodulin-independent activity of the autophosphorylated enzyme (41) to further test the requirement for calmodulin binding. KN-93 has the same differential effect on kinase made Ca\(^{2+}\)/calmodulin-independent by the Thr\(^{286}\) to aspartate mutation; it did not block the constitutive activity of the heteromultimer (Fig. 3C, lanes 1–4) but did block activation of wild type kinase (Fig. 3C, lanes 5–8). However, despite its inability to block phosphorylation of exogenous substrates by the heteromultimer, KN-93 completely inhibited autophosphorylation of the K42M subunit in the multimer (Fig. 3A, compare lanes 2 and 4), suggesting that the calmodulin-binding site of the substrate subunit must be occupied in order for it to be phosphorylated by the kinase subunit.

We further examined the requirement for calmodulin binding by disabling the calmodulin-binding site of the substrate subunit using the double T305D/T306D point mutation shown previously to block Ca\(^{2+}\)/calmodulin binding (Fig. 1B). Indeed, substrate subunits consisting of mutant T305/306D, which are unable to bind calmodulin (data not shown), cannot be phosphorylated when coexpressed and coassembled with either tag-T286/305/306D (Fig. 4, lanes 1 and 2) or tag-wild type CaM kinase II (Fig. 4, lanes 3 and 4). Wild type CaM kinase II subunits in the heteromultimer can phosphorylate themselves (Fig. 4A) or an exogenous substrate (Fig. 4C) in the presence of Ca\(^{2+}\)/calmodulin but cannot phosphorylate coassembled substrate T305/306D subunits, which are incapable of binding calmodulin (Fig. 4A). Similar results were achieved when triple mutant K42M/T305D/T306D CaM kinase II subunits were substituted for T305/306D subunits in heteromultimers (data not shown).

Since CaM kinase II can be autophosphorylated at multiple sites, it was important to determine whether all of the substrate-directed incorporation of \(^{32}P\) was occurring at Thr\(^{286}\), the only site whose phosphorylation generates autonomous activity (13, 35). We used tag-T286/305/306D as the obligate kinase subunit and compared its phosphorylation of coexpressed mutants K42M or K42M/T286A as substrate (Fig. 5). In fact, no \(^{32}P\) incorporation was detected when the substrate subunit contained the T286A mutation (Fig. 5A, compare lanes 2 and 4). These data, along with the demonstration that Thr\(^{286}\) is phosphorylated, using phosphoselective antisera (Fig. 2), strongly indicate that the substrate-directed effect of calmodulin is to enable phosphorylation of Thr\(^{286}\).

Results presented above are consistent with the idea that Thr\(^{286}\) is shielded from autophosphorylation in the native conformation of CaM kinase II when Ca\(^{2+}\)/calmodulin is not bound to the enzyme (28, 30). Soderling and colleagues (30, 42) have suggested that mutation of Cys\(^{289}\) to proline disrupts the autoinhibitory domain to expose Thr\(^{286}\) and makes the enzyme Ca\(^{2+}\)-independent. Therefore, we examined whether this mutation in substrate subunit would obviate the need for Ca\(^{2+}\)/calmodulin binding to this subunit during autophosphorylation. We analyzed the autophosphorylation of mutant K42M/C289P, as the obligate substrate subunit with a potentially Ca\(^{2+}\)/calmodulin-independent conformation near Thr\(^{286}\) in heteromultimers with either tag-T286/305/306D as obligate kinase or tag-wild type as both Ca\(^{2+}\)-dependent kinase and substrate (Fig. 5, lanes 5–8). Autophosphorylation of K42M/C289P occurred in a Ca\(^{2+}\)/calmodulin-independent fashion (Fig. 5A, lanes 5 and 6) in contrast to the requirement for Ca\(^{2+}\)/calmodulin seen when substrate does not have a proline residue to disturb the secondary structure near Thr\(^{286}\) (Fig. 5A, lanes 1 and 2). When K42M/
FIG. 4. Disabling calmodulin binding of obligate substrate subunits blocks their phosphorylation. Two different heteromultimers were expressed in COS cells by cotransfections and identical samples were analyzed for 32P incorporation, subunit expression, and kinase activity. Recombinant CaM kinase II holoenzymes consisted of T(305/306)D coassembled with tag-T(286/305/306)D subunit (lanes 1 and 2) and T(305/306)D coassembled with tag-wild type subunit (lanes 3 and 4). Positions of the T(305/306)D subunits and of the tagged subunits in A and B are indicated by the lower and upper arrows, respectively. A, autoradiograph of autophosphorylation in either the absence (-) or presence (+) of Ca2+/calmodulin. B, immunoblot with monoclonal antibody CB-α2 as described in the legend to Fig. 1. C, relative kinase activities toward autocamtide-3 in either the absence (-) or presence (+) of Ca2+/calmodulin, as indicated in A.

C289P is coassembled with wild type CaM kinase II subunits, its phosphorylation does require Ca2+/calmodulin, but in this case calmodulin is providing a kinase-directed function to activate the kinase subunits whose activation and autophosphorylation require Ca2+/calmodulin (Fig. 5, A and C, lanes 7 and 8). These data support a substrate-directed function for calmodulin in CaM kinase II Thr286 autophosphorylation, a function that is not necessary when Thr286 of the substrate subunit is already exposed, e.g. by a nearby mutation.

DISCUSSION

It has been suggested that Ca2+/calmodulin has two independent functions during CaM kinase II autophosphorylation (27). Ca2+/calmodulin serves a conventional kinase-directed function in which it relieves the inhibitory interaction between the autoinhibitory domain and the catalytic domain, thereby activating the kinase (43). The observation that phosphorylation of inactive truncated CaM kinase II mutants by previously activated CaM kinase II monomers still required calmodulin suggested a requirement for a second calmodulin (27). We have now delineated a second function for calmodulin in intact holoenzymes of the kinase by demonstration that the calmodulin must bind directly to a substrate subunit in order for Thr286 autophosphorylation to proceed, a substrate-directed function.

As shown in Fig. 3, phosphorylation of Thr286 remains Ca2+/calmodulin-dependent in heteromultimers composed of a constitutively active kinase that is unable to bind calmodulin coassembled with an obligate substrate subunit.

Autophosphorylation within mutant heteromultimers appears to be similar to that in wild type CaM kinase II holoenzymes, i.e. it occurs through an intraholoenzyme and intersubunit mechanism (Fig. 2). Although CaM kinase II is able to autophosphorylate at a number of distinct sites, we employed reaction conditions that limit autophosphorylation to Thr286 (see "Experimental Procedures"). This is illustrated by the lack of 32P incorporation into substrate subunits containing a T286A mutation (Fig. 5).

Even when the substrate subunit contains Thr286, this residue is not phosphorylated if the calmodulin-binding site is disabled by substitution of aspartate for threonines 305 and 306 (Fig. 4). Thus, calmodulin binding is likely an inherent requirement for both the substrate and kinase subunits involved in each autophosphorylation reaction. Our results agree with the previous suggestion by Soderling and colleagues (30, 42) that Thr286 is shielded from phosphorylation in the absence of bound calmodulin. This conclusion stemmed from their observation that mutant C289P CaM kinase II homomultimers...
displayed some activity and autophosphorylated at Thr^{286} in the absence of Ca^{2+}/calmodulin (42).

Our findings provide a mechanistic basis for requiring concurrent calmodulin binding to at least two proximate subunits of CaM kinase holoenzymes for initial autophosphorylation to occur. The requirement explains the Hill coefficients of −1.6 for generating autonomous CaM kinase II activity in response to increasing levels of calmodulin (24, 29) whereas kinase activation toward exogenous substrates is not cooperative (24). It is likely that features of the kinase that take advantage of its multimeric structure, such as its dual requirements for Ca^{2+}/calmodulin in autophosphorylation, which in turn alters calmodulin binding kinetics, termed calmodulin trapping (19), and produce an autonomous activity, contribute to its ability to act as a frequency detector of Ca^{2+} oscillations with high sensitivity to frequency and calmodulin levels (24). Our findings also refine the molecular basis for a second feature of frequency-dependent activation, i.e. a molecular switch in sensitivity to Ca^{2+} oscillations such that conversion of some subunits to an autonomous species facilitate subsequent increments in autonomous activity or sensitize the enzyme to lower frequencies of stimulation (24). As suggested (24), phosphorylation of Thr^{286} in one subunit relieves it of a requirement for calmodulin and thereby facilitates Thr^{286} phosphorylation in a second subunit, creating a functional cooperativity. Moreover, our results confirm that a constitutive kinase subunit does not require interaction with calmodulin to phosphorylate Thr^{286} on a neighboring subunit. For example, removal of the calmodulin-binding requirement in the substrate subunit K42M/C289P, which may expose Thr^{286} without binding calmodulin, enables constitutive autophosphorylation of this subunit (Fig. 5).

The need for calmodulin binding onto two distinct subunits for CaM kinase II autophosphorylation is somewhat reminiscent of systems where multiple effects of Ca^{2+}/calmodulin are exerted on both an upstream (e.g. CaM kinase I kinase) and a downstream kinase (in this instance, CaM kinase I), thus bringing acute sensitivity to the system in the presence of the ligand (31). As these authors point out, however, effects of CaM kinase I phosphorylation and CaM kinase II autophosphorylation is somewhat reminiscent of systems where multiple effects of Ca^{2+}/calmodulin are exerted on both an upstream (e.g. CaM kinase I kinase) and a downstream kinase (in this instance, CaM kinase I), thus bringing acute sensitivity to the system in the presence of the ligand (31). As these authors point out, however, effects of CaM kinase I phosphorylation and CaM kinase II autophosphorylation are different.

To demonstrate that the mechanism of Thr^{286} phosphorylation requires both subunits to have bound calmodulin, we created heteromultimers with distinct kinase and substrate subunits. In vivo, of course, any subunit of wild type CaM kinase II should be able to assume either role. It would be interesting to determine whether the mechanism of subunit activation and subunit presentation, the kinase- and substrate-directed steps of autophosphorylation, involve distinct conformations or interactions with calmodulin.

Note Added in Proof—It has recently been shown using the Drosophila homologue of CaM kinase II that autophosphorylation of Thr^{287} (equivalent to Thr^{286} in rat) CaM kinase II) requires bound calmodulin acting in a substrate-directed mechanism (Wang, Z., Palmer, G., and Griffith, L. C. (1998) J. Neurochem. 71, 378–387).