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Structural studies on the regulation of Ca²⁺/calmodulin dependent protein kinase II

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Abstract

Ca²⁺/calmodulin dependent protein kinase II (CaMKII) is a broadly distributed metazoan Ser/Thr protein kinase that is important in neuronal and cardiac signaling. CaMKII forms oligomeric assemblies, typically dodecameric, in which the calcium-responsive kinase domains are organized around a central hub. We review the results of crystallographic analyses of CaMKII, including the recently determined structure of a full-length and autoinhibited form of the holoenzyme. These structures, when combined with other data, allow informed speculation about how CaMKII escapes calcium-dependence when calcium spikes exceed threshold frequencies.

Introduction

The pioneering investigations of Bruce Kemp and colleagues introduced the pseudosubstrate hypothesis for how calcium-responsive protein kinases are regulated by Ca²⁺/calmodulin (Ca²⁺/CaM) or its paralogs [1]. These Ca²⁺-dependent kinases have C-terminal autoinhibitory segments that resemble the protein substrates of the enzyme in certain respects, but lack the canonical phosphorylation sites found in true targets. Because these segments are present at high local concentration with respect to the active site, they bind to it and block substrate access. Adjacent to the pseudosubstrate motif, or spanning it, is a recognition element for Ca²⁺/calmodulin, or paralogs such as S100 and troponin C. When Ca²⁺ levels rise, the Ca²⁺/CaM complex (or a related one) binds to the autoinhibitory segment of the kinase and displaces it, thereby activating the enzyme.

Beginning with the structural and biochemical analyses of the giant protein kinase twitchin by Kemp and co-workers [2,3] and followed by the determination of the structure of phosphorylase kinase [4], CaMKI [5] and titin kinase [6], the general features of this mechanism have been validated. The autoinhibitory segments in these different enzymes do

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not always block the site of phosphate transfer, but in all cases the entrance groove to the catalytic center, as first defined by the structure of cAMP-dependent protein kinase (PKA) bound to a peptide inhibitor (PKI) [7], is blocked by the autoinhibitory segment.

For most of these kinases, such as twitchin and CaMKI, the catalytic activity is directly related to Ca^{2+} levels, rising and falling as the Ca^{2+} levels increase and decrease. In contrast, Ca^{2+} /CaM dependent protein kinase II (CaMKII) has the ability to acquire Ca^{2+} independence, referred to as autonomy, when activated strongly by Ca^{2+} [8,9]. This step is sensitive to the frequency of the Ca^{2+} spike trains that activate CaMKII and is due to autophosphorylation [10]. If subjected to short Ca^{2+} spikes at low frequency (*e.g.*, < 1 Hz), CaMKII reverts to a quiescent state in the absence of Ca^{2+} . If, however, the Ca^{2+} spike train is at higher frequency (*e.g.*, >10 Hz) with the same total exposure to Ca^{2+} , the enzyme acquires Ca^{2+} independence and is able to phosphorylate substrates even when Ca^{2+} levels subside. CaMKIV, a monomeric Ca^{2+} /CaM-dependent protein kinase, also has the ability to acquire some calcium independence upon phosphorylation by Ca^{2+} /CaM-dependent protein kinase kinase (CaMKK) [11]. The mechanism by which CaMKIV is regulated is quite different from that of CaMKII, and is not discussed further.

The acquisition of autonomy prolongs the active state of CaMKII and is likely to be critical for the generation of long-term potentiation (LTP), a strengthening of synaptic connections that underlies synaptic plasticity in learning and memory [12]. Transgenic mice deficient in neuronal CaMKII or mutated at critical phosphorylation sites within the autoinhibitory segment have limited LTP generation and display impairments in learning and memory [13,14].

Early electron microscopic images revealed that CaMKII forms symmetric holoenzyme assemblies, usually dodecameric (Fig. 1a) [15–17]. Each subunit of CaMKII within the holoenzyme is comprised of a kinase domain, a regulatory segment, and a hub domain (also referred to as the association domain) (Fig 1b). The hub domain is necessary for oligomerization, and it acts as a central scaffold point for the kinase domains. There exists a diversity of CaMKII isoforms in mammals; four CaMKII genes in humans, termed α , β , γ , and δ , give rise to ~40 isoforms through alternative splicing [18]. The kinase and hub domains of the four human isoforms are closely related in sequence (~95% and ~80% identity, respectively), and the most striking difference between these isoforms is in the length of the linker connecting the kinase domain to the hub domain, which ranges from no residues to ~30 residues. The α and β isoforms are found predominantly in neurons, while the γ and δ isoforms are found throughout the rest of the body.

The importance of CaMKII in neuronal processes is well documented [19–21]. Important insights into how it functions have been obtained by directly visualizing CaMKII in neurons, where it is one of the most highly expressed proteins. One striking example is a study in which fluorescently labeled CaMKII was shown to translocate to the pre-synaptic sites of dendrites upon stimulation by glutamate, which activates specific receptors at the synapse, including the NMDA receptor [12]. Active CaMKII binds the NMDA receptor, which locks CaMKII in an active conformation [22]. A particularly impressive study used laser-pulsing and caged glutamate to induce Ca^{2+} spikes in dendrites, with simultaneous observation of the activation of CaMKII, using a FRET-based reporter [23]. It is clear from these studies and others, (see, for example, [24–29]) that CaMKII has the ability to respond to the frequency and not just the amplitude of Ca^{2+} spikes, and this property is likely to be important for its role in LTP.

CaMKII is important for the mechanical and electrical properties of cardiac cells, where the α isoform is prevalent [30]. Increased CaMKII autophosphorylation, along with increased

transcription and expression, is associated with atrial fibrillation, arrhythmias, hypertrophy and heart failure [31–35]

Considering the size of the CaMKII holoenzyme (~700 kDa), the first steps toward understanding its atomic-level structure were to break it down into more manageable pieces. Thus, the crystal structures of the hub domain assembly and kinase domain alone were the first determined separately [36,37]. Recently, the crystal structure of an intact holoenzyme assembly was determined in the autoinhibited form, in the absence of $\text{Ca}^{2+}/\text{CaM}$ [38]. Electron microscopy (EM) [15–17] and small angle X-ray scattering (SAXS) [36] studies have also been critical for piecing together the properties of the holoenzyme.

Structures of the CaMKII kinase domain

CaMKII has a canonical Ser/Thr kinase domain, but one distinguishing feature is that the activation loop of CaMKII does not contain a phosphorylation site. In contrast to canonical kinases, in which the activation loop is stabilized in an active conformation by phosphorylation, in CaMKII this loop adopts an active conformation without phosphorylation. Instead, phosphorylation control in CaMKII is mediated by the regulatory segment, which occludes the active site in the absence of $\text{Ca}^{2+}/\text{CaM}$ or when unphosphorylated.

The regulatory segment contains the CaM binding domain as well as three key sites of regulatory autophosphorylation: Thr 286, Thr 305 and Thr 306 (mouse isoform numbering) (Fig 1b). Thr 286 is autophosphorylated after $\text{Ca}^{2+}/\text{CaM}$ binding, which results in autonomous $\text{Ca}^{2+}/\text{CaM}$ -independent activity [39,40] (Fig. 1c). The subsequent dissociation of $\text{Ca}^{2+}/\text{CaM}$ leads to autophosphorylation of residues 305 and 306, which prevents reassociation of $\text{Ca}^{2+}/\text{CaM}$ because these residues lie within the CaM binding interface [41]. Interestingly, Met 281 (as well as Met 282 in some isoforms), which lies in the regulatory segment, is sensitive to an oxidation that generates an autonomous kinase with functional implications in cardiac tissue [42,43].

Several crystal structures of autoinhibited forms of CaMKII kinase domains have been determined. Before proceeding to a discussion of these structures, we note that there is some controversy in the literature about the interpretation of the role of dimerization of the CaMKII kinase domain in autoinhibition. Our group determined the first structure, that of *C. elegans* CaMKII, and showed that the regulatory segments form a coiled-coil dimer in the crystal [36]. We had noted in our original paper that there is no evidence that the isolated autoinhibited kinase domain dimerizes in solution, and so the potential relevance of the crystallographic dimer might manifest itself only in the context of the assembled holoenzyme, where the local concentration of kinase domains is extremely high. Subsequent crystallographic analysis of autoinhibited mammalian CaMKII kinase domains [40] and electron paramagnetic resonance (EPR) analysis of the isolated *C. elegans* CaMKII kinase domain [44] found no evidence for coiled-coil formation of the regulatory segment, but this may simply be a consequence of the lack of assembly into an intact holoenzyme. The CaMKII assembly is highly dynamic, and we feel that it is premature to conclude that the dimer seen in the *C. elegans* structure is a crystal artifact, as has been suggested [44]. Resolution of this apparent disparity awaits further dissection of the role of the regulatory segment in intact CaMKII holoenzymes.

Crystal structures of the isolated kinase domain of CaMKII can be classified into two types: those with the active site blocked by the regulatory segment and those with released regulatory segments [38,40]. In the first category, there is the structure of *C. elegans* kinase domain with the full-length regulatory segment (Fig. 2a) (PDB: 2BDW). As noted above, the *C. elegans* kinase domain forms a dimer in the crystal, mediated by a coiled-coil formed

by the regulatory segments. There are also several structures of human CaMKII kinase domains bound to small molecule inhibitors in this category, and these do not form similar dimers (isoform crystallized: PDB code, : 2VZ6, : 3BHH, : 2VN9 (Fig. 2b), : 2V7O) [40]. There is also a structure of the *C. elegans* kinase domain bound to a peptide inhibitor (PDB: 3KL8). In the second category, there is a structure of the human isoform crystallized in the presence of $\text{Ca}^{2+}/\text{CaM}$ (PDB: 2WEL), in which the portion of the regulatory segment spanning Thr 286 of one kinase is bound to the active site of another. Autophosphorylation at Thr 286 in the regulatory segment requires two kinases, one serving as the enzyme and one as the substrate [45]. This structure represents the enzyme-substrate complex that is formed during autophosphorylation, upon release of the regulatory segment by $\text{Ca}^{2+}/\text{CaM}$. A similar enzyme-substrate complex has been obtained for *C. elegans* CaMKII, but without bound $\text{Ca}^{2+}/\text{CaM}$ (PDB: 3KK8 and 3KK9).

Dimerization of the autoinhibited kinase domain by the coiled-coil formed by the regulatory segment, as seen in the structure of autoinhibited *C. elegans* CaMKII, is expected to keep the kinase inactive even when the kinases are released from the hub (Fig. 2c) [36]. In particular, such a dimeric arrangement would prevent autophosphorylation of Thr 305 and Thr 306, which could otherwise occur in *cis* (slow autophosphorylation of Thr 305 does occur under basal conditions [46]). Formation of such a dimer would resist activation because the CaM binding region is occluded in the coiled-coil [5,47]. The physiological relevance of this dimer has yet to be demonstrated, and the importance of the coiled-coil interface is difficult to probe by mutation because it contains residues that are critical for $\text{Ca}^{2+}/\text{CaM}$ binding. Additionally, the high concentrations achieved in crystallization may mimic what is imposed by the dodecameric structure, a condition that is difficult to achieve *in vitro* with the kinase domain alone.

The other crystal structures of the CaMKII kinase domain do not reveal dimerization interfaces between kinase domains. Although dimerization of the kinase domains has been shown *in vivo* in the context of the holoenzyme [48,49] and *in vitro* with the kinase domain alone [40], evidence for coiled-coil formation is lacking. It should be noted that two of these structures of the autoinhibited kinases (PDB: 2VZ6 and 3BHH) are truncated constructs, which exclude portions of the regulatory domain that would comprise the coiled-coil.

A particularly interesting structure is that of the autoinhibited CaMKII kinase domain (PDB: 2VN9), which does contain the entire regulatory sequence. The CaM binding region is no longer helical and, interestingly, Thr 307 (corresponding to 306 in the standard numbering) in this construct is placed precisely where a threonine residue in a substrate would be located during a phosphorylation reaction (Fig. 2b). This structure resembles the pseudosubstrate model originally proposed by Kemp, and a detailed analysis by EPR of the regulatory segment in the isolated *C. elegans* kinase domain supports the idea that autoinhibition involves interactions between the regulatory segment and the ATP-binding region of the kinase [44]. It should be noted, however, that in contrast to a strict pseudosubstrate model, the regulatory segment in this structure behaves like a proper substrate, and the adoption of this conformation is likely to result in phosphorylation of Thr 307. Phosphorylation of Thr 307 would block $\text{Ca}^{2+}/\text{CaM}$ binding, so this configuration presumably occurs at a later stage, after autonomy has been achieved. Conversely, in the autoinhibited dimer structure of the *C. elegans* enzyme, the corresponding threonine residue (306) is far from the active site and its phosphorylation within the dimer is prevented by the coiled-coil (Fig. 2c). Establishing the importance of these different conformations of the regulatory segment in the context of the complete reaction cycle of CaMKII awaits further study.

The activation of CaMKII by $\text{Ca}^{2+}/\text{CaM}$ is highly cooperative [50]. One component of this cooperativity arises from interactions between the regulatory segment in one kinase and the peptide-recognition groove of another [51]. In structures where this interaction is seen (PDB: 2WEL, 3KK8, 3KK9), the portion of the regulatory segment that is upstream of Thr 286 interacts in *trans* with the substrate-binding groove of another kinase (this groove is occupied by the portion of the regulatory segment that is downstream of Thr 286 in autoinhibited forms of CaMKII). This suggests a substrate-capture mechanism in which the binding of $\text{Ca}^{2+}/\text{CaM}$ and consequent release of the autoinhibitory segment in one subunit (the “enzyme”) facilitates the capture of the regulatory segment from another subunit to which $\text{Ca}^{2+}/\text{CaM}$ has not yet bound (the “substrate”) (Fig. 3). This would facilitate binding of $\text{Ca}^{2+}/\text{CaM}$ to the “substrate” subunit, leading to cooperativity. Biochemical analysis supports the existence of such a mechanism [51].

The central hub

The kinase domains in the CaMKII holoenzyme are presented as protrusions from the central hub, forming a double-layered ring. Such an organization, in which a central hub displays catalytic domains that are splayed outwards, appears to be unusual, although EM reconstructions of the apoptosome display an architecture somewhat similar to that of CaMKII [52,53].

There are several crystal structures of the hub domain of CaMKII, which show two different stoichiometries. Crystallization of mouse CaMKII and *C. elegans* hub domains alone revealed a tetradecameric assembly of two 7-membered rings stacked head to head [37,54]. Importantly, EM images of the full-length mouse CaMKII holoenzyme demonstrated that the assembly has sixfold symmetry, consistent with it being a dodecamer [54]. When the kinase domains were released from the hub by proteolytic cleavage, the resulting hub assembly was shown to have seven-fold symmetry. Thus, the hub assembly can convert from a dodecameric assembly to a tetradecameric one when the kinase domains are removed. These findings suggest there is some strain imposed on the hub domain ring by the kinase domains. The human CaMKII isoform hub domain has been crystallized in both dodecameric and tetradecameric forms [40]. Overlaying these two structures reveals that the structure of the hub domains is unchanged, with the conversion from dodecameric to tetradecameric forms involving a small change in the angle between each hub subunit. The physiological significance of this interconversion between oligomeric states is unclear at present.

Holoenzyme structure

Although CaMKII has been shown to adopt different oligomeric states [40,48,54,55], it is likely to be predominantly dodecameric in solution. The first purification of CaMKII in 1983 from rabbit skeletal muscle showed that SDS separation yielded a product of ~58 kDa, while analytical ultracentrifugation yielded a product of 696 kDa, indicating a dodecameric complex [17]. Initial EM studies reported in this early paper captured CaMKII in circular formations with petal-like extensions that have six-fold symmetry.

This dodecameric assembly was later corroborated by negative stain and cryo-EM studies [15,16]. Although both studies concluded that rat neuronal CaMKII holoenzyme is comprised of two stacked hexameric rings, the two reconstructions look quite different. The fundamental difference between their resulting reconstructions is the orientation of the kinase domains relative to the hub domains. In one reconstruction, the kinase domains protrude above and below the plane of the hub, resulting in a taller structure when viewed from the side. In the other study, the reconstruction shows the kinase domains extending outwards in the same plane as the hub, resulting in a wider structure. We now appreciate that

these two reconstructions of CaMKII may represent alternative configurations of the CaMKII holoenzyme.

A human CaMKII holoenzyme with a very short linker was recently crystallized in an inhibited conformation (Fig. 1a) [38]. Three tricks were used to obtain crystals of the holoenzyme, which diffracted X-rays anisotropically to 4.0/3.6 Å. First, a small molecule inhibitor of the c-Abl tyrosine kinase, found previously to inhibit CaMKII adventitiously, helped stabilize the holoenzyme [56]. Second, Thr 306 was mutated to Val, in order to reduce phosphorylation heterogeneity. Finally, the linker connecting the kinase domain to the hub was shortened to the maximal extent to restrict movement of the kinase domain (the construct used for the linker region corresponds to a naturally occurring short linker isoform, 7, of CaMKII) [57].

The crystal structure of the CaMKII holoenzyme is likely to represent a maximally autoinhibited form of the enzyme. This structure reveals a compact state in which each kinase domain is nestled between its own hub domain and the one adjacent to it (Fig. 4a), making them completely inaccessible to peptide substrates. The regulatory domain is not completely helical; instead, the end of the helix leading into the hub melts into a short turn and random coil. This turn, termed the α -clip, is comprised of some of the residues required for Ca^{2+} /CaM binding, and makes contacts with a β -sheet of its own hub domain. The α -clip interaction effectively buries the residues corresponding to Thr 305 and Thr 306 and ultimately yields the regulatory segment completely inaccessible to Ca^{2+} /CaM. These and other interactions hold the kinase domains slightly above and below the plane of the central hub.

The compact nature of the crystallized holoenzyme assembly is very different from the more extended conformations observed for isoforms with longer linkers, using EM [15,16] or SAXS [36,38]. This raises the question as to whether the compact arrangement is specific to isoforms with short linkers. Mutations in the hub domain that are expected to disrupt the docking of the kinase domain onto the hub affect the cooperativity of activation by Ca^{2+} /CaM for both short and long linker isoforms, suggesting that the compact assembly is relevant for both (Fig. 4b). Interestingly, the effects of these mutations on the long linker isoforms are seen only under molecular crowding conditions, suggesting that under dilute conditions the long linker isoforms prefer a more extended conformation in which the kinase domains are not docked onto the hub [38].

Our current thinking is that the autoinhibited form of the CaMKII holoenzyme undergoes a dynamic equilibrium between compact and extended conformations (Fig. 5). The crystal structure of the holoenzyme provides a clear picture of the compact state, but the extended state remains nebulous due to the lack of direct structural evidence within the context of the holoenzyme. Once the kinase domains are popped out from the hub, they may adopt the coiled-coil inhibited structure or remain as monomers with the regulatory segment blocking the ATP binding site, or they may dimerize in a different conformation altogether. The equilibrium between the compact and extended states is tuned by the linker length. Longer linker lengths favor an extended autoinhibited form, which is more easily activated by Ca^{2+} /CaM, and has a lower frequency threshold for activation by Ca^{2+} spikes. Such a mechanism provides an easy way for nature to tune the responsiveness of different isoforms without much variation in the sequences of the kinase and hub domains.

The results of SAXS analyses of various CaMKII isoforms are consistent with the linker length controlling the balance between compact and extended assemblies [38]. SAXS reconstructions of a short linker construct reveal a compact structure, while reconstructions of a long linker constructs reveal an extended structure with six knobs protruding from the

central hub. The reconstruction for the short linker isoform can be switched from compact to extended by making a point mutation in the hub domain at the docking site for the kinase domain (I321E). Likewise, the reconstruction for the *C. elegans* holoenzyme, which has a long linker, can be switched from extended to compact by shortening the linker (Fig. 4c, d).

We believe that the extended form promotes Ca^{2+} /CaM binding and thereby facilitates activation. It is clear that Ca^{2+} /CaM binding induces a large conformational change within the kinase that releases inhibition by the regulatory segment. EPR studies have shown that upon activation, there is a transition between an inhibited (closed) state and an activated (released) state as suggested from the crystal structure of the activated truncated kinase [40,44]. This conformational change has been cleverly exploited to create FRET probes for activation [58–60] that have been used *in vivo* to study CaMKII function [23,28].

Frequency dependent activation must be a consequence of the kinetics of activation within a CaMKII holoenzyme. Simulation studies have shown that this phenomenon could be achieved without holoenzyme formation, if the various rate constants and concentrations are tuned appropriately (see, for example, [61,62]). An important role for the oligomeric structure is probably to bring the enzyme and substrate CaMKII into close proximity so that the rates of transphosphorylation and release of inhibition are matched appropriately to the Ca^{2+} spike frequency. Importantly, the duration of each Ca^{2+} pulse will affect the frequency threshold – a shorter pulse duration will necessitate a higher threshold frequency for activation and vice versa. Once some subunits within the holoenzyme have been autophosphorylated at or above the threshold frequency, lower frequencies are sufficient to achieve further activation [10]. Additionally, the Ca^{2+} /CaM dissociation rate is decreased by 10,000-fold after Thr 286 phosphorylation [63], which, in combination with cooperativity, acts to perpetuate activation.

Conclusion

Our knowledge of CaMKII structure and regulation has improved tremendously in the past decade, but various important aspects of the structure still await further clarification. A detailed picture of the extended autoinhibited state is unavailable. At the same time, the development of sophisticated fluorescence-based tools now allows the interrogation of CaMKII function in cells with unprecedented control over experimental parameters such as the frequency of Ca^{2+} spikes. These advances make it feasible to eventually close the gap between our deepening understanding of the molecular architecture of CaMKII and its many functions in brain, cardiac, and other tissues.

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Figure 1. Structural organization of CaMKII. a) Crystal structure of an intact CaMKII holoenzyme. The hub assembly is shown as a surface representation (gray) and the kinase domains are shown as cartoons. Each kinase domain is nestled between two hub domains, making contacts with each. The CaM binding region is buried by interactions with the hub. b) Each CaMKII subunit is comprised of a kinase domain (blue), regulatory segment (yellow), variable linker region (green), and a hub domain (gray). The regulatory segment contains three critical phosphorylation sites: Thr 286 is located within the R1 region of the regulatory segment, and Thr 305, Thr 306 are located within the CaM binding region (pink). The CaM binding footprint spans part of the R2 region (docking site for the catalytic domain) and the

entire R3 region within the regulatory segment. c) Activation by Ca^{2+} /CaM displaces the regulatory segment from its docking site, thereby freeing the substrate binding site and exposing Thr 286 for *trans* phosphorylation by an adjacent CaMKII kinase. This figure was adapted from (35).

Figure 2.

Crystal structures of kinase domains of CaMKII variants. a) *C. elegans* CaMKII. The regulatory segment is completely helical and projects Thr 306 far from the active site. PDB code: 2BDW

b) Human CaMKII crystallized bound to a small molecule inhibitor, Bisindolylmaleimide IX. The residues of the CaM binding region form a flexible loop instead of a helix as seen in (a). Colors correspond to those in (a). PDB code: 2V7O

c) Crystallized dimer of *C. elegans* CaMKII. The regulatory segments of two kinases form a coiled-coil, burying the CaM binding region.

Figure 3.

Substrate capture during activation within the CaMKII holoenzyme. In the inactive state, each kinase is autoinhibited. Upon Ca²⁺/CaM binding, activation occurs cooperatively within the holoenzyme via a substrate capture mechanism. An enzyme kinase captures a substrate kinase (both bound to CaM) and results in Thr 286 *trans* autophosphorylation. This figure was adapted from (39) with permissions.

Figure 4.

SAXS analysis of the CaMKII holoenzyme. a) The human short-linker construct, used for the crystallization of the full-length holoenzyme, has a compact SAXS envelope. b) A mutation (I321E) introduced to the short-linker construct at the docking site between the kinase and hub domain shows a significantly larger SAXS envelope. This may represent an extended form of the autoinhibited holoenzyme where the kinases are popped out from the hub domain. Additionally, the I321E has a left-shifted EC50 value for CaM binding, which corroborates the model that the extended conformation is more accessible to CaM than the compact conformation observed in the crystal structure. c,d) *C. elegans* CaMKII also converts from a large to small SAXS envelope when the variable linker is shortened. These may also represent the extended and compact conformations of CaMKII, respectively. This figure was adapted from (35).

An adjustable equilibrium set by the
length of the variable linker region

Figure 5.

Conformational changes in the holoenzyme. The CaMKII holoenzyme has been shown to adopt both compact (crystallization, SAXS) and extended (SAXS) autoinhibited states. An equilibrium exists between these states, and is sensitive to the length of the variable linker connecting the kinase and hub domains. In the compact autoinhibited conformation, residues comprising the CaM binding region make contacts with the hub domain, thereby making it highly inaccessible to CaM. In the extended autoinhibited conformation, the CaM binding region is more exposed, though the structural details of this extended state have yet to be elucidated. Upon Ca^{2+} /CaM binding, the regulatory segment is released from the kinase domain, thereby relieving inhibition. Active kinases then *trans* autophosphorylate adjacent kinases and subsequently phosphorylate downstream targets. Colors correspond to those in Fig. 1. This figure was adapted from (35).