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Structural Dynamics of the Activation of Elongation Factor 2 Kinase by Ca²⁺-Calmodulin

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Abstract

Eukaryotic elongation factor 2 kinase (eEF-2K), the only known calmodulin (CaM) activated α -kinase, phosphorylates eukaryotic elongation factor 2 (eEF-2) on a specific threonine (Thr-56) diminishing its affinity for the ribosome and reducing the rate of nascent chain elongation during translation. Despite its critical cellular role, the precise mechanisms underlying the CaM-mediated activation of eEF-2K remain poorly defined. Here, employing a minimal eEF-2K construct (TR) that exhibits activity comparable to the wild-type enzyme and is fully activated by CaM *in vitro* and in cells, and using a variety of complimentary biophysical techniques in combination with computational modeling, we provide a structural mechanism by which CaM activates eEF-2K. Native mass analysis reveals that CaM, with two bound Ca²⁺ ions, forms a stoichiometric 1:1 complex with TR. Chemical crosslinking mass spectrometry (XLMS) and small angle X-ray scattering (SAXS) measurements localize CaM near the N-lobe of the TR kinase domain and the spatially proximal C-terminal helical repeat. Hydrogen deuterium exchange mass spectrometry (HXMS) and methyl NMR indicate that the conformational changes induced on TR by the

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Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article (14 figures) can be found online at <https://>

engagement of CaM are not localized but are transmitted to remote regions that include the catalytic site and the functionally important phosphate binding pocket. The structural insights obtained from the present analyses, together with our previously published kinetics data, suggest that TR, and by inference, wild-type eEF-2K, upon engaging CaM undergoes a conformational transition resulting in a state that is primed to efficiently auto-phosphorylate on the primary activating T348 *en route* to full activation.

Graphical Abstract

Keywords

Eukaryotic elongation factor 2 kinase; calmodulin-dependent kinase; mass spectrometry; solution NMR spectroscopy; small angle X-ray scattering

Introduction

Elongation, the second of three broadly classified phases of translation^{1–3}, is influenced by three major protein factors in eukaryotes: elongation factor-1 α (eEF-1 α), elongation factor-2 (eEF-2) and elongation factor-3 (eEF-3)^{4;5}. While eEF-1 α and eEF-3 are involved in recruiting aminoacyl-tRNAs to the ribosome, eEF-2 is responsible for the GTP-dependent translocation of the nascent chain from the ribosomal A-site to the P-site^{4–6}. The addition of each new amino-acid residue to the elongating peptide chain requires the hydrolysis of GTP by eEF-2, to generate the energy for translocation⁷. Phosphorylation of eEF-2 on a specific threonine residue, Thr-56^{8–13}, by eukaryotic elongation factor 2 kinase (eEF-2K) significantly reduces its affinity for the ribosome and results in an overall reduction in the rate of protein synthesis^{12;14;15}. eEF-2K^{8;9} is a member of the α -kinase family¹⁶ that is largely unrelated in sequence to canonical protein kinases. eEF-2 is the only validated physiological substrate of eEF-2K, though other targets have recently been suggested¹⁷.

The kinase activity of eEF-2K is upregulated by its interactions with calmodulin (CaM)^{10;11}, making it the only known CaM-regulated α -kinase. However, this mode of activation contrasts that seen in other CaM-dependent kinases e.g. CaM kinase II (CAMKII), that involves displacement of a regulatory “pseudo-substrate” segment from its kinase docking region upon Ca²⁺-CaM binding exposing critical phosphorylation sites and the binding site for substrate^{18;19}. This “release-of-inhibition” mechanism^{20;21} appears not to be operative in eEF-2K, which utilizes a unique two-step allosteric activation mechanism²² that is illustrated

schematically in Figure S1. Using comprehensive kinetic and biochemical techniques we have established that these steps are – (**Step 1**) the binding of Ca^{2+} -CaM to eEF-2K results in a conformational change to generate a state that enables efficient auto-phosphorylation on a primary activating threonine, T348^{22;23}; (**Step 2**) the engagement of the phosphorylated T348 in a phosphate binding pocket (PBP) on the kinase domain leads to a second conformational change and results in a state with the highest activity towards the substrate eEF-2. However, in the absence of structural information for full-length eEF-2K and for the CaM•eEF-2K complex, the nature of the conformational transitions that facilitate these steps are not known.

Several biochemical and truncation studies^{22;24;25} have defined the domain organization of eEF-2K (725 residues, 82.2 KDa), as shown schematically in Figure 1a. The N-terminus of eEF-2K comprises of a non-canonical CaM-binding domain (CBD); we have recently determined the structure of this region bound to Ca^{2+} -CaM (Figure 1b)²⁶. The structure of the catalytic kinase domain (KD) that follows the CBD has been solved but the corresponding co-ordinates are not publicly available. The fact that the eEF-2K KD shares significant overall sequence identity with other α -kinases^{27;28} has allowed the construction of a robust homology model (Figure 1b)²⁹. Bridging the KD and the C-terminal region (CTR; defined as the region between residues 490–725) that contains several α -helical repeats (HRs)³⁰, is the regulatory loop (R-loop) that contains several positive and negative-regulatory phosphorylation sites³¹, in addition to the aforementioned T348. We have determined the structures of two different C-terminal constructs, CTR_{627–725} (this includes the final helical repeat, hence termed HR3)³² and CTR_{562–725} (that includes the last two helical repeats, hence termed HR23; Piserchio et. al, in preparation). The HR23 construct (Figure 1b) represents approximately 70% of the CTR.

As eEF-2K plays a critical role in modulating a central physiological process, protein translation, it is tightly regulated through multiple signals. These include its primary activation by Ca^{2+} -CaM and higher order regulation of activity by additional phosphorylation events mediated by kinases such as PKA³³ and AMPK³⁴, by pH³⁵ and by cellular Ca^{2+} levels³⁶. However, a necessary first step towards understanding this complex regulation of eEF-2K is to decipher in atomic detail the mechanism of its primary mode of activation that is mediated by Ca^{2+} -CaM. Towards this goal, we developed a minimal construct of eEF-2K that lacks 70 residues at the N-terminus and a section (359–489) of the R-loop (Figure 1a), but contains both the primary activating auto-phosphorylation site (T348) and a second activating auto-phosphorylation site, S500, that has been shown to play a key role under basal Ca^{2+} concentration³⁶. Here, we show that this construct, that we term the TR construct of eEF-2K (TR in short; 530 residues including the 6-glycine linker, 60.2 KDa), is similarly activated by Ca^{2+} -CaM as full-length eEF-2K *in vitro* and in cells. Further, we utilized a variety of mass spectrometric techniques including native mass spectrometry, hydrogen deuterium exchange mass spectrometry (HXMS), chemical crosslinking mass spectrometry (XLMS) together with solution NMR spectroscopy and small angle X-ray scattering (SAXS) to propose a structural mechanism by which Ca^{2+} -CaM interacts with, and activates TR, and by inference, full-length eEF-2K.

Results

TR forms a stable 1:1 complex with Ca^{2+} -CaM

All studies described below (with the obvious exception of the functional studies *in vitro* and in cells) have been performed using fully-dephosphorylated TR i.e. inactive TR. Therefore, in the present manuscript, all the biophysical studies described below, probe events involved in **Step 1** (described above and represented schematically in Figure S1) to generate a conformation that enables efficient chemistry i.e. auto-phosphorylation on T348.

Since TR contains the primary CaM-binding region²⁶ (the CBD, Figure 1) of eEF-2K, we surmised that it would form a stoichiometric complex with Ca^{2+} -CaM as in the case of full-length eEF-2K (FL). We relied on size-exclusion chromatography to characterize TR and its complex with Ca^{2+} -CaM. Interestingly, TR, its complex with Ca^{2+} -CaM, and Ca^{2+} -CaM itself, all appear at positions corresponding to higher molecular masses than expected (Figure S2). This most likely results from the non-spherical overall shape of the species probed (as will be described in greater detail below). Indeed, Ca^{2+} -CaM has been shown to have a radius of gyration (R_G) that is larger than that expected for a spherical species of the same molecular weight³⁷.

In order to confirm the stoichiometry of the Ca^{2+} -CaM•TR complex, we relied on native electrospray ionization mass spectrometry (ESI-MS), a technique that involves injecting aqueous protein into the gas phase under non-denaturing conditions for intact mass analysis. This soft ionization technique maintains non-covalent interactions such those in molecular complexes and enables the accurate determination of the mass and hence of the stoichiometry^{38–40}. In order to enable native mass analysis of TR and the Ca^{2+} -CaM•TR complex, the eluate (Figure S3) from a size-exclusion LC column in each case was injected directly into an electrospray ionization mass spectrometer (SEC/ESI-MS)⁴¹. Ion peaks in the ESI-MS spectra of TR alone and in complex with Ca^{2+} -CaM dominate the higher mass/charge (m/z) region of the spectra (Figure 2). The lower charge-density ions, centered around the +16 charge state for apo TR (mass = 60291 Da estimated by deconvolution of the +17 charge state; consistent with that expected for a monomeric species) and the +17 charge state corresponding to the Ca^{2+} -CaM•TR complex (mass = 77328 Da estimated from the +18 charge state; consistent with that expected for a 1:1 complex), represent compact, natively folded conformations. It should be mentioned the number of charges acquired by the molecular species depends on the exposed surface area⁴². Thus, unfolded/disordered regions of proteins would be expected to pick up a larger number of charges than a folded domain and the former would show a wider distribution of charged species than the latter. It is notable that the spectra of TR alone and the Ca^{2+} -CaM•TR complex do show the presence of higher charge-density ions (shown enclosed by the red rectangles in Figure 2). These result from partially disordered structures with significant solvent accessibility. The relative abundance of these peaks is higher in the mass spectrum of TR alone (top panel in Figure 2a) in comparison to the spectrum of the Ca^{2+} -CaM•TR complex (bottom panel in Figure 2b). This is not surprising since the N-terminal CBD is likely to be disordered in the absence of Ca^{2+} -CaM.

In addition to peaks corresponding to the 1:1 Ca^{2+} -CaM•TR complex, charge states that correspond to Ca^{2+} -CaM transiently dissociated from the complex were also observed. A close inspection of these peaks reveals an isotopic distribution corresponding to CaM with 0, 1 and 2 bound Ca^{2+} ions but none with 3 or 4 Ca^{2+} ions (see inset of Figure 2b and Figure S4). This is in line with the presence of only two Ca^{2+} ions seen in the structure of CaM in complex with a peptide corresponding to the CBD²⁶. In contrast, free CaM, that was added in a slight excess to ensure the stability of the Ca^{2+} -CaM•TR complex, and that appears at a higher elution volume on the size exclusion column (Figure S3) reveals peaks corresponding to 0, 1, 2, 3 and 4 bound Ca^{2+} ions, as expected (Figure S4).

TR is active *in vitro* and in cells

Having confirmed the ability of TR to form a stoichiometric complex with CaM, we tested whether it retains the ability to be activated by Ca^{2+} -CaM by measuring its efficiency in phosphorylating pep-S, an efficient peptide substrate of FL⁴³. No phosphorylated product was seen in the absence of CaM in a Ca^{2+} -containing buffer (Figure 3a). However, pep-S was efficiently phosphorylated by TR upon the inclusion of CaM with a k_{obs} of $16.3 \pm 0.9 \text{ s}^{-1}$ compared with a k_{obs} of $14.5 \pm 0.3 \text{ s}^{-1}$ for FL (Figure 3b), suggesting that the kinase activity of TR is stimulated by Ca^{2+} -CaM to a similar extent as FL *in vitro*.

To evaluate the behavior of TR in cells, vectors encoding either vector alone (CV), TR or FL were transfected into MCF-10A (eEF-2K^{-/-}) cells²² that lack endogenous eEF-2K. While the cellular levels of TR were found to be ~3-fold lower than that of FL (Figure 3c), the relative levels of eEF-2 phosphorylated on Thr-56 were found to be comparable in the TR and FL transfected cells (Figure 3c, left panel). This suggests that the activity of TR is significantly higher than that of FL in cells (Figure 3c, right panel). This is not surprising since the negative regulatory phosphorylation sites on the R-loop e.g. S359 and S366 (along with the positive regulatory site, S398) are missing in TR³¹. Nevertheless, taken together with the *in vitro* data, our results suggest that TR retains its ability to be upregulated by Ca^{2+} -CaM and to site-specifically phosphorylate eEF-2 on Thr-56.

Ca^{2+} -CaM binding leads to greater solvent protection in specific regions of TR

We used hydrogen deuterium exchange mass spectrometry (HXMS) to estimate the extent of solvent exposure of backbone amide positions in TR by measuring the degree of ^2H incorporation upon incubation with D_2O for varying lengths of time (5, 10, 30 and 240 minutes). About 35% of the amide protons of TR were replaced by ^2H within the first 5 minutes of incubation. This was followed by slower incorporation of an additional ~11% over four hours, suggesting that more than half the amide positions in TR were well shielded from solvent (Figure S5). Absent a correction for back exchange, this number represents a lower bound of the TR protons that are solvent-exchangeable over this timeframe. While HXMS data for the Ca^{2+} -CaM•TR complex (MS analysis following 5, 10, 30, 60 and 240 minutes of incubation in D_2O) revealed similar overall trends as for TR alone, the presence of Ca^{2+} -CaM resulted in increased overall protection (Figure S5). This decreased solvent accessibility can be attributed to increased steric shielding (due to Ca^{2+} -CaM binding), decreased dynamics, or both.

In order to determine the specific regions of TR that show increased protection in the presence of Ca^{2+} -CaM, we relied on local HXMS measurements. TR in the apo state, or that in the context of the Ca^{2+} -CaM•TR complex, was digested using an immobilized pepsin column after incubation in D_2O for varying lengths of time (5, 10, 30 and 60 minutes). The peptides generated were subjected to HPLC-ESI-MS/MS analysis. In all, data for 196 overlapping peptides (representing 90.4% coverage of the TR sequence; see Figure S6 for a representative dataset) could be analyzed. Of these, a total of 40 peptides displayed statistically significant increase in protection induced by Ca^{2+} -CaM (Figures 4 and 5; see Figure S7 for examples of peptides showing no statistically significant differences in protection between the two states; see Figure S8 for representative raw datasets for peptides with increased protection in the Ca^{2+} -CaM•TR complex). None of the peptides for which data could be analyzed showed a statistically significant decrease in protection in the presence of Ca^{2+} -CaM. The largest overall increase in protection was seen in the β -barrel on the N-lobe of the KD including $\beta 2$, $\beta 3$, $\beta 4$ and $\beta 5$ (Figure 4). Increased protection was also seen on the phosphate-binding P-loop and at the C-terminal end of αC , that along with the αD (that also shows somewhat increased protection; see peptide 7 in Figure 4), constitutes the PBP, a pocket proposed to accommodate phosphorylated T348, following its auto-phosphorylation. Occupancy of the PBP by phosphorylated T348 drives the second conformational transition (as part of **Step 2**, described above; Figure S1) that facilitates the formation of the fully-active form of eEF-2K²². Peptides corresponding to the αE helix (peptide 8 in Figure 4) and the $\beta 5$ - αC loop (that connects the N-lobe β -barrel to αC ; peptide 5) also showed increased protection in the presence of Ca^{2+} -CaM.

We have previously shown that the N-terminal CBD comprises the primary CaM binding region of eEF-2K (and by inference, of TR)²⁶. However, a single peptide (77–86) that includes only one (W85) of the critical CaM binding residues (CBD engages CaM in a 1-5-8 binding mode: W85-I89-A92; Figure 1b), albeit the most important one, could be resolved in our local HXMS measurements (see Figure S6). While this peptide showed an overall trend suggesting increased protection in the presence of Ca^{2+} -CaM, the differences between the free and bound states were within the error bounds (not shown). This observation could be due to reasons that are mundane - the lack of sufficient peptide coverage of this region preventing us from fully characterizing its dynamics, or more fundamental – this region remains dynamic (on the timescale of the exchange) in the complex. Additional measurements are needed for further clarification.

Some parts of the CTR also showed statistically significant changes in protection upon Ca^{2+} -CaM binding. Increased protection was seen on three contiguous helices ($\alpha 3'$, $\alpha 4'$ and $\alpha 5'$; Figure 5) belonging to the second (HR2) and third (HR3) helical repeats. Other regions of TR did not show any statistically significant changes in protection in the presence of Ca^{2+} -CaM.

Specific cross-links indicate the relative spatial location Ca^{2+} -CaM and the structural domains of TR

While the spatial organization of the structural domains of intact TR is unknown in the absence of a three-dimensional structure, it is difficult to conceive that all of the regions that

show increased protection in the HXMS studies, discussed above, could simultaneously interact with CaM in the context of a heterodimeric complex. For example, it seems unlikely that the N-lobe of the KD and the PBP could both directly interact with CaM without large-scale distortions in the structures of TR, CaM, or both. Thus, in order to parse the effects of direct interactions with CaM on the HXMS-determined solvent protection from those that result from structural rearrangements within TR as a consequence of CaM-binding i.e. long-range allosteric effects, we relied on the chemical cross-linking mass spectrometry (XLMS) measurements^{44;45}.

We utilized the “zero-length” carbodiimide cross-linker EDC that links primary amines (N-terminus and lysine sidechains) to glutamate, aspartate sidechains and the C-terminus⁴⁶. The cross-linked species were digested with trypsin and the resulting peptides were analyzed using ESI-MS/MS. Several cross-linked peptides could be identified but only 7 could be localized to within segments on the interacting partners (the KD and CTR of TR and CaM) that were short enough to be useful as constraints (see Figures S9 and S10 for specific fragmentation patterns).

As shown in Table 1, a total of four inter-protein (Inter) cross-links between TR and CaM could be identified, in addition to three inter-domain cross-links within TR (IntraTR). Of the cross-links between CaM and TR, both ends of the cross-link (using a one-letter code for TR residues and a three-letter code for CaM residues) could be uniquely identified for two - E145-Lys30 (Inter-2 in Table 1) and K715-Glu87 (Inter-4 in Table 1). In addition, K132 and K162 on TR were identified as forming cross-links with acidic residues on CaM (Inter-1 and Inter-3 in Table 1). It is notable that E145, K132 and K162 are all localized on the N-lobe of the TR KD in regions that display increased protection in the presence of Ca²⁺-CaM (Figure 4) in the HXMS measurements. Given that the CBD, which is critical for the recognition of Ca²⁺-CaM, is located immediately N-terminal to the N-lobe of the KD, it stands to reason that CaM bound at the CBD is likely to be in physical proximity to the N-lobe of the KD and indeed poised to interact with it in the Ca²⁺-CaM•TR complex. Thus, this increased protection seen for the N-lobe of the KD, in the HXMS studies described above, most likely results from a direct interaction with Ca²⁺-CaM. Indeed, our previous studies have indicated a somewhat reduced affinity of a peptide encoding only the CBD (K_D ~245 nM) towards Ca²⁺-CaM compared to that of full-length wild-type eEF-2K (K_D ~17 nM)²⁶, suggesting that additional stabilizing contacts could exist between Ca²⁺-CaM and eEF-2K. On the other hand, it is difficult to conceive of a scenario where Ca²⁺-CaM engaged by the CBD and docked at the N-lobe of the KD could simultaneously interact with the PBP. Therefore, the increased protection seen at the PBP in the presence of Ca²⁺-CaM likely results from a conformational change that is transmitted from the CaM docking site at the N-lobe of the KD; this is explored in greater detail below.

The inter-domain cross-links within TR provide a means to assess the spatial locations of the various domains of TR with respect to each other; the most useful amongst these is the unambiguous K93-D685 crosslink (IntraTR-1 in Table 1) that places the C-terminal region of TR (specifically HR3) close to the N-lobe of the KD. It is notable that K93 lies immediately C-terminal to residue 8 (A92; see Figure 1b) of the CBD segment that engages CaM in a 1-5-8 binding mode. This would suggest that the HR3 is also proximal to CaM, an

arrangement that is supported by the observed cross-link between K715 and Glu87 (Inter-4 in Table 1). It is notable, however, that this region shows only a small increase in protection upon Ca^{2+} -CaM binding in the HXMS measurements suggesting that while in spatial proximity to CaM, it does not participate in a direct interaction with it, unlike the N-lobe of the KD.

Next, we tested the utility of the cross-links listed in Table 1 as inputs for the generation of a model of the Ca^{2+} -CaM•TR complex using the homology model of eEF-2K KD²⁹ together with the NMR structures of HR23 construct (Piserchio et al., in preparation) and the Ca^{2+} -CaM•CBD complex²⁶. First, we needed to assess whether the individual structures of the KD, HR23 and Ca^{2+} -CaM•CBD complex (Figure 1b) were maintained in the context of the Ca^{2+} -CaM•TR complex. We used a set of cross-links within the KD and CTR modules of TR and within CaM itself to validate the structures. As evident from Table 2, the residues found to be cross-linked in the context of the Ca^{2+} -CaM•TR complex were within reasonable distances of each other (generally $< 23 \text{ \AA}$)⁴⁷ in the structure of isolated HR23, and to a large extent, for the homology model of the KD. This suggests that the structures of these modules are largely maintained in the context of the Ca^{2+} -CaM•TR complex. The situation was drastically different for CaM, with the distances between cross-linked pairs calculated using the structure of the Ca^{2+} -CaM•CBD complex were found to be too large to allow efficient cross-linking if the structure were to be maintained. This scenario is best embodied by the observed cross-link between Lys30 on the N-lobe of CaM with Glu127 on its C-lobe. These residues have a C α -C α distance of $\sim 37 \text{ \AA}$ in the context of the Ca^{2+} -CaM•CBD complex, placing them at a distance that would not enable cross-linking. In contrast, the intra-C-lobe distances between the cross-linked residues Lys94 and Glu127/Asp129 (Table 2) are far more reasonable at $\sim 17\text{--}20 \text{ \AA}$. These data suggest that the inter-lobe orientation in the Ca^{2+} -CaM•TR complex is quite different from that represented in the Ca^{2+} -CaM•CBD NMR ensemble. Given this scenario, it is not surprising that while our efforts to model the KD•CTR_{496–725} complex (see Materials and Methods section for details) yielded converged structures (Figure S11), our attempts to include the Ca^{2+} -CaM•CBD structure in this model did not lead to convergence.

As shown in Figure 6, the structural model for the KD•CTR_{496–725} complex is consistent with all the observed inter-domain crosslinks within TR. Indeed, the model is also consistent with the crosslinks with segments of TR that were not explicitly included in the model itself e.g. it is completely plausible for D685, that is spatially proximal to the N-terminus, to cross-link with K93, a part of the N-terminal CBD (Figure 1b), that is not represented in the model. This suggests that the computational model represents a plausible organization of the domains of TR in the context of the Ca^{2+} -CaM•TR complex. While this analysis does not provide a structural model for CaM within the complex, the locations of K132, E145, K162 and K715, all of which cross-link with CaM (Figure 6b), suggest a spatial localization of CaM near the N-lobe of the KD and the C-terminus of the CTR near HR3.

Ca^{2+} -CaM binding leads to allosteric effects in the TR kinase domain

For the computational model described above, there are regions of TR that show increased protection from solvent in the presence of Ca^{2+} -CaM in the HXMS studies but are not found

to be spatially proximal to it (Figure 6c). These regions include the PBP and parts of the CTR in which increased protection from solvent likely results from conformational changes upon the engagement of the CBD and the N-lobe of the KD by Ca^{2+} -CaM. For an additional orthogonal test of this hypothesis, we utilized the $\delta 1$ positions of the 20 Ile residues of TR as NMR probes of conformational changes upon Ca^{2+} -CaM binding. The Ile $\delta 1$ resonances were assigned by comparison of $^{13}\text{C}, ^1\text{H}$ correlation spectra of wild-type $^{13}\text{C}, ^1\text{H}$ -Ile- $\delta 1$ -labeled TR with corresponding spectra of point mutants where a particular Ile was replaced by Leu (except I232A and I522S). Using this approach 15/20 Ile- $\delta 1$ resonances were unambiguously assigned (Figure S12). Resonances corresponding to I89 and I349 could not be uniquely identified but were attributed to a single cluster along with I237. I131 and I215 are proximal in space on the N-lobe of the KD and mutation of one altered the resonance position of the other, though this exercise allowed narrowing down the corresponding assignments to a pair of well-resolved resonances.

As expected, several Ile resonances showed significant perturbations in the presence of Ca^{2+} -CaM (Figure 7a). These include I131 and I215, located on the N-lobe of the KD, as well as I251, I271, I275 and I287 all of which lie at or near the PBP and the G-loop. Resonances corresponding to residue I232 that lies near the catalytic site and the spatially adjacent I209 are not identifiable in the bound state spectra, likely due to extremely large chemical shift changes. This suggests substantial conformational rearrangements at the catalytic center. An inspection of Figure 7b reveals that the perturbed Ile residues form a connected network extending from the N-lobe of the KD to the PBP. This indicates a likely pathway by which conformational changes induced by the engagement of the N-lobe of the KD by Ca^{2+} -CaM could be transmitted to remote sites including the PBP. The likely existence of this long-distance communication network is underscored by the fact that a T348A mutation leads to a ~10-fold decrease in the affinity of eEF-2K for CaM²². In contrast to the KD, most of the Ile residues on the CTR are not located in regions that show significant protection in the presence of Ca^{2+} -CaM (Figure S12) in the HXMS measurements. The resonance corresponding to I614, that lies in this region, does show some broadening but this perturbation is not as significant as that seen for the KD resonances.

SAXS measurements suggest an elongated structure for the Ca^{2+} -CaM•TR complex

We used small angle X-ray scattering to determine the overall hydrodynamic features of the Ca^{2+} -CaM•TR complex. The complex showed no signs of aggregation over more than a 3-fold range of concentration (from 1.9 mg/mL to 6.4 mg/mL; Figure 8a). This contrasts the behavior of TR alone that showed significant aggregation at moderate-to-high concentration (Figure S13). Analysis of the SAXS data produced an R_G of ~37 Å (see Table 3 for details) and a D_{max} of ~130 Å (Figure 8b) suggesting that the complex forms an elongated structure in solution. This is also borne out by the molecular envelope calculated from the SAXS data that indicates a species that is approximately 130 Å by 92 Å by 34 Å in solution (Figure 8c). Using the modeled structure of the KD•CTR₄₉₆₋₇₂₅ complex (described above) in addition to that of Ca^{2+} -CaM•CBD tethered to the N-terminus of the KD using a flexible linker, we used the SAXS data to generate model for the Ca^{2+} -CaM•TR complex (see Materials and Methods for details). As shown in Figure 8c, the model shows CaM localized near the N-

lobe of the KD and the C-terminal end of CTR. In addition, the various structural modules are accommodated well into the SAXS-determined molecular envelope. Thus, TR forms a hatchet-shaped complex with Ca^{2+} -CaM, the former comprising the blade and the latter, the handle, as shown in Figure 8c.

Discussion

We have developed a minimal construct of eEF-2K lacking a significant segment of its N-terminus and most of the regulatory R-loop linking the kinase domain to the C-terminal region. The presence of the N-terminal CaM-binding domain and the section of the regulatory loop including T348, the site of primary activating auto-phosphorylation, ensures that this minimal construct (TR) is activated by Ca^{2+} -CaM to a similar extent as full-length enzyme *in vitro* while retaining its ability to efficiently phosphorylate eEF-2 on Thr-56 in cells. Analysis of chemical crosslinking data using mass spectrometry and computational modeling suggest a topology for TR in which the C-terminus of the CTR (HR3) is spatially proximal to the N-lobe of the KD, and where both of these regions lie in close proximity to CaM in the Ca^{2+} -CaM•TR complex. Increased protection against ^2H exchange with solvent in HXMS studies suggests that CaM, that is anchored to its primary binding site at the N-terminal CBD, is likely stabilized further by additional interactions with the N-lobe of the KD. The importance of these latter interactions is also borne out by the fact that the affinity of Ca^{2+} -CaM towards a peptide encoding the CBD is ~15-fold lower than that compared to full-length eEF-2K. It is also notable that an affinity of ~1 μM remains even upon mutation of the key CaM-anchor residue on the CBD (W85S)²⁶. A preponderance of validated cross-links between the N-lobe of CaM (3 of 4 cross-links with TR involve the N-lobe of CaM; see Table 1) and the TR KD seems to suggest that the N-lobe of CaM is more proximal to TR than the CaM C-lobe. Though additional data are needed to confirm this hypothesis.

It is noteworthy that regulatory interactions involving the N-lobe of the kinase domain are not unprecedented in α -kinases. The KD of the Ca^{2+} -dependent TRP channel is active as a head-to-head domain swapped dimer that forms through the interactions of an N-terminal helical segment of one protomer that packs against the αC helix of the second (Figure S14)²⁷. This interaction involves significant contacts between the N-lobe β -barrel regions of the two protomers. Truncation/deletion of this N-terminal segment or mutation of specific residues therein leads to loss of dimerization or kinase activity or both⁴⁸. It has been previously proposed that the N-terminal CBD of eEF-2K could play a similar role, with CaM perhaps substituting for the second protomer⁴⁸ in activating the enzyme.

The established view for the activation of CaM-regulated kinases is the so-called “release of inhibition” mechanism¹⁹ in which the active site is blocked by a “pseudo-substrate” moiety that also contains a CaM recognition element; engaging Ca^{2+} -CaM releases this inhibition and allows kinase activation. As mentioned previously, eEF-2K appears to be activated by a different mechanism. It has been shown by Tavares et al.²² that the presence of Ca^{2+} -CaM has a drastic effect on the intrinsic catalytic activity (>2000-fold increase in k_{cat}) without major changes in the affinity for a peptide substrate (~2-fold decrease in the apparent K_{M}) and a modest decrease in the affinity for ATP (~8-fold increase in the apparent K_{M}). Our HXMS results are indeed consistent with these data with no decrease in solvent protection

seen at the catalytic cleft of TR (or for that matter, for any other region) upon engaging Ca^{2+} -CaM. The HXMS data that shows increased protection in regions on the KD that are well separated in space including on the PBP and indeed on the regions of the CTR that are spatially proximal to the kinase active site in our computational model, suggest significant conformational changes in TR upon Ca^{2+} -CaM binding. These data are in line with the NMR analysis that reveals spectral perturbations spreading across most of the KD in the presence of Ca^{2+} -CaM. We suspect that this set of conformational changes, that enhances the intrinsic catalytic activity of eEF-2K, represents the first of the two conformational steps in its activation pathway predicted in the kinetics studies of Tavares et al.²² (Figure S1). This change likely involves a remodeling of the catalytic cleft, enabled by a series of conformational transitions propagating from the N-lobe of the KD, the site of engagement with CaM, as suggested by our NMR analysis. This remodeling is perhaps also facilitated by a rearrangement of the relative positions of the KD and CTR. The role of the CTR in modulating the activity of eEF-2K is highlighted by the previous observation that the isolated KD is incapable of phosphorylating not only eEF-2, but also a substrate peptide. However, this ability can be restored *in trans* by the CTR⁴⁹. Our studies also highlight the fact that Ca^{2+} -CaM binding induces greater order at the PBP. This change could be perceived as a conformational rearrangement in preparation to accommodate phosphorylated T348 leading to the second conformational transition predicted in the kinetics studies resulting in a state with the highest activity towards substrate²². While our current studies provide insight into the overall organization of the structural domains of TR and of CaM in the context of the Ca^{2+} -CaM•TR complex and evidence of significant conformational changes (Figure 9) induced by Ca^{2+} -CaM binding to TR priming it to enable efficient auto-phosphorylation on T348, the precise nature of these transitions await high resolution structures of the complex and more detailed analyses of dynamics using NMR and/or computational approaches. In this regard, the stripped-down yet functional TR construct provides an added benefit for future structural studies - since it is missing many of the segments that are expected to be disordered in full-length eEF-2K, it could, in principle, be an excellent candidate for crystallization trials. Our extensive efforts to obtain crystals of full-length eEF-2K have not yielded any positive results. Further, the smaller size of TR (60 kDa) should also ease the performance of detailed NMR analyses that would have been more problematic in full-length eEF-2K (82 kDa).

We note that the current studies probe events involved with **Step 1** of the two-step activation process of eEF-2K (Figure S1). Thus, our analyses are sensitive to all conformational changes following the engagement of Ca^{2+} -CaM that lead to a state capable of efficiently auto-phosphorylating on T348. An additional set of conformational transitions are expected after T348 phosphorylation and engagement of the PBP by phospho-T348²² (**Step 2**). These latter events will be probed by future studies on T348-phosphorylated TR.

Materials and Methods

Expression and purification of His₆-SUMO-tagged TR

A His₆-SUMO tagged TR construct was cloned into a pET-15b expression vector (Novagen) between the NdeI and BamHI restriction sites and transformed into BL21 DE3 *E. coli* cells

(New England Biolabs) for expression. The cells were plated on ampicillin plates and incubated overnight at 37 °C. A single colony was inoculated into 10 mL of LB medium (containing 100 mg/L of ampicillin) and grown overnight at 37 °C. The overnight culture was inoculated into 0.4 L of LB medium and grown at 37 °C until the culture reached an A_{600} of 0.8, at which point the temperature was reduced to 18 °C and expression was induced by the addition of IPTG to a final concentration of 0.4 mM. Post induction, the cells were grown for 16 hours and harvested by centrifugation at $2820 \times g$ for 30 minutes. The cell pellet was re-suspended in 50 mL of cold lysis buffer (20 mM Tris, 200 mM NaCl, 0.1% β -mercaptoethanol, 0.1% Triton X-100, 10 mM imidazole and 1 tablet of Roche Complete Mini EDTA-free inhibitor tablet, at pH 7.5); all subsequent steps described below were carried out at 4 °C. The cells were lysed by sonication and cell debris was removed by centrifugation at $10800 \times g$ for 30 minutes. The soluble lysate was added to a 5 mL (bed volume) Ni-NTA affinity column (Qiagen) previously equilibrated with lysis buffer and incubated for 1 hour with end-over-end rotation. The column was then washed extensively with washing buffer (20 mM Tris, 200 mM NaCl, 0.1% β -mercaptoethanol, 20 mM imidazole, pH 7.5) and the bound protein was subsequently eluted with 50 mL of elution buffer (20 mM Tris, 200mM NaCl, 0.1% β -mercaptoethanol, 250 mM imidazole, pH 7.5). The His₆-SUMO tag was cleaved by the addition of ULP1 protease (1 μ g per 50 mL of elution) and dephosphorylated with λ -phosphatase (New England Biolabs) during overnight dialysis into cleavage buffer (20 mM Tris, 200 mM NaCl, 0.1% β -mercaptoethanol, 1 mM MnCl₂, pH 7.5). The effectiveness of cleavage was monitored by 12% SDS-PAGE gels. The cleavage reaction was quenched by addition of 0.1 mM AEBSF. The cleaved protein was further purified via ion exchange chromatography using a 5 mL Hitrap Q HP column (GE Life Sciences) applying a linear NaCl gradient from 0.2 to 0.8 M in a buffer containing 20 mM Tris, 2 mM DTT at pH 7.5. The purified protein was then concentrated using spin columns and further purified by gel filtration chromatography using a Superdex 200 10/300 GL (GE HealthCare Biosciences) column pre-equilibrated with a buffer containing 20 mM Tris, 200 mM NaCl, 2 mM DTT, 1 mM CaCl₂ at pH 7.5 (referred to as SEC buffer)

Calmodulin was expressed and purified as described previously²⁶. To generate the Ca²⁺-CaM•TR complex, purified CaM in a SEC buffer was added to the TR samples in the same buffer in a 1:2 molar ratio followed by gel filtration (as above) to purify the 1:1 complex. Fractions containing the pure Ca²⁺-CaM•TR heterodimer were collected and concentrated using spin columns to final concentrations ranging from 40 to 50 μ M for hydrogen exchange mass spectrometry and chemical crosslinking experiments.

Analysis of TR activity *in vitro*

Measurement eEF-2K activity against a peptide substrate (pep-S) was carried out as described previously, in the following buffer: 25 mM HEPES, pH 7.5; 2 mM DTT; 10 μ g/mL BSA; 50 mM KCl; 10 mM MgCl₂; 100 μ M EGTA; 150 μ M CaCl₂; 2 μ M CaM; 150 μ M pep-S; 1.0 mM [γ -³²P]-ATP; and 2.5 nM wild-type eEF-2K (FL) or TR. Calmodulin was omitted from this buffer for assays in the absence of CaM^{23;50}. All experiments were performed in duplicate.

Generation of the TR-pcDNA3-HA-FLAG construct

Overlap-extension mutagenesis was used to directly generate TR starting with the wild-type eEF-2K-pcDNA3-HAF construct described previously²². Primers were designed to incorporate ends compatible with ligation-independent cloning (LIC). The mutagenesis product and amplified pcDNA3-HAF vector were digested with T4 DNA polymerase in the presence of dATP or dTTP, respectively. The digested products were annealed at room temperature and transformed into DH5 α , purified, and sequenced.

Analysis of TR activity in MCF-10A eEF-2K^{-/-} cells

MCF-10A cells lacking endogenous eEF-2K (eEF-2K^{-/-})²² were plated at a density of 150,000 cells per well in 6-well tissue culture plates. The following day, cells were transfected using Lipofectamine 3000, according to the manufacturer instructions (using 2 μ L P3000 reagent per μ g DNA, and 2.5 μ g DNA per well), with pcDNA3-HAF vectors encoding either wild-type eEF-2K or TR, or an empty control vector (CV). Transfection was carried out for 6 hours in DMEM/F12 lacking any supplements, after which time the media was changed to complete MCF-10A media. Cells were then allowed to recover for 48 hours to allow for expression prior to lysis. Lysis and Western blotting for eEF-2K activity were carried out as described previously²². The following primary antibodies were used: eEF-2P^{Thr56}, #2331, Cell Signaling Technologies (diluted 1:2000); eEF-2, Millipore (diluted 1:20000); eEF-2K (C-12), Santa Cruz Biotechnology (diluted 1:500); Actin, Millipore (diluted 1:20000). The following fluorescent secondary antibodies (Li-COR) were used at a 1:15000 dilution: Goat anti-Rabbit, 926–32211; Goat anti-Mouse, 926–68070. Blots were imaged on a Li-COR Odyssey-Sa.

Inline size exclusion chromatography and native electrospray mass spectrometry

The TR and Ca²⁺-CaM•TR complex for native ESI-MS was prepared as described above. The samples were injected onto a MABPac SEC-1 Size Exclusion LC column pre-equilibrated with 50 mM ammonium acetate with a continuous flow rate of 50 μ L/minute. Elution from the SEC column was directly introduced into a Bruker maXis-II ETD ESI-QqTOF mass spectrometer. Spectral averaging of TR and the Ca²⁺-CaM•TR complex were performed using the Bruker COMPASS software package. The mMass software was then used for baseline correction and smoothing with a 5 Da Gaussian smoothing function. The free CaM spectra were similarly processed except the use of a 0.05 Da Gaussian smoothing function.

Hydrogen/deuterium exchange mass spectrometry (HXMS)

Protein samples (TR in the apo state and the Ca²⁺-CaM•TR complex) for HXMS experiments were purified as described above. To obtain the deuterium uptake on the global level, exchange was initiated by the addition of 2 μ L of the sample (either free TR or the Ca²⁺-CaM•TR complex) in buffer A to 98 μ L of exchange buffer (20 mM Tris, 200 mM NaCl, 2 mM DTT pH 7.5 in 99.9% D₂O) to a final protein concentration of 2 μ M. Samples were then incubated at room temperature for the desired exchange time (5, 10, 30 and 240 minutes for TR and 5, 10, 30, 60 and 240 minutes for the Ca²⁺-CaM•TR complex). The exchange was quenched by 2 minutes of incubation on ice followed by the addition 100 μ L

of quench buffer (0.1 M guanidinium hydrochloride, 0.4% formic acid) to reduce the pH to 2.4. All subsequent steps were performed in an ice bath. The quenched samples were desalted using a 10 μ L C8 Opti-lynx II trap cartridge (Optimize Technologies) at a flow rate of 0.12 mL/minute using 0.25% formic acid as the mobile phase. The protein was then eluted using a gradient from 10% acetonitrile, 0.25% formic Acid (Buffer A) to 95% acetonitrile, 0.25% formic acid (Buffer B) at a flow rate of 0.1 mL/minute. The desalted protein was then eluted directly into a maXis-II ETD ESI-QqTOF spectrometer at a flow rate of 0.04 mL/minute for mass determination. The average mass of the protein was determined using the Bruker COMPASS software package. To determine the deuterium uptake at each time-point, the average mass of a non-deuterated reference sample was subtracted from the average mass of each deuterated sample.

To acquire local exchange profiles (D_2O incubation for 5, 10, 30 and 60 minutes was used for both TR and the Ca^{2+} -CaM•TR complex), the quenched protein sample was immediately injected over an Enzymate BEH Pepsin Column (Waters) with the resulting peptides being desalted by a 10 μ L C8 Opti-lynx II trap cartridge (Optimize Technologies). The digestion with subsequent desalting was performed at a flow rate of 0.12 mL/minute using 0.25% formic acid as the mobile phase. The mixture of peptides was then resolved with a 50×1 mm Hypersil Gold C18 column (Thermo Scientific) and eluted directly into the maXis-II ETD ESIQqTOF spectrometer using a flow rate of 0.04 mL/minute. The resulting peptides were identified using the Bruker COMPASS software package. The extent of exchange was assessed using the commercial software HDExaminer (Sierra Analytics). HDExaminer identified exchanged peptides from raw MS data based on peptide retention times from the COMPASS analysis of an unlabeled reference sample. HDExaminer then calculated the deuterium uptake of each peptide after specified exchange times. The percent deuterium uptake relative to the theoretical maximum for each peptide was then averaged across replicate experiments for each time point. The difference between the average percent deuterium uptake of apo TR and the Ca^{2+} -CaM•TR heterodimer peptides was then taken to identify regions that experience changes in protection upon CaM binding. All experiments were conducted in triplicate, though some peptides could not be unambiguously identified in some replicates. All peptides used in the analysis were observed in duplicate or triplicate for each time point.

Chemical crosslinking mass spectrometry (XLMS)

Proteins for chemical crosslinking were purified through size exclusion chromatography (as above) using columns pre-equilibrated with a buffer containing 50 mM MES, 200 mM NaCl, 1 mM $CaCl_2$ at pH 6.5) immediately prior to carrying out chemical crosslinking experiments. The crosslinking reaction was carried out according to manufacturer's instructions. Briefly, 0.4, 0.8 or 1.6 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1.1, 2.2 or 4.4 mg of N-hydroxysulfosuccinamide (sulfo-NHS), respectively, were dissolved directly into a 1 mL sample of 50 μ M Ca^{2+} -CaM•TR and incubated at room temperature. After 15 minutes, β -mercaptoethanol was added to a final concentration of 0.14%. The samples were then incubated at room temperature for 30 minutes, after which a 300 μ L aliquot was removed and the reaction was quenched on ice with 50 mM glycine. Another 300 μ L aliquot was similarly collected and quenched at 1 hour

of reaction time. The remaining 400 μ L was incubated for an additional hour and similarly quenched. The samples were then digested for 16 hours with 10 μ g/mL of MS grade trypsin (Fisher) at 37 °C. The protease digestion was quenched with 1 mM PMSF protease inhibitor. The resulting mixture of peptides was subjected to HPLC-ESI/MS analysis using Acclaim 300 2.1 $\times$ 150 mm C18 column (Thermo Scientific) and a maXis-II ETD ESI-QqTOF mass spectrometer. A database of expected crosslinked peptides was generated with xComb⁵¹ and a MASCOT server (Matrix Science) was used to search the XLMS data for the predicted peptides. False positive peptides were identified by comparison of the crosslinked data sets to an untreated control sample and analysis of CID fragmentation patterns.

Production of ¹³C, ¹H Ile- δ_1 -labeled TR

Plasmids encoding His₆-SUMO-TR and corresponding point mutants were transformed into *E. coli* C41 (DE3) cells. Each Ile in TR was mutated one at a time to Leu (except I232 that was mutated to Ala, and I522 that was mutated to Ser) by site-directed mutagenesis using the Phusion Master Mix with HF Buffer and Q5 High-Fidelity 2 $\times$ Master Mix (New England BioLabs). Fresh C41 (DE3) colonies were inoculated into starter LB cultures supplemented with ampicillin (100 mg/L), grown at 37 °C until the A₆₀₀ reached 0.6, centrifuged (4000 $\times$ g for 15 minutes at 4 °C). The cell pellet was re-suspended in a starter M9 medium prepared in D₂O and grown overnight. The overnight culture was inoculated into a 0.5 L D₂O-based M9 medium supplemented with 1 g/L ¹⁵NH₄Cl and 2 g/L ²H-glucose and grown at 37 °C. When the A₆₀₀ reached 0.5, 50 mg/L (methyl-¹³C, 3,3-d₂) α -ketobutyric acid (Cambridge Isotope Laboratories) was added to the growth that was incubated for 1 hour, followed by induction with 0.5 mM IPTG at 18 °C for 22 hours. Cells were harvested by centrifugation (2820 $\times$ g for 30 minutes at 4 °C) and re-suspended in lysis buffer (50 mM Tris at pH 7.8, 500 mM NaCl, 150 mM KCl, 5 mM β -mercaptoethanol, 20 mM imidazole, and 1 mM CaCl₂). After lysis by sonication, the lysate was centrifuged (15,000 $\times$ g for 30 minutes at 4 °C). The supernatant was extracted and incubated with Ni-NTA beads (Qiagen) for 1 hour at 4 °C. The beads were poured into an Econo-Column (Bio-Rad), washed with 10 column volumes of lysis buffer, and eluted with elution buffer (lysis buffer containing 250 mM imidazole). ULP1 protease (1:1000 ULP1 to fusion mass ratio) and λ -phosphatase (0.25 nanomoles) were added into the eluate, which was then dialyzed against 4 L dialysis buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 5 mM β -mercaptoethanol, and 1 mM MnCl₂) at 4 °C for 16 hours, using 12–14 kDa MWCO dialysis bag (Spectra/Por). Dialyzed samples were centrifuged (15,000 $\times$ g for 30 minutes at 4 °C), filtered (0.22 μ m), and purified over a 5 mL HiTrap Q HP (GE Healthcare Biosciences) column using a linear gradient of 0.2 – 0.8 M NaCl (20 mM Tris at pH 7.5, 5 mM β -mercaptoethanol). The TR sample was further purified using a gel filtration column (Superdex 200 10/300 GL, GE Healthcare Biosciences) pre-equilibrated with NMR buffer (20 mM BisTris at pH 6.8, 150 mM KCl, 10 mM CaCl₂, 5 mM β -mercaptoethanol, and 0.1 % NaN₃).

NMR titrations

All NMR experiments were performed at 25 °C on a Bruker Avance spectrometer operating at 800 MHz equipped with a cryogenic probe capable of applying pulsed field gradients along the z-axis. A 37 μ M sample of ¹³C, ¹H-Ile- δ_1 , U-[¹⁵N, ²H]-labeled TR in NMR buffer prepared in D₂O was used in the CaM-titration experiments using ¹³C, ¹H HMQC spectra

(64 and 512 complex points in the ^{13}C and ^1H dimensions, respectively, with corresponding sweep-widths of 7 and 12.5 ppm). In addition to apo TR, data were obtained in the presence of 0.25, 0.5, 1, 2, and 4 molar equivalents of U- ^2H -labeled CaM. All spectra were processed using NMRPipe⁵² and analyzed using NMRViewJ⁵³. Assignment of the Ile- δ_1 resonances were obtained using ^{13}C , ^1H SOFAST-HQMC⁵⁴ spectra of ^{13}C , ^1H -Ile- δ_1 -labeled TR mutants at 700 MHz (64 or 96 or 128 and 512 complex points in the ^{13}C and ^1H dimensions, respectively, with corresponding sweep-widths of 7 and 13.3 ppm) in the absence/presence of various molar ratios of unlabeled CaM. The concentrations of the various Ile mutants of TR in the NMR samples varied from 11.4 to 46 μM . The SOFAST-HMQC experiments ensured the rapid collection of correlation spectra for the mutants which had varying degrees of stability in solution.

Generation of a computational model of the KD•CTR complex

For the first step in the generation of the KD•CTR_{496–725} complex, the structure of a C-terminal fragment encompassing residues L496-E725 was generated utilizing the NMR structure of the HR23 construct (G562-E725) together with *ab initio* modelling⁵⁵ and RosettaDock⁵⁶ as implemented in the Rosetta3.7 suite. Fragment libraries for *ab-initio* modeling were generated using the Robetta server⁵⁷. Initially, a fragment corresponding to residues V502-S575 (CTR_{502–575}) was modeled *ab initio*. The calculation resulted in partial convergence for the fragment encompassing residues E541-S575 (CTR_{541–575}) that was consistently modeled as containing two antiparallel helices. Next, from the lowest energy structure belonging to the HR23 NMR ensemble, the folded portion that corresponds to CTR_{597–725} was extracted. A loop spanning residues Q624-C631 was also removed, since it could favor a different conformation in the presence of the neighboring regions that were missing in the NMR construct. The previously described CTR_{541–575} fragment was then docked onto the CTR_{597–725} fragment using two harmonic restraints and a 15 Å tolerance between the N-terminal residue (G597) of CTR_{597–725} and the C-terminal residue of CTR_{541–575} (S575), and between residues L564 of CTR_{541–575} and D609 of CTR_{597–725}, in order to preserve the expected anti-parallel orientation of the helices. The predicted loop that links these two regions (S575-G597), as well as the Q624-C631 loop were then modeled using the kinematics closure (KIC)⁵⁸ method implemented in Rosetta, resulting in the final model for CTR_{541–725}. It is notable that the newly constructed Q624-C631 loop assumed a conformation that was similar to that observed in the experimental NMR structure of the HR23 construct.

Next, we modeled a CTR_{496–562} fragment; it should be noted that this *in-silico* construct shares the 541–562 helical region with the previously described CTR_{541–725} model. For CTR_{496–562} however, the native eEF-2K sequence ⁵⁴¹EKGEEWDQESAVFHLEHAANLG⁵⁶² (that forms the N-terminal helix of CTR_{541–725}) was modified using RosettaRemodel⁵⁹, such that the hydrophobic residues that contact the neighboring helix on the C-terminal side (bold residues) (in the context of the modelled CTR_{541–725}) were converted to polar residues to form an artificial capping interface in the context of CTR_{496–562}. This was done in order to prevent packing of the native hydrophobic face into the hydrophobic interior of CTR_{496–562} and thus resulting in non-native contacts. This led to the following sequence: ⁵⁴¹EKGEEWDQESQVFHKEHKSNQS⁵⁶². With these

mutations in place, an *ab initio* model of CTR_{496–562} was then constructed. The resulting CTR_{496–562} was then superimposed onto the CTR_{541–725} model using their overlapping regions (residues E541–G562). At this point the redundant E541–G562 fragment within the CTR_{496–562} construct was removed, together with the neighboring G536–C540 loop. On the CTR_{541–725} model, the disordered E541–D546 fragment was also removed. A continuous CTR_{496–725} was obtained by modelling the missing loops using the KIC methodology. Finally, the resulting structure of CTR_{496–725} was relaxed in the Rosetta force field using default parameters.

Next, the CTR_{496–725} fragment was docked onto the modeled KD (H103–P330) using RosettaDock. The observed inter-domain crosslinks (K516/K517–D248 and K517/K520–D248/D249; IntraTR-2, 3 in Table 1) were used as ambiguous restraints using a flat harmonic scoring function with zero penalty range of 15 Å. A total of 20000 calculations were attempted, of these, 1205 resulted in no violations of restraints and successful structure output. Docked decoys with interface energies ranging from –5 to –10 (Rosetta Energy Units) were used for further analysis (264 total). The interface RMSD values of the decoys were calculated within RosettaDock using the local refinement protocol with one round of minimization which leads to rescoring of all decoys with the lowest energy decoy as reference.

Small angle X-ray scattering (SAXS)

The Ca²⁺-CaM•TR complex for SAXS experiments was purified as described above. Samples were concentrated using Amicon Ultra 10K MWCO spin concentrators (Millipore) to final concentrations of 1.9 or 6.4 mg/mL (for Ca²⁺-CaM•TR). Flow through from the concentrator was used as a reference to subtract the buffer contribution from the scattering data. All experiments were performed on a Rigaku BioSAXS 1000 instrument equipped with a Dectris Pilatus 100K detector attached to one port of an FRE+ Superbright rotating copper anode X-ray generator.

Scattering data was analyzed using the ATSAS 2.8.3 software package⁶⁰. The particle distribution function was calculated using GNOM⁶¹ and the molecular envelopes were calculated using DAMMIF⁶². In all 20 structures were calculated for each concentration and selected based on normalized spatial deviation values (NSD) and an average envelope was determined using DAMAVER⁶³. The frequency map and the average model computed by DAMAVER was utilized in a DAMMIN⁶⁴ run to generate the final refined molecular envelope. In addition, SAXS data was also collected for TR alone at concentrations of 3 mg/mL, 5 mg/mL and 10 mg/mL. No detailed analysis was performed in these cases since an initial inspection suggested concentration dependent aggregation.

The 6.4 mg/mL SAXS dataset was used together with the structures of the Ca²⁺-CaM•CBD complex²⁶ (a representative structure was chosen from the NMR ensemble) and the KD•CTR_{496–725} complex (from above) were to generate the overall topology of the Ca²⁺-CaM•TR complex using CORAL⁶⁵. To allow some flexibility between modules, residues W99 and A100 were removed from the C-terminus of CBD in the Ca²⁺-CaM•CBD complex and the new C-terminal residue (P98) was linked to A108 of the KD (after removing the H103–I107 segment at the N-terminus) in the KD•CTR_{496–725} complex using a 9-glycine

linker. The C-terminal P330 of the KD was then linked to the N-terminal L496 of the CTR both in the context of the KD•CTR_{496–725} complex using a 40-glycine linker (including the 6-glycine linker native to TR). Calculations were performed using the entire SAXS dataset or the range listed in Table 3 ($q=0.0238\text{--}0.2293$) using different orientations of Ca^{2+} -CaM•CBD relative to the KD•CTR_{496–725} complex, including one in which the former was placed at a significant distance without linking the C-terminus of the CBD to the N-terminus of the KD in the starting configuration. Overall similar organization of domains was obtained in all cases irrespective of starting configuration. The final structure generated by CORAL was superimposed on the DAMMIN refined molecular envelope (from above) using SUPCOMB⁶⁶.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- eEF-2K phosphorylates eEF-2 leading to a suppression of translational elongation.
- A minimal, fully-functional construct, TR, of eEF-2K has been developed.
- The architecture of the complex between calmodulin and TR has been defined.
- Engagement of calmodulin leads to significant conformational changes in TR including at remote sites.
- This study provides insight into the calmodulin-mediated activation of eEF-2K.

Figure 1.

(a) Schematic representation of the full-length (FL) and truncated (TR) constructs of eEF-2K. TR is constructed by deleting 70 N-terminal residues and a section (359–489) of the regulatory loop (R-loop) of FL. The N-terminal calmodulin-binding domain (CBD) and the kinase domain (KD) are retained in TR, as are the C-terminal helical repeats (HR1, HR2 and HR3) along with the primary activating phosphorylation site (T348). The deleted segment of the R-loop (359–489) is replaced by a 6-glycine (G_6) linker in TR. **(b)** The relevant modules for which validated structures are available are shown. The KD including residues H103-P330 is a homology model (blue), the HR23 construct of the CTR (G562-E725, purple) and the Ca^{2+} -CaM•CBD complex (CaM in light blue, CBD, S74-A100, in orange) are NMR structures. The insets show the key residues (K205, R252 and T254) of the phosphate binding pocket (PBP) on the KD and the 1-5-8 binding mode (W85, I89 and A92) of the CBD on CaM (that contains 2 Ca^{2+} ions at its N-lobe binding site; Ca^{2+} ions are absent from the C-lobe sites). The N- and C-termini of each module are indicated.

Figure 2.

ESI-MS analysis of free TR **(a)** and the Ca^{2+} -CaM•TR complex **(b)** averaged over specific fractions eluted from a gel filtration column (see Figure S3). Major charge states are labeled and states of higher charge corresponding to disordered regions (red rectangle) are also shown. For the Ca^{2+} -CaM•TR complex shown in **(b)**, charge states corresponding to a minor population of a 2:2, rather than a 1:1, complex are labeled in blue. Also shown, labeled in green, are the +7 and +8 charge states of free Ca^{2+} -CaM dissociated from the Ca^{2+} -CaM•TR complex. The inset shows an expansion of the +7 charge state with the isotopic distributions resulting from 0, 1 and 2 bound Ca^{2+} ions. Also see Figure S4.

Figure 3.

(a) The activity (picomoles of product per picomole of enzyme) of TR as ascertained through its ability to phosphorylate an optimal peptide substrate (pep-S) is stimulated by calmodulin (CaM) in the presence of Ca^{2+} (red). TR cannot phosphorylate pep-S in the absence of CaM (green). (b) TR (blue) and FL (red) show similar activity towards pep-S in the presence of Ca^{2+} -CaM. The inset shows the k_{obs} values, determined using the initial rate regime, for the two cases. Each datum represents an average of duplicate measurements. (c) Western blot analyses show lower levels of TR compared to FL in MCF-10A (eEF-2K^{-/-}) cells following transient transfection, but comparable levels of eEF-2 phosphorylated on Thr-56 (all measurements in duplicate). eEF-2 levels in cells transfected with vector alone (CV), or in those expressing TR or FL, are shown. Pan-actin is used as loading control. The right panel shows the relative activities of TR and FL (as measured through Thr-56 phosphorylated eEF-2 levels; averaged over duplicate measurements) normalized to the cellular levels of each construct.

Figure 4.

(a) Results of a representative HXMS dataset using a 60-minute D₂O-incubation period mapped onto the homology model of the KD of eEF-2K. Specific regions of the KD discussed in the text are indicated. Each residue has been colored according to a Δ_{av} value (using a cyan to magenta gradient; residues with no peptide coverage have been colored black) defined as:

$$\Delta_{av}(\%) = 100 \left(\frac{2H_{-CaM}^{inc} - 2H_{+CaM}^{inc}}{2H_{max}^{inc}} \right)_{peptides}$$

Where ${}^2H_{+CaM}^{inc}$ and ${}^2H_{-CaM}^{inc}$ represent 2H incorporation in the presence or the absence of CaM for a given peptide and ${}^2H_{max}^{inc}$ represents the maximum possible 2H incorporation for that peptide. The $\langle \rangle$ denotes an average over all peptides that contain a given residue e.g. in Figure S6, N77 is contained in 2 peptides, W85 in 1 peptide, L104 in 7 peptides. Note that > 0 indicates increased protection i.e. reduced 2H incorporation in the presence of CaM. **(b)** Time-course of 2H incorporation for selected peptides that show statistically significant differences in the absence (black) and the presence (red) of Ca^{2+} -CaM. The data represent average and standard deviation values determined from three separate measurements. The location of the peptides on the homology model of the KD are indicated using specific colors (the corresponding colors are used in numbering the peptides on the 2H incorporation curves). The bound ADP and sidechains of K132, E145 and K162 that have been shown to cross-link with CaM are shown in stick representation and labeled.

Figure 5.
(a) Δ_{av} values from a selected 60-minute dataset mapped onto a representative structure from the NMR ensemble of HR23 construct using the same approach as in Figure 4. Only the region (G597-E725) that is ordered in the NMR structure is shown. The coloring scheme is the same as in Figure 4a. **(b)** ^2H -incorporation time courses for peptides from the CTR that show statistically significant differences in the presence (red) and absence (black) of Ca^{2+} -CaM. The location of the peptides on the HR23 structure are indicated using specific colors (the corresponding colors are used in numbering the peptides on the ^2H incorporation curves). The sidechain of K715 that cross-links with CaM is shown in stick representation.

Figure 6.

(a) Computational model of the KD•CTR₄₉₆₋₇₂₅ complex using a homology model of the KD (shown in blue), the NMR structure of CTR₅₉₇₋₇₂₅ (extracted from HR23, shown in purple); the structure of the remaining fragment has been modelled using Rosetta (shown in grey). The N- and C-termini of each fragment are labeled. (b) Sidechains of the residues that form cross-links between the KD and the CTR (IntraTR-2 and IntraTR-3 in Table 1) are shown in stick representation. Sidechains of cross-linked residues are shown red (peptide 1) and cyan (peptide 2), respectively. These cross-links were used as constraints in generating the model for the KD•CTR₄₉₆₋₇₂₅ complex. Note that D685 (shown in blue) cross-links (IntraTR-1 in Table 1) with the N-terminal K93 (that belongs to the CBD, in orange) that is not part of the model. Sidechains of residues that cross-link with CaM listed in Table 1 are shown and labeled in magenta. (c) Locations of the peptides that show statistically

significant differences in protection in the HXMS measurements in the presence of Ca^{2+} -CaM are colored red (peptides 1–8 from Figure 4 and peptides 1–3 in Figure 5).

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Figure 7.

(a) ^{13}C , ^1H HMQC spectra (800 MHz) of ^{13}C , ^1H -Ile- δ 1, ^2H -labeled TR in the absence (green) or presence (magenta) of CaM in a Ca^{2+} -containing buffer. The resonances that show significant perturbations are labeled in blue. The resonances corresponding to the spatially contiguous I131 and I215 (enclosed by the black rectangle) that could not be uniquely assigned yet are both perturbed (one disappears and the other shows a significant change in resonance position) are labeled using light blue lettering. (b) The spatial distribution of the perturbed residues is indicated on the homology model of the KD using spheres, colored and labeled using the same scheme as in (a).

Figure 8.

(a) Plot of the SAXS data of the Ca^{2+} -CaM•TR complex at two different concentrations, 1.9 mg/mL (red) and 6.4 mg/mL (blue). The data have not been normalized with respect to concentration. The corresponding Guinier regions are shown in the inset. (b) Concentration normalized pair distribution functions for the SAXS data from (a). (c) The hatchet shaped molecular envelope generated from the SAXS data for the 6.4 mg/mL sample showing the approximate dimensions based on the diameters along the three principal axes of the inertia tensor calculated using UCSF Chimera. The spatial locations of CaM (light blue), CBD (orange), KD (dark blue) and CTR₄₉₆₋₇₂₅ (purple) in the context of the KD•CTR₄₉₆₋₇₂₅ complex determined using CORAL ($\chi^2=1.52$) are shown.

Figure 9.

A model for **Step 1** of the two-step activation²² of eEF-2K by Ca^{2+} -CaM is shown schematically. The CBD (orange) is engaged by Ca^{2+} -CaM (light blue) with the loss of 2 Ca^{2+} ions (from the C-lobe of CaM). This primary interaction is supplemented by additional weak interactions of CaM (most likely through the CaM N-lobe) with the N-lobe of KD (blue). The docking interaction between CaM and the eEF-2K KD leads to conformational changes that are transmitted to remote sites on the latter (represented schematically by the yellow dashed arrows) including the phosphate binding pocket (PBP, cyan), the catalytic center (grey oval) and the CTR (purple). This results in a conformation (extreme right) that is primed to efficiently phosphorylate on T348 (yellow with black outline). Thus, Ca^{2+} -CaM binding to eEF-2K may be conceived as leading to the formation of a transient “excited-state” configuration (the first virtual state in Figure S1) of the binary complex that subsequently relaxes to a stable pre-chemistry complex primed to efficiently phosphorylate on T348.

Table 1.List of cross-links between TR and CaM and between the domains of TR^a

Number	Peptide 1	Region	Peptide 2	Region	Crosslinked Residues
Cross-Links between TR and CaM					
Inter-1	¹¹⁷ YNAVTVG EWLDDDEVL <u>I</u> K MASQPF GR ¹⁴⁰	KD	³⁸ SLGQNPT <u>E</u> A <u>ELQ</u> DMINEVD ADGNGTIDFPE FLTMMAR ⁷⁴	CaM (N-lobe)	K132: Glu45/Glu47/Asp50 ^b
Inter-2	¹⁴¹ GAMR <u>E</u> C FR ¹⁴⁸	KD	²² DGDGTIT <u>T</u> K ELGTVMRSLG QNPTEAELQD MINEVDADGN GTIDFPEFLTM MAR ⁷⁴	CaM (N-lobe)	E145:Lys30
Inter-3	¹⁵¹ KLSNFLH AQQW <u>K</u> ¹⁶²	KD	³⁸ SLGQNPT <u>E</u> A <u>ELQ</u> DMINEVD ADGNGTIDFPE FLTMMAR ⁷⁵	CaM (N-lobe)	K162: Glu45/Glu47/Asp50
Inter-4	⁷⁰⁸ LANQYY Q <u>K</u> AEEAW AQMEE ⁷²⁵	CTR	⁸⁷ <u>E</u> AFR ⁹⁰	CaM (C-lobe)	K715: Glu87
Cross-links between the domains of TR					
IntraTR-1	⁸⁷ HAIQK <u>A</u> ⁹³	CBD	⁶⁸⁵ <u>D</u> PQR ⁶⁸⁸	CTR	K93:D685
IntraTR-2	⁵⁰⁹ LNALDLE <u>K</u> K ⁵¹⁷	CTR	²⁴⁸ <u>D</u> DNIR ²⁵²	KD	K516/K517: D248
IntraTR-3	⁵¹⁷ <u>K</u> IG <u>K</u> ⁵²⁰	CTR	²⁴⁸ <u>D</u> DNIRLTPQ AFSHFTFER ²⁶⁵	KD	K517/K520:D248/D249

^aThe cross-linked residues for both peptides (1 and 2) (either unambiguously or ambiguously identified) are underlined on their respective sequences (columns 2 and 4) and indicated in column 6. Cross-linked residues that could be unambiguously identified are indicated in bold in all cases.

^bCaM residues are indicated by three letter codes and eEF-2K residues by single letter codes in column 6.

Table 2

List of cross-links within the domains of TR and within CaM

Residue 1	Residue 2	Ca-Ca Distance (Å)
TR		
K150 (KD)	E174/D177(KD)	24.0/24.6
K170 (KD)	D177 (KD)	13.7
K594 (CTR)	D629 (CTR)	17.1±1.0 ^a
K684 (CTR)	E717/E718/E725 (CTR)	16.4±0.2/14.6±0.3/15.9±0.9 ^a
CaM		
Lys30 (N-lobe)	Glu127 (C-lobe)	36.6±1.7 ^b
Lys77 (C-lobe)	Glu31 (N-lobe)	26.1±0.2 ^b
Lys94 (C-lobe)	Glu127/Asp 129 (C-lobe)	20.1±0.6/17.7±0.4 ^b

^a Average distance based on an NMR structural ensemble (20 structures) of eEF-2K_{562–725} (PDB: to be submitted)

^b Average distance based on the NMR structural ensemble of the Ca²⁺-CaM•eEF-2K_{CBD} complex (PDB: 5J8H)

Table 3.Results of SAXS analysis on the Ca^{2+} -CaM•TR complex

Concentration (mg/mL)	1.9	6.4
Guinier Analysis		
I_0/c	1.34±0.01	1.26±0.00
R_G (Å)	35.5±0.4	34.7±0.4
$q_{\min}$	0.01234	0.01665
$q_{\max}RG$	1.25	1.27
Molecular Mass Analysis (Da)		
MW from Q_p ($q_{\max}$)	76955 (0.1971)	74302 (0.2185)
MW from V_c (V_c ; $q_{\max}$)	68580 (548; 0.3011)	66871 (534; 0.3011)
Bayesian credibility interval (MW)	67900–75300 (72400)	69650–75300 (72400)
P(r) Analysis		
R_G (Å)	37.45±1.36	36.53±1.29
$D_{\max}$ (Å)	130	128
q range	0.0195–0.2250	0.0238–0.2293
χ^2	0.8294	0.8360
DAMMIF (20 runs, default parameters)		
q range	0.0195–0.2250	0.0238–0.2293
Symmetry/anisotropy	PI/unknown	PI/unknown
NSD	1.384±0.183	1.166±0.113
χ^2 range	0.765–0.767	0.971–0.977
Resolution from SASRES (Å)	42±3	37±3
MM (0.5× v_{volume})(Da)	69140±390	66870±315
DAMMIN		
q range	0.0195–0.2250	0.0238–0.2293
χ^2	0.7928	0.9723