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Intersubunit capture of regulatory segments is a component of cooperative CaMKII activation

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The dodecameric holoenzyme of calcium–calmodulin-dependent protein kinase II (CaMKII) responds to high-frequency Ca^{2+} pulses to become Ca^{2+} independent. A simple coincidence-detector model for Ca^{2+}-frequency dependency assumes noncooperative activation of kinase domains. We show that activation of CaMKII by Ca^{2+}–calmodulin is cooperative, with a Hill coefficient of ~3.0, implying sequential kinase-domain activation beyond dimeric units. We present data for a model in which cooperative activation includes the intersubunit 'capture' of regulatory segments. Such a capture interaction is seen in a crystal structure that shows extensive contacts between the regulatory segment of one kinase and the catalytic domain of another. These interactions are mimicked by a natural inhibitor of CaMKII. Our results show that a simple coincidence-detection model cannot be operative and point to the importance of kinetic dissection of the frequency-response mechanism in future experiments.

Calcium–calmodulin-dependent protein kinase II (CaMKII) is unique among the protein kinases because it is known to respond not just to the strength of the activation signal but also to its frequency[1]. This property, which is manifest in the ability of CaMKII to escape calcium dependence at high calcium-spike frequency, is likely to underlie the essential role for CaMKII in the strengthening of synaptic connections between neurons by long-term potentiation[2] and in the response to and control of heart rate[3]. The frequency response of CaMKII relies on the Ca^{2+}–calmodulin-dependent phosphorylation of a specific threonine residue (Thr286 in the mouse α isoform) in one kinase domain by another once a critical spike frequency is crossed, resulting in calcium-independent activity (autonomy)[1]. Mutation of Thr286 in CaMKII has dramatic effects, most notably the impairment of spatial learning in mice that bear this mutation[4,5].

Another unique feature of CaMKII is its assembly into large symmetrical holoenzymes in which 12 kinase domains are tightly packed around a central ring-shaped scaffold[6–8]. This raises a conceptual challenge in understanding how the enzyme is regulated. Because transphosphorylation of Thr286 is a key step in the activation process, it is difficult to understand how uncontrolled phosphorylation is prevented in such a holoenzyme assembly, which concentrates kinase domains to an extraordinary extent. One model for frequency decoding posits that CaMKII is a 'coincidence detector' in which the activation of kinase domains within a holoenzyme ring occurs stochastically as calcium levels rise[9]. As adjacent kinase domains need to bind Ca^{2+}–calmodulin in order to switch on[10,11], the time required for this to happen would be linked to the onset of transphosphorylation and the consequent acquisition of calcium independence[12–15] (see **Fig. 1a**).

The simplest form of a coincidence-detection model (illustrated in **Fig. 1a**) is based on the fact that isolated and autoinhibited CaMKII kinase domains, separated from the holoenzyme assembly, bind to Ca^{2+}–calmodulin in a 1:1 stoichiometry and are activated without cooperativity. It has also been observed, in studies using a particular synthetic peptide substrate known as autocamtide, that the activation of the holoenzyme by Ca^{2+}–calmodulin occurs without apparent cooperativity[16]. This result was interpreted to mean that Ca^{2+}–calmodulin binding to one kinase subunit within a holoenzyme is independent of binding to other subunits. The uncorrelated binding of Ca^{2+}–calmodulin to the holoenzyme is the essential feature of this model that introduces the time delay before transphosphorylation can occur (**Fig. 1a**).

Despite the elegance of this model, other findings concerning CaMKII make it difficult to understand how such a coincidence-detection mechanism might actually work. The binding of calcium-saturated calmodulin to the CaMKII holoenzyme shows clear evidence for positive cooperativity[7,8]. This suggests that the binding of one molecule of Ca^{2+}–calmodulin to a kinase subunit, which is expected to activate it, also potentiates adjacent kinase subunits for binding to Ca^{2+}-calmodulin (**Fig. 1b**). A crystal structure of the autoinhibited kinase domain of CaMKII revealed that the autoinhibitory segment, which contains the Ca^{2+}-calmodulin binding site, forms an antiparallel coiled coil within dimeric kinase domains[8]. The formation of autoinhibited dimers within the holoenzyme would explain why Ca^{2+}–calmodulin binds cooperatively, because the first binding event would necessarily disrupt the coiled coil formed by the autoinhibitory segment and would thereby increase the binding affinity of the holoenzyme for a

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second Ca^{2+}–calmodulin molecule (**Fig. 1b**). These studies did not analyze the cooperativity of kinase activation by Ca^{2+}–calmodulin, but because kinase activation results from Ca^{2+}–calmodulin binding, it follows that activation should also be cooperative with respect to Ca^{2+}–calmodulin. The acquisition of autonomy (in other words, the phosphorylation of Thr286 following activation) is indeed cooperative with respect to Ca^{2+}–calmodulin[1], which is also difficult to understand in terms of the simplest coincidence-detector model.

In this paper, we re-examine the cooperativity of CaMKII activation by Ca^{2+}–calmodulin by measuring the activity of the holoenzyme for two peptide substrates. One of these is autocamtide, which was used in the previous studies and is based on the sequence of a portion of the regulatory segment of CaMKII[17]. The other, referred to as syntide, is based on the sequence of a CaMKII phosphorylation site in glycogen synthase[18]. Our measurements using these substrates show that both the enzyme from *Caenorhabditis elegans* and the mammalian γ isoform are activated cooperatively by Ca^{2+}–calmodulin. The Hill coefficients that we obtained from these measurements are as high as 4.3, depending on the construct used, suggesting that the binding of the first Ca^{2+}–calmodulin molecule to one kinase subunit potentiates several other kinase subunits for Ca^{2+}–calmodulin binding (**Fig. 1c**). Thus, binding and activation follow the same pattern, and the CaMKII subunits do not behave independently. Based on these results, CaMKII is unlikely to function as a simple coincidence detector.

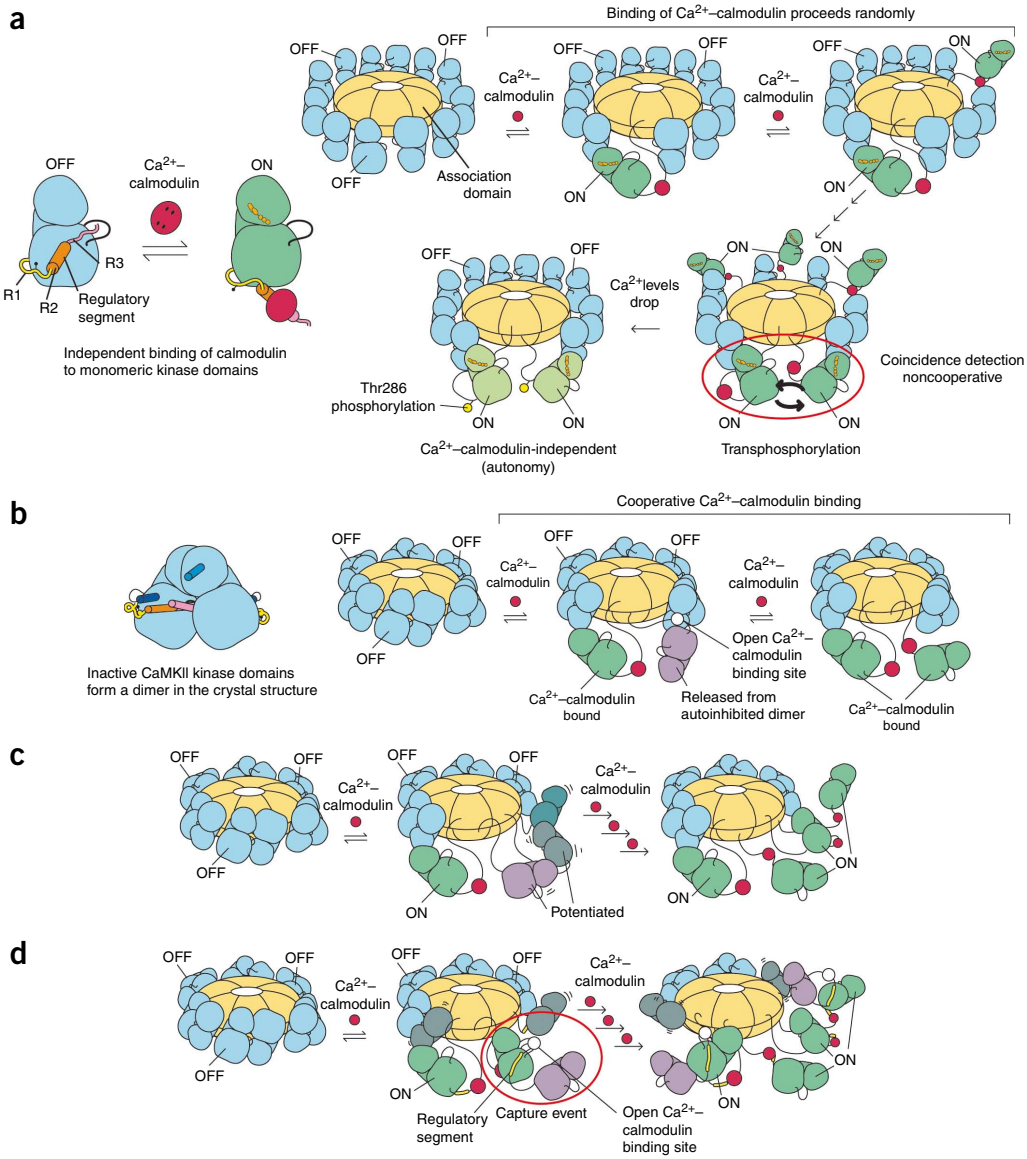

Figure 1 Cartoon schematic of models for CaMKII activation. (**a**) In a coincidence-detection model, Ca^{2+}–calmodulin binding and activation occurs stochastically. Activation without cooperativity allows for CaMKII transphosphorylation to occur only in response to coincident and adjacent Ca^{2+}–calmodulin binding events. It is not known if autophosphorylation is bidirectional for nearest-neighbor subunits. (**b**) Binding of Ca^{2+}–calmodulin is cooperative[7,8], and autoinhibited kinase domains form a dimer in the crystal[8]. In a holoenzyme comprising autoinhibited dimers, Ca^{2+}–calmodulin binding to one kinase in the dimer would release the Ca^{2+}–calmodulin binding site of a second kinase domain. (**c**) This work demonstrates that activation of CaMKII is cooperative; thus, CaMKII is not a simple coincidence detector. The level of cooperativity observed indicates sequential activation of kinase domains beyond dimers, where activation of a kinase subunit potentiates activation beyond a second subunit (shown as gray-blue subunits). (**d**) The capture of regulatory segments as substrates for adjacent kinase domains increases the cooperativity of activation. Only the activation and potentiation through subunit capture are shown, with any subsequent autophosphorylation omitted for simplicity.

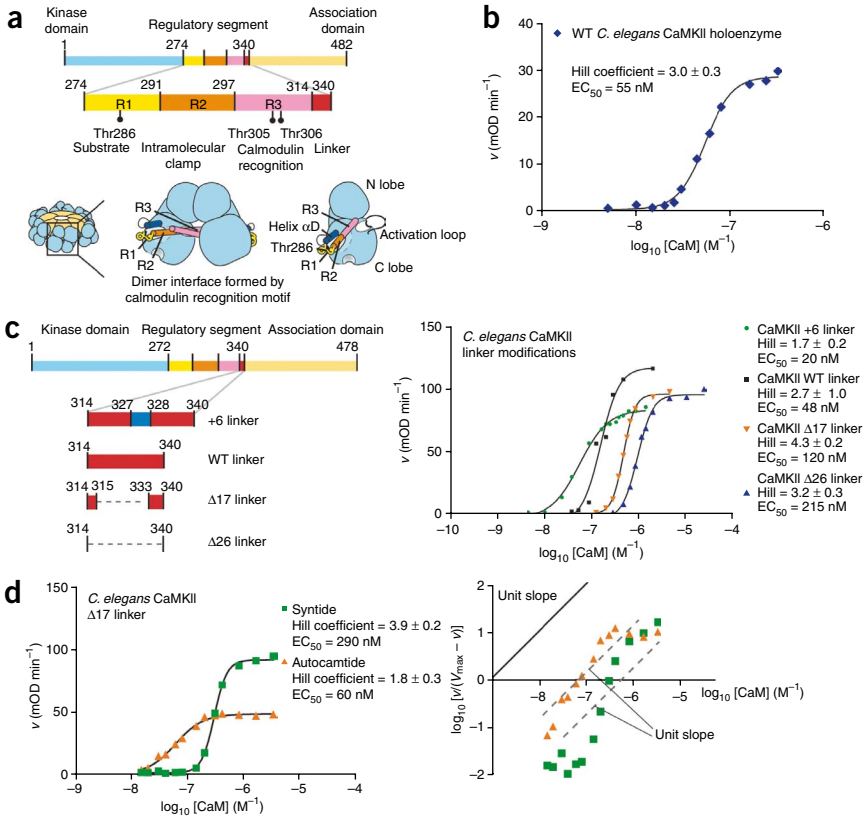

Figure 2 Cooperativity of CaMKII activation by Ca^{2+}–calmodulin. (**a**) Schematic diagrams of the CaMKII domain structure and regulatory segment arrangement in the autoinhibited state. Left, domain structure of CaMKII. The regulatory segment is enlarged, highlighting three elements: the R1 element, which contains the regulatory phosphorylation site Thr286; the R2 element, which clamps the regulatory segment to the kinase domain in the autoinhibited state; and the R3 element, which includes the calmodulin-recognition motif. Below, schematic diagram of the structure of the autoinhibited kinase domain: schematic of arrangement of autoinhibited dimers of the kinase domain in the CaMKII holoenzyme, with individual kinase domain enlarged. In the autoinhibited state, the R1 element is sequestered in a cleft below helix αD, and the R2 element positions helix αD to prevent substrate access to the active site. (**b**) Cooperative activation of *C. elegans* CaMKII holoenzyme occurs with a Hill coefficient of 3.0 ± 0.3. *C. elegans* holoenzyme phosphorylation of the peptide syntide was measured as a function of calmodulin concentration. (**c**) The Hill coefficient for *C. elegans* CaMKII activation for constructs with various linker lengths. The velocity of substrate phosphorylation at varying calmodulin concentrations is shown. The linker modifications within residues 314–340 are indicated in the schematic at left. In the +6 linker, six flexible residues were introduced to the middle of the linker region. In the Δ17 linker, 17 residues were deleted from the middle of the linker region. In the Δ26 linker, all 26 residues were removed from the linker region. (**d**) Cooperativity of calmodulin activation is reduced by autocamtide for the *C. elegans* CaMKII Δ17 deletion construct. Activity of CaMKII Δ17 was measured toward syntide and autocamtide and plotted on a velocity (v) versus $\log_{10}$[CaM] plot (right) and a $\log_{10}$ [$v/(V_{max} - v)$] versus $\log_{10}$ [CaM] plot (left). All error bars and ± terms expressed are s.e.m.

Why did the earlier reports conclude that activation of CaMKII by autocamtide was not cooperative? We find that although both autocamtide and syntide show cooperative activation, the observed degree of cooperativity is lower for autocamtide. As this peptide is based on the regulatory segment of CaMKII, we turned to an analysis of this segment's possible role in the cooperativity of the activation process. We have determined a new crystal structure of a truncated form of CaMKII in which the regulatory segment of one kinase (the substrate, bearing the critical Thr286 residue) is bound at the active site of the other (the enzyme). The structure shows that the regulatory segment from the substrate interacts extensively with the kinase domain of the enzyme. This raises the possibility that Ca^{2+}–calmodulin-bound subunits with open active sites can capture the regulatory segments of adjacent autoinhibited kinase domains, thereby causing the activation to 'spread' by potentiating the sequential binding of additional Ca^{2+}–calmodulin in a manner extending beyond dimeric units (**Fig. 1d**). We present biochemical evidence that supports a role for such a substrate-capture mechanism in increasing the cooperativity of Ca^{2+}–calmodulin binding to CaMKII. The reduction of apparent cooperativity that is seen with autocamtide might arise from interference with this mechanism, and the cooperativity that is actually present might simply have been missed in previous analyses.

How does the enzyme prevent runaway transphosphorylation upon Ca^{2+} stimulation, which would short-circuit frequency detection? By showing that the simple coincidence-detection model cannot be operative, our work emphasizes the need for future experiments aimed at explaining how the activity of the kinase domains is controlled in a way that sets the timing for transphosphorylation appropriately.

RESULTS

CaMKII activation is cooperative with exogenous peptide substrate

The essential component of CaMKII activation is displacement by Ca^{2+}–calmodulin of a regulatory segment that otherwise blocks the active site[9]. The regulatory segment follows the kinase domain and contains three elements denoted R1, R2 and R3 (**Fig. 2a**). Unlike many protein kinases, the catalytic activity of CaMKII does not depend on phosphorylation of the activation loop, located near the active site[19]. Instead, the regulatory segment blocks the active site in the absence of Ca^{2+}–calmodulin and sequesters the R1 element, bearing Thr286, in a channel adjacent to helix αD of the catalytic domain[8] (**Fig. 2a**). The sequence motif recognized by calmodulin begins seven residues downstream of Thr286 and extends through the R3 element. The R2 and R3 elements form an α helix, with the R2 element clamping the regulatory segment to the kinase domain.

Phosphorylation of Thr286 requires two Ca^{2+}–calmodulin binding events: one resulting in activation of the subunit that serves as the enzyme and the other releasing Thr286 in the substrate subunit[10,11]. The crystal structure of the autoinhibited CaMKII kinase domain shows a dimer in which the R2 and R3 elements of the regulatory segment form an intermolecular, antiparallel coiled coil[8]. The C-terminal lobes (C lobes) of the two kinase domains in the dimer are at either end of the ~40-Å-long coiled coil, which serves to keep the Thr286 residue in one kinase domain far away from the active site of the other (**Fig. 2a**). Isolated kinase domains without the association domain are monomeric in solution, even when the regulatory segment is present. The crystallographic dimer may only be formed in the holoenzyme, where kinase domains are at high local concentration.

Calmodulin binding releases the regulatory segment from the kinase domain, thereby exposing Thr286 and making it available for phosphorylation. The rebinding of the regulatory segment to the catalytic domain is prevented by Thr286 phosphorylation, even in the absence of Ca^{2+}–calmodulin[20]. The R3 element also contains two autophosphorylation sites, Thr305 and Thr306, which prevent rebinding of Ca^{2+}–calmodulin when phosphorylated.

We measured the activity of full-length wild-type *C. elegans* CaMKII holoenzyme (residues 1–482) toward two different peptide substrates as a function of Ca^{2+}–calmodulin concentration, using a continuous spectrophotometric assay to measure reaction progress (see Online Methods). One of these peptides, known as autocamtide, was used in the earlier work, and its sequence (KKALRRQETVDAL, with phosphorylation site underlined) is derived from the Thr286 autophosphorylation site of CaMKII[17]. The other peptide, known as syntide (PLARTLSVAGLPGKK), is derived from glycogen synthase, an exogenous CaMKII substrate[18]. We used saturating levels of Ca^{2+} (200 μM) so that the measurements report on the cooperativity of Ca^{2+}–calmodulin binding to CaMKII rather than on that of Ca^{2+} binding to calmodulin. Although the activation of CaMKII under subsaturating Ca^{2+} concentrations is likely to be relevant[21,22], it has been shown that the frequency response of CaMKII is preserved under conditions where calmodulin is saturated with Ca^{2+} (ref. 1). We used standard Hill analysis to determine cooperativity (**Supplementary Discussion**), and the Hill coefficient was determined by a numerical fit to the reaction velocity as a function of Ca^{2+}–calmodulin concentration (see Online Methods).

The EC_{50} value for Ca^{2+}–calmodulin was 55 ± 1.0 nM when syntide was used as a substrate, with a Hill coefficient of 3.0 ± 0.3 (**Fig. 2b**). For a system with two coupled binding sites, positive cooperativity results in Hill coefficients greater than 1.0 but less than 2.0 (**Supplementary Discussion**). The observation that the Hill coefficient is greater than 2.0 indicates that three or more subunits of the holoenzyme are coupled in the activation process.

There are more than 20 different forms of mammalian CaMKII as a result of alternative splicing leading to insertions and deletions in the linker region between the kinase and association domains[23]. Different isoforms are expressed in a tissue-specific and developmentally timed manner[9] and have different Ca^{2+} frequency responses to Ca^{2+}–calmodulin[24]. To examine the effect of linker length, we made constructs of *C. elegans* CaMKII in which we shortened the linker between the regulatory segment and the association domain, either by deleting 17 residues or by deleting the linker completely. We also lengthened the linker by inserting six residues that are expected to be flexible (with the sequence SAGSAS) between residues 327 and 328 (as indicated in **Fig. 2c**). The length of the linker has a marked effect on the Hill coefficient, with the shorter linkers yielding higher Hill coefficients (**Fig. 2c**). We obtained the maximum value of the Hill coefficient for the CaMKII Δ17 construct, in which 17 residues were deleted (Hill coefficient of 4.3 ± 0.2 compared to 2.7 ± 1.0 for the wild-type enzyme). Making the linker longer and more flexible decreases the Hill coefficient to 1.7 ± 0.2. The linker length varies in different isoforms of mammalian CaMKII, and our results suggest that this may reflect the tuning of the Ca^{2+} responsiveness between different isoforms.

We measured the activity of full-length mammalian CaMKII (*Sus scrofa* γ isoform) toward syntide as a function of increasing Ca^{2+}–calmodulin concentration. The Hill coefficient was 2.0 ± 0.2, showing that cooperative activation for syntide is also observed in a mammalian isoform (**Supplementary Fig. 1a**). The *S. scrofa* γ-isoform linker region is 15 residues longer than the wild-type *C. elegans* CaMKII, consistent with the observed inverse correlation between linker length and Hill coefficient (**Fig. 2c**). We also measured the activity of *C. elegans* CaMKII with all the major regulatory phosphorylation sites (Thr286, Thr305 and Thr306) mutated to alanine. The Hill coefficient for Ca^{2+}–calmodulin activation of this mutant form was 2.9 ± 0.6, indicating that the cooperative activation process does not require autophosphorylation (**Supplementary Fig. 1b**).

Previous measurements of the activation of the mouse α isoform of the CaMKII holoenzyme by Ca^{2+}/calmodulin used an end-point radiometric assay and autocamtide as a substrate and yielded an EC_{50} value of 48 ± 6 nM and a Hill coefficient of 1.1 ± 0.2 (ref. 16). By repeating the syntide measurements with a radiometric assay, we verified that our observation of positive cooperativity for syntide phosphorylation was not due to differences between the spectrophotometric and radiometric assays (**Supplementary Fig. 1c**). We used the spectrophotometric assay to compare the degree of cooperativity in Ca^{2+}/calmodulin activation of the *C. elegans* Δ17 construct with syntide and autocamtide as substrates. The Hill coefficient derived from these measurements was 3.9 ± 0.2 for syntide and 1.8 ± 0.3 for autocamtide (**Fig. 2d**). Thus, our new data show that the activation of CaMKII by Ca^{2+}/calmodulin is cooperative for both substrates, although the apparent degree of cooperativity is lower with autocamtide.

To visualize the degree of cooperativity more directly, a graph of $\log_{10}(f/[1-f])$ versus $\log_{10}[\text{CaM}]$, where f is the fraction of maximal specific activity, is shown for the activity of the *C. elegans* CaMKII Δ17 construct toward syntide (**Fig. 2d**). The Hill coefficient is the slope of $\log_{10}(f/[1-f])$ versus $\log_{10}[\text{CaM}]$, and the graph confirms that the Hill coefficient is ~3.0 for syntide as a substrate and ~2.0 for autocamtide.

Table 1 Data collection and refinement statistics

	Substrate complex form A (PDB 3KK8)	Substrate complex form B (PDB 3KK9)	CaMKIINtide complex (PDB 3KL8)
Data collection			
Space group	$P6_522$	$P2_1$	$P2_1$
Cell dimensions			
a, *b*, *c* (Å)	79.4, 79.4, 175. 5	37.7, 60.5, 70.2	72.4, 83.1, 145.1
α, *β*, *γ* (°)	90, 90, 120	90, 93.9, 90	90, 101.9, 90
Resolution (Å)	68.7–1.7 (1.8–1.7)[a]	50.00–3.2 (3.3–3.2)[a]	83.1–3.4 (3.6–3.4)[a]
R_{sym}	5.4 (59.6)[b]	12.7 (39.9)	16.1 (83.1)
$I/\sigma I$	33.7 (2.8)	9.93 (2.3)	5.3 (1.2)
Completeness (%)	99.6 (94.9)	97.7 (87.7)	98.3 (99.9)
Redundancy	8.8 (6.1)	3.5 (3.1)	3.4 (3.3)
Refinement			
Resolution (Å)	68.7–1.7 (1.8–1.7)	50.00–3.2 (3.3–3.2)	83.1–3.4 (3.6–3.4)
No. reflections (unique/measured)	35,411/578,066	5,171/8,043	21,523/282,717
R_{work} / R_{free}	17.2%/20.7%	27.0%/32.9%	23.3%/28.5%
No. atoms			
Protein	4,493	2,204	10,867
Water	258	–	80
B-factors			
Protein	37.34	91.22	75.71
Water	44.06	–	42.22
R.m.s. deviations			
Bond lengths (Å)	0.010	0.004	0.009
Bond angles (°)	1.086	0.798	1.266

[a]Data were collected from one crystal for each data set. [b]Values in parentheses are for highest-resolution shell.

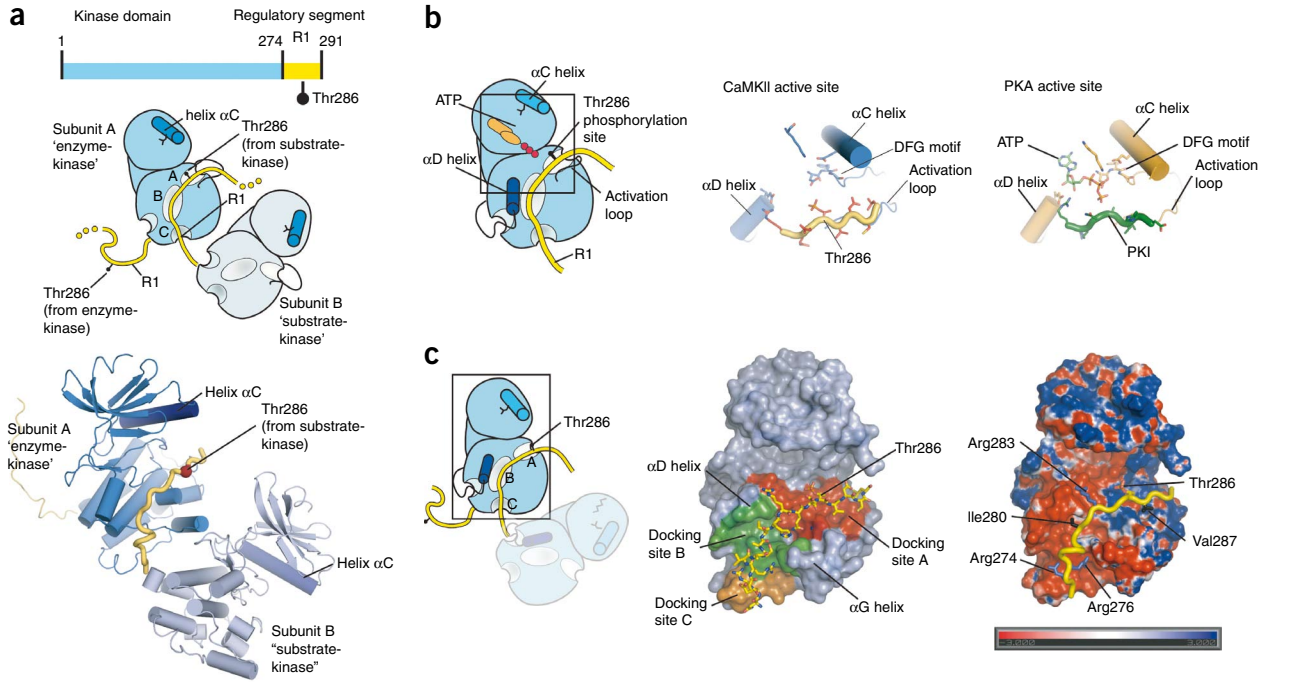

Figure 3 Crystal structure of the CaMKII enzyme–substrate complex. (**a**) Schematic diagram of the crystallized CaMKII enzyme–substrate complex. The construct contains the kinase domain of CaMKII and the R1 portion of the regulatory segment. The structure shown (at right) is that of crystal form A. (**b**) The CaMKII enzyme–substrate-complex active site is in the active conformation. Comparison of the major active-site components of the CaMKII enzyme–substrate complex with that of protein kinase A in the active state[32] (PDB 1ATP). (**c**) Docking sites used by the R1 element are indicated as docking sites A, B and C and are colored red, green and gold, respectively, on a surface representation of the kinase domain. The R1 element is shown in a sticks representation. At right, an electrostatic surface potential representation (produced with Adaptive Poisson-Boltzmann Solver tools[39]) of the CaMKII kinase domain in the enzyme–substrate complex illustrates a negatively charged region (red) encompassing docking sites B and C that is used by residues in the R1 element (basic residues shown in blue, hydrophobic residues shown in black).

We also measured the activity of *C. elegans* CaMKII holoenzyme toward autocamtide and syntide at saturating concentrations of Ca^{2+}–calmodulin. The K_M values for CaMKII toward autocamtide and syntide are 6.4 ± 0.8 μM and 150.0 ± 24 μM, respectively (**Supplementary Fig. 1d**). These results show that the reduction in cooperativity observed for autocamtide is correlated with a lower K_M value. Both sequences contain the core canonical serine/threonine recognition motif RXX(T/S)Φ (where X represents any residue and Φ represents a hydrophobic residue), indicating that interactions outside this motif are responsible for a lower K_M value for autocamtide as a substrate. Autocamtide is an artificial substrate based on the regulatory segment of CaMKII[17], so we wondered whether the observed differences between autocamtide and syntide could reflect a role for the regulatory segment in cooperativity. As noted above, phosphorylation of Thr286 is not required for cooperative activation, so the regulatory segment is not necessarily acting as a substrate in whatever process underlies cooperativity. The studies discussed below were aimed at dissecting the role of the regulatory segment in cooperativity.

The R1 element mediates intersubunit interactions upon activation

A CaMKII construct containing the kinase domain and intact regulatory segment (but lacking the association domain) was monomeric in solution at concentrations below 100 μM, as observed previously[8]. The addition of Ca^{2+}–calmodulin to this construct shifted the population toward a predominantly dimeric form, as determined by multiangle light scattering coupled to gel filtration (**Supplementary Fig. 2**). When the C-terminal portions of the regulatory segment (the R2 and R3 elements) were removed, the shorter construct (kinase and R1 element) was a multimer. A construct containing only the kinase domain, with the R1 element also removed, was monomeric at 100 μM concentration.

Structure of an enzyme–substrate complex of CaMKII

The light-scattering measurements suggest that release of the R1 element from the autoinhibitory interaction by Ca^{2+}–calmodulin allowed it to form an alternative intermolecular interaction. To define the nature of this interaction, we crystallized a CaMKII construct containing the kinase domain and the R1 element of the regulatory segment but lacking the R2 and R3 elements. This construct crystallized readily in multiple crystal forms, and the structures of two crystal forms were determined in the absence of nucleotide (forms A and B, **Table 1**). The structure in crystal form A was determined at 1.7-Å resolution and was refined to a conventional *R* value of 17.2% (free *R* value of 20.7%). The structure in crystal form B was refined at 3.2 Å (conventional and free *R* values of 27.0% and 32.9%, respectively).

In both structures, the R1 element of the regulatory segment of one kinase domain is presented for phosphorylation at the active site of another (**Fig. 3a**). This interaction is repeated in a chain throughout the crystal lattice, with the R1 element of one kinase (referred to as the 'substrate-kinase') inserted into the active site of the next one (referred to as the 'enzyme-kinase'). The in *trans* interaction of the regulatory segment with a second kinase domain was essentially the same in the two crystal forms, although the orientation between the enzyme-kinase and the substrate-kinase was different. Unambiguous features in electron-density maps indicate that the Thr286 residue was phosphorylated, something that presumably occurred during protein expression or purification.

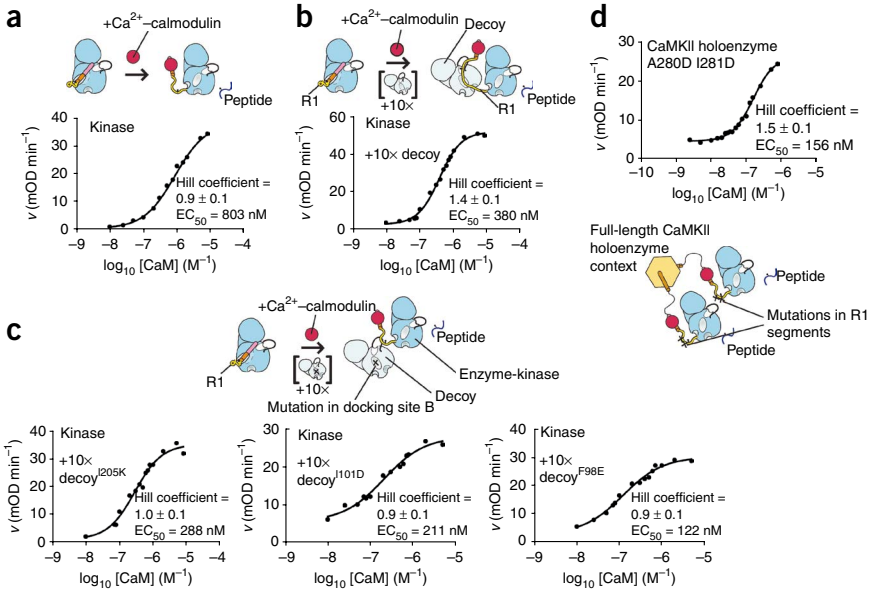

Figure 4 Capture of the regulatory segment results in cooperative activation of a monomeric kinase domain. (**a**) The activity of monomeric *C. elegans* CaMKII kinase domain toward syntide at varying calmodulin concentrations. The monomeric kinase domain does not show cooperative activation (data from a representative Ca^{2+}–calmodulin activation response shown). (**b**) Presence of a decoy kinase domain that is competent for capture of the R1 element results in cooperativity in the calmodulin activation of monomeric kinase domain. The decoy was added in 10× molar excess (100 nM) and has no enzyme activity because of mutations (D135N and K42M) introduced in the active site. (**c**) Mutations in docking site B of the decoy eliminate cooperative activation of the monomeric kinase domain. Data for three mutations (I205K, I101D and F98E, each introduced separately) are shown. (**d**) Mutation of residues (A280D and I281D, introduced together) in the R1 element in the *C. elegans* CaMKII holoenzyme results in a reduction in the Hill coefficient for Ca^{2+}–calmodulin activation. All error bars and ± terms expressed are s.e.m.

All 18 residues of the R1 element (residues 274–291) are visualized in electron-density maps, allowing identification of the connection between this element and the main body of the kinase domain from which it emanates. Of these residues, 16 (Arg276–Val291) made contacts with the C lobe of the enzyme-kinase. Asn273 formed a pivot between the substrate-kinase and the R1 element. This pivot is flexible, as indicated by comparison with the form B crystal structure.

While this manuscript was being prepared, a structure of a complex between calmodulin and a human δ CaMKII kinase-domain construct with all three regulatory elements present was deposited in the Protein Data Bank (PDB 2WEL) by the Structural Genomics Consortium. Intriguingly, this structure reveals a very similar chain of enzyme-substrate interactions, although the segment connecting the R1 element to the kinase domain (residues 275–280) is apparently disordered. Calmodulin is bound to the R3 element (which was not present in our structure) and does not make significant contact with the kinase domain. In considering this, along with other structures determined by our group and the Structural Genomics Consortium, a consistent picture of the interactions made by the regulatory segment emerges. When the intact regulatory segment (R1, R2 and R3) is present, but without calmodulin, the regulatory segments form a dimeric coiled coil[8]. When the calmodulin-recognition element (R3) is deleted but R1 and R2 are present, the R2 element enters and blocks the active site because a coiled coil can no longer be formed (seen in PDB 2VN9, 2VZ6, 2V7O and 3BHH). If calmodulin is bound to the R3 element, or if the R2 and R3 elements are deleted, an activated form of the kinase is obtained (the structures discussed in this paper and PDB 2WEL).

The kinase domain is in an active conformation[25,26] in our new structures (**Fig. 3b**). The last eight residues of the R1 element of the substrate-kinase (Arg283–Leu291) bind to the active site of the enzyme-kinase, as seen for other kinase-substrate complexes[26]. The last eight residues of the R1 element therefore form a canonical substrate-docking interaction, and we refer to the region of the kinase domain that engages this portion of the R1 element as docking site A (**Fig. 3c**).

There are two other interactions made by the R1 element on the enzyme-kinase. A hydrophobic pocket is located in the C lobe of the kinase domain, between helix αD and the C-terminal end of the activation loop, and it cradles Ile280 from the R1 element (**Fig. 3c**). This site, referred to as docking site B, is occluded in the autoinhibited form of the kinase due to rotation of helix αD[8].

A cluster of acidic residues is located at the very base of the kinase domain, near the C-terminal end of helix αD and alongside helix αG, and this cluster interacts with Arg274 and Arg276 of the R1 element. This region, referred to as docking site C, orients the R1 element as it leaves the substrate-kinase and enters the channel leading into the active site of the enzyme-kinase (**Fig. 3c**). Autocamtide contains several basic residues that are absent in syntide, and we speculate that these residues interact with docking sites B and C and are responsible for the lower K_M value and (to anticipate the discussion that follows) a reduction in cooperativity for autocamtide as a substrate. Basic residues N-terminal to the canonical recognition sequence are also found in high-affinity cellular CaMKII substrates, such as the *N*-methyl-D-aspartate (NMDA) glutamate receptor[27,28]. Use of docking site C in a high-affinity substrate interaction is consistent with the observation that autocamtide can compete with binding of the NMDA receptor tail, whereas syntide cannot[29].

Capture of regulatory segments results in cooperative activation

The extensive interactions between the R1 element of the substrate-kinase and the three docking sites on the enzyme-kinase suggest that the R1 element can perform two distinct roles in the regulation of CaMKII. In addition to its role in sequestering Thr286 in the autoinhibited state, the R1 element in one subunit might serve as a molecular grappling hook that can be captured by an adjacent activated kinase domain. Such a capture would potentiate Ca^{2+}–calmodulin binding to the first subunit, increasing the cooperativity of the process.

To test this idea, we measured the activity of isolated catalytic subunits without the association domain. Note that the isolated kinase domain, even with the regulatory elements R1 to R3 present, is monomeric in solution[8,30,31]; monomeric subunits are not capable of Thr286 transphosphorylation at the low concentrations (10 nM) we used in our assays.

We measured activity toward syntide substrate peptide for the construct lacking the association domain, obtaining an EC_{50} value of 803 nM with no cooperativity (Hill coefficient of 0.9 ± 0.1; **Fig. 4a**). MS analysis showed that, although our preparations of holoenzyme were not phosphorylated, constructs of the kinase domain lacking

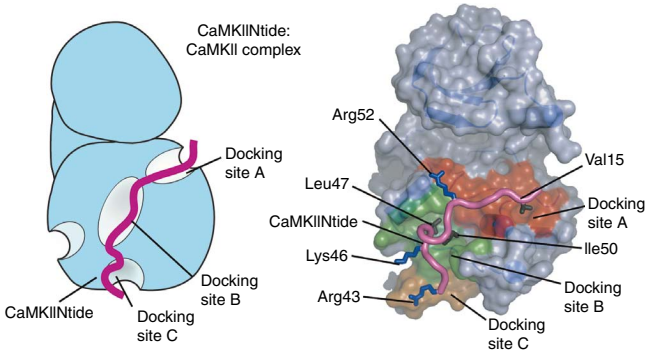

Figure 5 Structure of the CaMKII inhibitor CaMKIINtide bound to the kinase domain. Left, schematic diagram of the CaMKII kinase domain and the docking regions occupied by CaMKIINtide. Right, surface representation of the CaMKII kinase domain with the CaMKIINtide peptide bound; critical residues are highlighted. Basic residues (Arg43 and Lys46 (rat numbering for CaMKIINtide[40])) occupy docking sites C and B, Leu47 and Ile50 occupy docking site B and a pseudosubstrate recognition mode of interaction is observed in docking site A (Arg52 at the P–3 site, Val56 at the P+1 site).

the association domain were partially phosphorylated on Thr305 and Thr306 (data not shown). This likely accounts for the increased value of EC_{50} for activation by Ca^{2+}–calmodulin, which was approximately tenfold higher than that observed for Ca^{2+}–calmodulin binding to a kinase-dead form of this construct[8].

We added a tenfold excess (100 nM) of a kinase domain, inactivated by mutation (D135N K42M), that lacks the regulatory segment. This 'decoy' kinase domain has docking sites A, B and C unoccupied and cannot bind to Ca^{2+}–calmodulin. The presence of the decoy lowers the EC_{50} values of Ca^{2+}–calmodulin to 380 nM, consistent with capture of the R1 element by the decoy and facilitation of Ca^{2+}–calmodulin binding to the autoinhibitor (**Fig. 4b**). Activation of the kinase domain shows apparent cooperativity with respect to the concentration of Ca^{2+}–calmodulin, with a Hill coefficient of 1.4 ± 0.1.

The nonhyperbolic response of the CaMKII kinase domain to Ca^{2+}–calmodulin in the presence of the decoy protein implies that the decoy protein and Ca^{2+}–calmodulin mutually facilitate the binding of each other to the enzyme. Binding of calmodulin to the enzyme releases its R1 element, which can then bind to the alternative docking sites on the decoy protein. In a reciprocal fashion, binding of the decoy protein to the R1 element releases the R2 and R3 elements for interaction with Ca^{2+}–calmodulin. We have modeled this system with a kinetic scheme, which predicts that the Hill coefficient for Ca^{2+}–calmodulin activation of the enzyme should be greater than 1.0 but less than 2.0 (**Supplementary Discussion**).

If the decoy subunit is mutated in docking site B to prevent binding of the R1 element (I205K, I101D and F98E, each mutation introduced separately), the activation by Ca^{2+}–calmodulin is no longer cooperative in the presence of the decoy (**Fig. 4c**). Mutation of residues in the R1 element that interact through docking site B (A280D and I281D, introduced together) in the full-length *C. elegans* holoenzyme likewise results in a reduction of the Hill coefficient for Ca^{2+}–calmodulin-dependent activation to 1.5 ± 0.1 (compared to a Hill coefficient of 3.0 ± 0.3 for the wild-type holoenzyme), consistent with capture of the R1 element being one component of cooperative activation (**Fig. 4d**).

At the high local concentrations of the subunits within the holoenzyme, the R1 element could block the access of exogenous substrates by acting as a product inhibitor. This is consistent with the observation that the activity of Thr286 phosphorylated CaMKII in the absence of Ca^{2+}–calmodulin is only 30–70% of that of fully activated Ca^{2+}–calmodulin-bound CaMKII[11].

A CaMKII inhibitor mimics interactions made by the R1 element

There is considerable similarity between the substrate-like binding mode of the R1 element of CaMKII and the docking of the protein kinase inhibitor PKI to protein kinase A (PKA)[32]. CaMKIINtide is a peptide inhibitor of CaMKII derived from the protein CaMKIIN, whose tissue-specific expression correlates tightly with that of CaMKII[33]. A peptide inhibitor corresponding to residues 281–309 of the regulatory segment inhibits the activity of the isolated CaMKII kinase domain toward syntide with a K_i value of 0.2 μM (ref. 20). We also measured CaMKIINtide inhibition of CaMKII kinase-domain activity toward syntide, and we obtained an IC_{50} value of 0.12 μM. We observe that CaMKIINtide is a competitive inhibitor of *C. elegans* CaMKII with respect to autocamtide (**Supplementary Fig. 3**), as reported by others for the mammalian enzyme[33]. Mutational analysis has indicated that CaMKIINtide interacts with docking site B[34], but the primary sequence of CaMKIINtide does not indicate an obvious mode of inhibition.

We determined the crystal structure of a 21-residue segment of CaMKIINtide bound to the kinase domain of CaMKII at 3.4-Å resolution, and we observed a mode of interaction very similar to that observed for the in *trans* interaction of the R1 element (**Fig. 5**). Instead

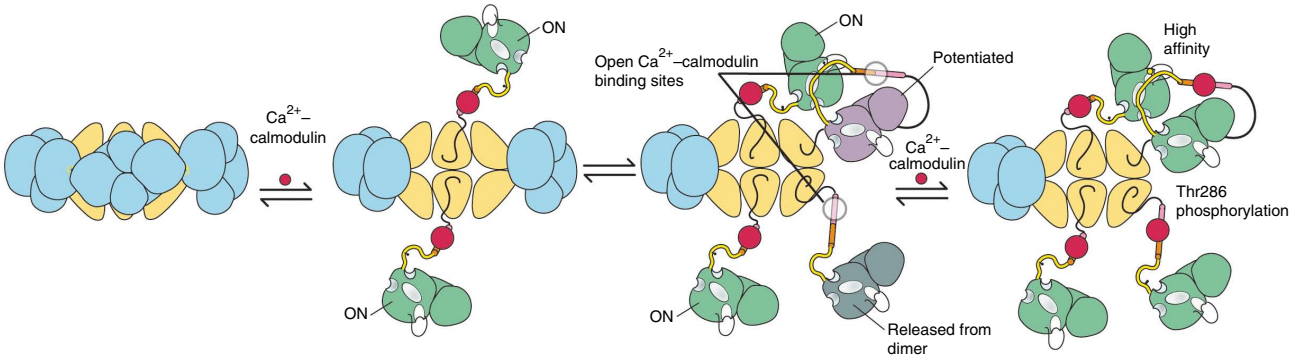

Figure 6 A hypothetical mechanism for CaMKII activation. If the time interval between Ca^{2+} spikes is long compared to the dissociation time of Ca^{2+}–calmodulin (low frequency), initial binding is presumed to lead to a separation of kinase domains; however, transphosphorylation of Thr286 does not occur before Ca^{2+}–calmodulin dissociates. If the interval between Ca^{2+} spikes is short compared to the Ca^{2+}–calmodulin dissociation time (high-frequency regime) Ca^{2+}–calmodulin remains bound to the first pair of kinase domains long enough for a second, slow step to occur, in which the activated kinase domains capture the regulatory segments of adjacent kinase domains. This potentiates the binding of Ca^{2+}–calmodulin to those domains with increased affinity, resulting in the phosphorylation of Thr286 and acquisition of autonomy (Ca^{2+}–calmodulin-independent activity).

of threonine, the inhibitor has an arginine residue at the P0 site and therefore cannot be phosphorylated. Several hydrophobic and basic residues are present in the N-terminal portion of the inhibitor and confer specificity for the CaMKII kinase domain using interactions in docking sites B and C (**Fig. 5**). The visualization of a mode of inhibition that is similar to the R1 interaction suggests that CaMKIINtide, in addition to inhibiting the activated subunit, would also block the capture of adjacent regulatory segments. Whereas CaMKIINtide can block peptide substrate phosphorylation, it apparently does not block autophosphorylation[34], suggesting that high CaMKIINtide concentrations would be necessary to block intersubunit capture interactions from occurring within the holoenzyme. At subsaturating levels of inhibition, it is conceivable that different expression levels of CaMKIINtide could tune the cooperative response of CaMKII.

DISCUSSION

The observation that the activation of CaMKII by Ca^{2+}–calmodulin is cooperative means that adjacent kinase domains are activated preferentially over those located more distantly within the holoenzyme. This rules out a simple coincidence-detection mechanism such as the one depicted in **Figure 1a**. Our previous analysis of the structural basis for cooperativity in CaMKII focused on the role of autoinhibited kinase-domain dimers in the process[8]. If the binding of two molecules of Ca^{2+}–calmodulin affects only two kinase subunits, then the value of the Hill coefficient should be between 1.0 and 2.0. Our new data, which show that the Hill coefficient for activation is greater than 2.0 for many CaMKII constructs, suggest that there are additional interactions between dimers in the holoenzyme, such that disruption of one dimer facilitates the disruption of additional dimers.

A reduction in the Hill coefficient was observed when autocamtide, the peptide derived from the regulatory segment, was used as a substrate. For the construct with the greatest degree of cooperativity (CaMKII Δ17; see **Fig. 2d**), the Hill coefficient was reduced from ~4.0 with syntide to ~1.8 with autocamtide. This is consistent with a role for autocamtide in weakening interactions between dimers. Our finding that the R1 element of the regulatory segment makes extensive interactions with another kinase domain in a *trans* configuration suggests that an activated kinase domain can capture the regulatory segment of an adjacent kinase domain, potentiating the binding of Ca^{2+}–calmodulin to it. This is supported by the results of experiments in which a decoy kinase domain provides open binding sites for the regulatory segment, resulting in cooperativity for the activation process. The higher affinity of autocamtide compared to syntide for the substrate binding site is expected to impair the efficiency of this process.

Our results bring to the forefront the question of how an appropriate time delay is introduced in the transphosphorylation of Thr286, a critical parameter in determining the sensitivity of the CaMKII holoenzyme to the frequency of Ca^{2+} spikes[1]. Resolution of this issue awaits further experimentation, but one clue is provided by dramatic structural differences between two states of CaMKII that have been noted in electron-microscopic reconstructions from different groups[35,36]. In one set of reconstructions, the kinase domains appear to be arranged in the central plane of the holoenzyme assembly[36]. This is consistent with SAXS analysis of inactive CaMKII[8], which led to a model in which dimeric autoinhibited kinase domains form an outer ring around the central hub of association domains. In another set of reconstructions, the kinase domains are located in two rings, one well above the central plane and one below it[35].

We speculated previously that Ca^{2+}–calmodulin binding and the subsequent disruption of kinase-domain dimers might result in these domains moving apart from each other, above and below the association domain ring[8]. This is consistent with the increased distance between kinase domains that results from activation, as inferred from FRET measurements[37], and is also consistent with the second set of electron-microscopic reconstructions[35]. In such a mechanism for setting an autophosphorylation time delay, the first pair of kinase domains that are activated are prevented from phosphorylating each other because they are located on opposite sides of the central plane (**Fig. 6**). Transphosphorylation would await the release of the next pair of kinase domains, aided by the substrate-capture mechanism that is suggested by our data. A clearer picture as to how the timing of this step is determined will emerge after the kinetic rate constants for the various steps are measured. One exciting possibility for the future is that the frequency-dependent process may be monitored directly in neuronal dendrites, as highlighted by a recent study on the *in situ* activation of CaMKII by laser-stimulated Ca^{2+} spikes[38].

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Accession codes. Protein Data Bank: Coordinates for substrate complex crystal form A, substrate complex crystal form B and CaMKIINtide complex were deposited with accession codes 3KK8, 3KK9 and 3KL8.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

L.H.C., P.P., H.S. and J.K. designed the experiments; L.H.C., P.P. and L.A.B. performed the experiments and analyzed the data; S.D. performed the Hill coefficient analyses; L.H.C. and J.K. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. We developed a bacterial expression system for CaMKII by coexpression with Lambda Phosphatase, using a strategy similar to that for bacterial expression of certain tyrosine kinases[41]. We obtained yields of 2.5 μg of pure active holoenzyme per liter of bacterial culture, compared to 5–10 μg per liter of insect cell culture[8]. We expressed CaMKII in *Escherichia coli* by coexpression with λ phosphatase (kind gift of J. Dixon) in Tuner(DE-3)pLysS cells (Novagen). We subcloned λ phosphatase into a pCDFDuet1 vector (Novagen) and C-terminally cloned hexahistidine-tagged CaMKII and its mutant forms into a pET-20b vector (Novagen). We induced protein expression in bacteria by the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside and 0.5 mM Mn^{2+}. Cells were grown for 18 hours at 20 °C and flash frozen until used. The average yield was 2 μg of protein per liter of cells at >95% purity as judged by SDS-PAGE and Coomassie stain. We fused autoinhibited constructs of CaMKII (residues 1–340) and other truncations (residues 1–274 and residues 1–291) of the *C. elegans unc-43* gene to an N-terminally cleavable hexahistidine tag and cloned them as described previously[8].

Our experiments focused on the *C. elegans* enzyme because crystal structures were determined initially using this enzyme[8]. We repeated key experiments using mammalian enzyme from *S. scrofa*. Crystal structures for truncated forms of the human α, β, δ and γ isoforms of the human CaMKII autoinhibited kinase domain, determined by the Structural Genomics Consortium, are also available (PDB codes 2VN9, 2VZ6, 2V7O and 3BHH, respectively). The sequence identities within the kinase domain and the association domain between the *C. elegans* and human enzymes are ~80% and ~50%, respectively.

We purified the CaMKII holoenzyme and the kinase domain constructs by nickel–nitriloacetic acid affinity chromatography using a HiTrap HisBind column (Pharmacia) followed by anion exchange and S200 size-exclusion chromatography. The final buffer from the gel filtration was 25 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.3), 250 mM potassium chloride, 10% (v/v) glycerol and 1 mM tris(2-carboxyethyl)phosphine. We also expressed wild-type, full-length *C. elegans* CaMKII (encoded by the *unc-43* gene) in Sf9 insect cells using a baculovirus expression system as described previously[8]. The enzyme purified from the bacterial source, used in the experiments described here, behaves similarly to that purified from insect cell culture, as assessed by MS, analytical gel filtration and enzyme activity assays (data not shown). We expressed and purified *Gallus gallus* calmodulin as previously described[42] and determined its final concentration by amino acid analysis (Molecular Structure Facility, University of California, Davis). We expressed the *S. scrofa* γ isoform of CaMKII in Sf9 cells using a baculovirus expression system[8] and purified it as described above.

Crystallographic analysis. We grew crystals of $CaMKII_{1–289}$ using sitting drop vapor diffusion. We obtained crystals with 0.1 M potassium chloride, 5 mM magnesium sulfate, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0) and 15% (v/v) 2-methyl-2,4-pentanediol. We grew crystals of $CaMKII_{1–272}$ and CaMKIINtide in 20% (v/v) 2-methyl-2,4-pentanediol. We cryoprotected crystals in 25% (v/v) glycerol before freezing them in liquid nitrogen. We collected X-ray diffraction data at the Advanced Light Source beamline 8.2.2 at 100 K at the wavelength 0.9537 Å.

We solved structures by molecular replacement with Phaser[43], using the autoinhibited kinase domain of $CaMKII_{1–278}$ as a search model. We performed refinement with CNS[44] and PHENIX[45] and we performed model building with O[46] and Coot[47].

Enzyme activity assays. We monitored kinase activity using a continuous spectrophotometric assay as described earlier[48]. In this assay, ADP that is produced as a result of phosphorylation by the enzyme is coupled to the oxidation of NADH to NAD^+, which produces a decrease in absorbance at 340 nm. We carried the assays out in 100 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), 150 mM potassium chloride, 0.2 mM calcium chloride, 10 mM magnesium chloride, 0.5 mM ATP, 1 mM phosphoenolpyruvate, 0.28 mM NAD, 89 units ml^{-1} pyruvate kinase, 124 units ml^{-1} lactate dehydrogenase, 0.3–0.5 mM of the peptide substrates autocamtide AC-3 (KKALHRQETVDAL) or syntide (PLARTLSVAGLPGKK) and various concentrations of calmodulin at 30 °C in a 150-μl reaction volume. D. King (Howard Hughes Medical Institute) prepared peptides. We initiated reactions by the addition of 10–20 nM CaMKII to the mix and monitored the decrease in absorbance at 340 nm at 30 °C in a microtiter-plate spectrophotometer (SpectraMax). The enzyme concentration is expressed in terms of the concentration of kinase units and not holoenzymes. In addition, we also measured CaMKII activity using a phosphocellulose filter–binding assay and [^{32}P]ATP as previously described[16].

We plotted and analyzed cooperativity curves using the program Prism (version 5, GraphPad Software). We fit the data to the Hill equation:

$$Y = Y_{\min} + \frac{(Y_{\max} - Y_{\min})}{1 + \left(\frac{10^{(\log_{10} \mathrm{EC}_{50})}}{10^{(\log_{10}[L])}} \right)^{n}}$$

where Y is the maximal velocity, EC_{50} is the concentration at half maximal velocity, $[L]$ is the ligand concentration of calmodulin and n is the apparent Hill coefficient.

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