Signal Integration at Elongation Factor 2 Kinase

THE ROLES OF CALCIUM, CALMODULIN, AND SER-500 PHOSPHORYLATION*

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Eukaryotic elongation factor 2 kinase (eEF-2K), the only calmodulin (CaM)-dependent member of the unique α-kinase family, impedes protein synthesis by phosphorylating eEF-2. We recently identified Thr-348 and Ser-500 as two key auto-phosphorylation sites within eEF-2K that regulate its activity. eEF-2K is regulated by Ca²⁺ ions and multiple upstream signaling pathways, but how it integrates these signals into a coherent output, i.e. phosphorylation of eEF-2, is unclear. This study focuses on understanding how the post-translational phosphorylation of Ser-500 integrates with Ca²⁺ and CaM to regulate eEF-2K. CaM is shown to be absolutely necessary for efficient activity of eEF-2K, and Ca²⁺ is shown to enhance the affinity of CaM toward eEF-2K. Ser-500 is found to undergo autophosphorylation in cells treated with ionomycin and is likely also targeted by PKA. In vitro, autophosphorylation of Ser-500 is found to require Ca²⁺ and CaM and is inhibited by mutations that compromise binding of phosphorylated Thr-348 to an allosteric binding pocket on the kinase domain. A phosphomimetic Ser-500 to aspartic acid mutation (eEF-2K S500D) enhances the rate of activation (Thr-348 autophosphorylation) by 6-fold and lowers the EC₅₀ for Ca²⁺/CaM binding to activated eEF-2K (Thr-348 phosphorylated) by 20-fold. This is predicted to result in an elevation of the cellular fraction of active eEF-2K. In support of this mechanism, eEF-2K knockout MCF10A cells reconstituted with eEF-2K S500D display relatively high levels of phospho-eEF-2 under basal conditions. This study reports how phosphorylation of a regulatory site (Ser-500) integrates with Ca²⁺ and CaM to influence eEF-2K activity.

Protein synthesis is one of the most energy consumptive processes in eukaryotic cells, accounting for over 30% of the energy cost of a differentiating mammalian cell (1). Precise regulation of the rates at which proteins are synthesized and degraded is critical in maintaining cellular homeostasis. Translation of mRNA into polypeptide chains is carried out on the ribosome, where eEF-2 is responsible for the GTP-dependent translocation of the nascent chain from the ribosomal A-site to the P-site (2–5). Addition of each amino acid to the growing polypeptide requires GTP hydrolysis by eEF-2, which provides the energy for translocation (6). Phosphorylation of eEF-2 on Thr-56 significantly reduces its affinity for the ribosome and its ability to promote protein synthesis (7–12). Control of eEF-2 activity provides a means to rapidly and temporally modulate the rate of protein synthesis (12–14). The kinase responsible for phosphorylating eEF-2 is eukaryotic elongation factor-2 kinase (eEF-2K, EC 2.7.11.20), a ubiquitously expressed calcium/calmodulin (Ca²⁺/CaM)-regulated protein kinase (7–14) and member of the α-kinase family, which is unrelated in primary sequence to classical protein kinases (15, 16).

eEF-2K mediates an adaptive response to nutrient deprivation in tumors, leading to the promotion of tumor growth (17–19), and it was recently suggested to play a role in regulating the Warburg effect in tumor cells by modulating the synthesis of protein phosphatase 2A (PP2A) and the degradation of c-Myc through the ubiquitin-proteosomal pathway (20). We previously demonstrated a pathological role for eEF-2K in a tumor model by showing that the silencing of eEF-2K reduced tumor growth in a nude mouse MDA-MB-231 xenograft model (19). Its pathological role in human cancer is further supported by several studies correlating eEF-2K expression with low survival rates of patients with various forms of cancer, e.g. glioblastoma, medulloblastoma (17), and breast cancer (18).

The activity of eEF-2K is tightly controlled by Ca²⁺/CaM levels as well as multiple upstream kinases (21–25), other modifying enzymes (26), autophosphorylation (27, 28), and cellular pH (29). How these signals converge on eEF-2K is poorly understood. For example, the mechanisms underlying the activation of eEF-2K by low pH or in tumors expressing the active

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The abbreviations used are: eEF-2K, eukaryotic elongation factor 2 kinase; CBD, CaM binding domain; SLR, SEL-1-like helical repeat; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; KD, kinase domain; Pep-S, peptide substrate.
mechanistic target of rapamycin and Ras (which can both inhibit eEF-2K) (30) are unknown. To understand the regulation of eEF-2K in detail requires careful biochemical and structural studies. These have been hampered by an absence of highly purified and active full-length enzyme. However, we recently developed a protocol that affords pure, monomeric, and tag-less eEF-2K (31). This facilitated the first detailed biochemical analysis of its kinetic mechanism (27, 31), the identification of Ca\(^{2+}/\)CaM-inducible autophosphorylation sites such as Thr-348 (which was shown to activate the kinase (27)), as well as an evaluation of its mechanism of activation by Ca\(^{2+}/\)CaM (32).

In lieu of structural data, several studies have attempted to define the domain structure of eEF-2K using deletions and site-directed mutagenesis (33, 34). This has led to the overall organization of eEF-2K shown in Fig. 1. The N terminus of eEF-2K contains a CaM binding domain (CBD, ~79–96) and a catalytic kinase domain (KD, ~116–326). We solved the structure of the CBD·Ca\(^{2+}-\)CaM (Protein Data Bank code 5J8H), and we found that the CBD engages the CaM C-lobe in an anti-parallel 1-5-8 mode through hydrophobic interactions reinforced by a pair of electrostatic contacts (35). A striking feature of this complex is the absence and presence of Ca\(^{2+}\) from the C- and N-lobe sites of CaM, respectively, even under high Ca\(^{2+}\) conditions, where Ca\(^{2+}\) appears to enhance the CaM/eEF-2K interaction by promoting weak CaM N-lobe-mediated interactions.

The kinase domain of eEF-2K shares overall sequence homology with other members of the α-kinase family, such as TRPM7 (36) and the Dictyostelium discoideum myosin heavy chain kinase A (37). The region immediately following the KD, referred to as the regulatory loop (R-loop), is predicted to be disordered. The R-loop contains Thr-348, the autophosphorylation of which is central to the activation of eEF-2K (27, 32), and Ser-500, which is a target for autophosphorylation (27) and PKA (38–41). Elevated cAMP levels correlate with the activation and ultimately the ubiquitin-mediated degradation of eEF-2K in mammalian cells (39, 42), a process thought to involve phosphorylation of Ser-500. Degradation of eEF-2K may also involve a predicted phosphodegron created by phosphorylation of Ser-441 and Ser-445 (43). The C-terminal region contains three predicted SEL-1-like helical repeats (SLRs), known to participate in protein/protein interactions and may be involved in engaging the substrate eEF-2 (44, 45). Functional data indicate that the C-terminal region is able to modulate the kinase activity of eEF-2K through interaction with the KD (45).

We recently described a mechanistic model for the Ca\(^{2+}/\)CaM-mediated activation of eEF-2K (Fig. 2) (32) where the fully active conformation of eEF-2K is attained through two consecutive steps, which are the binding of Ca\(^{2+}/\)CaM followed by the autophosphorylation of Thr-348. In this study, we focus on understanding how Ca\(^{2+}\), CaM, and the post-translational phosphorylation of Ser-500 integrate to regulate and maintain the activity of eEF-2K in the context of this mechanism. We show the following: 1) CaM is absolutely essential for the activity of eEF-2K toward a peptide substrate, and bound apo-CaM almost fully activates eEF-2K; 2) Ca\(^{2+}\) enhances the binding affinity of eEF-2K toward CaM; 3) phosphorylation of Ser-500 enhances the rate of activating Thr-348 phosphorylation, as well as the dose response of the activated eEF-2K to CaM, but it has no effect on \(k_{cat}\); 4) eEF-2K undergoes autoprophosphorylation at Ser-500 both in vitro and in cells; and 5) the phosphomimetic Ser-500 to aspartic acid (S500D) mutation induces constitutive eEF-2K activity. This represents the first study to provide a testable framework for understanding how eEF-2K integrates upstream signals to serve as a critical cellular nutrient sensor, to control the rate of the elongation phase of protein synthesis.

**Results**

eEF-2K Is Completely Dependent on CaM and S500D Promotes Substrate Phosphorylation by Enhancing the Concentration Response of eEF-2K (Phosphorylated on Thr-348) to Apo-CaM—Recently, we reported that recombinant eEF-2K undergoes Ca\(^{2+}/\)CaM-stimulated autophosphorylation at five major sites in vitro, including Thr-348 and Ser-500 (27). We showed that phosphorylation of Thr-348 is rapid and that the T348A mutant exhibited a 25-fold higher activity than the wild type enzyme.
Phosphorylated Ser-500 Regulates eEF-2K Activation

(27). Not only did this study provide the first indication that the phosphorylation of Ser-500 could directly affect the activity of eEF-2K, it also provided the first evidence that eEF-2K could be regulated by apo-CaM (27).

Ser-500 has also been identified as one of two major sites phosphorylated by PKA in vitro (40). Although PKA activation was known to correlate with increased eEF-2K activity (41, 42), the molecular basis for this was unknown. As both phosphorylation by PKA (38, 40) and autophosphorylation (46, 47) were reported to induce a Ca\(^{2+}\)-independent activity of eEF-2K, we examined the possibility that phosphorylation of Ser-500 underlies this behavior. We first assessed whether autophosphorylation of Ser-500 correlated with the appearance of enhanced eEF-2K activity in the presence of 500 nM CaM and the Ca\(^{2+}\) chelator EGTA at pH 7.5. Thus, eEF-2K (500 nM) was allowed to autophosphorylate in the presence of CaM (5 \(\mu\)M) and Ca\(^{2+}\) (50 \(\mu\)M free) before being diluted 5-fold into a buffer containing EGTA (final concentration of 5 mM) to sequester Ca\(^{2+}\) and was assayed 1 min post-quench using a peptide substrate (Pep-S) (48). A strong correlation (over 0–180 min) between the incorporation of phosphate at Ser-500 (as determined by immunoblotting using a phospho-Ser-500-specific antibody) and the post-quench activity of eEF-2K against Pep-S was observed (Fig. 3A). Autophosphorylation enhanced the activity of eEF-2K 20-fold in the absence of Ca\(^{2+}\) but was unable to induce a similar enhancement in the activity of eEF-2K S500A (Fig. 3B). Autophosphorylation of eEF-2K also enhanced the activity of eEF-2K against the WT, suggesting that the rate enhancement was not limited to the phosphorylation of a peptide substrate (Fig. 3C). When we separated CaM from the autophosphorylated enzyme following an EGTA quench (separation scheme depicted in Fig. 3D), no activity was detectable against Pep-S (150 \(\mu\)M) (Fig. 3E). Upon re-addition of CaM (2 \(\mu\)M) to the enzyme, an increase in activity was observed, corresponding to \sim 10% of the maximal activity observed in the presence of Ca\(^{2+}\) (Fig. 3E).

To understand how the S500D mutant sensitized eEF-2K to apo-CaM, we compared the apo-CaM dependence of eEF-2K and S500D (both monophosphorylated on Thr-348 (32)) using Pep-S as substrate. Fig. 3F shows that \(k_{\text{obs}}\) versus [CaM] increases and then plateaus for both eEF-2K WT and S500D when the concentration of CaM is increased in the kinetic assay. The best fits through the data according to Equation 2 correspond to \(E_{\text{CaM}} = 37 \pm 1.1 \, \text{\mu M}\) and \(k_{\text{app}} = 11.0 \pm 0.13 \, \text{s}^{-1}\) for the WT enzyme, and \(E_{\text{CaM}} = 1.5 \pm 0.03 \, \text{\mu M}\) and \(k_{\text{app}} = 25.7 \pm 0.14 \, \text{s}^{-1}\) for S500D. This indicates that apo-CaM robustly activates eEF-2K when it is bound to eEF-2K and that the S500D mutant exhibits a 2.3-fold higher \(k_{\text{cat}}\) and a 25-fold lower \(E_{\text{CaM}}\) to achieve half-maximal activity. The introduction of the phosphomimetic S500D also promotes the apo-CaM-mediated activity of eEF-2K toward eEF-2 (Fig. 3G). These data show that eEF-2K is completely dependent on CaM for activity against eEF-2 and suggest that phosphorylation of Ser-500 reduces \(E_{\text{CaM}}\) by 25-fold to 1.5 \(\mu\)M, which is comparable with the concentrations reported for uncomplexed apo-CaM in several mammalian cells (49, 50). Thus, phosphorylation of Ser-500 may allow eEF-2K to become sensitive to apo-CaM in cells.
phospho-Thr-348 and thus are not capable of adopting a fully "active" conformation (32), were either unable to autophosphorylate Ser-500 (eEF-2K T254A) or did so at a much slower rate (eEF-2K K205A and eEF-2K R252A). Previously, we showed that the overall rate of autophosphorylation of eEF-2K is independent of enzyme concentration (27), supporting the notion that the majority of sites are phosphorylated through an intramolecular mechanism. To definitively test whether the autophosphorylation of Ser-500 is intramolecular, we tested whether eEF-2K S500A (which cannot be phosphorylated at Ser-500) is able to phosphorylate the catalytically inactive form of eEF-2K D284A (54) in the presence of saturating CaM.

FIGURE 3. Ca2+/CaM-stimulated autophosphorylation sensitizes eEF-2K to CaM through phosphorylation at Ser-500. A, eEF-2K Ca2+/CaM-independent activity and the extent of Ser-500 phosphorylation against the time of autophosphorylation. eEF-2K (500 nM) was allowed to autophosphorylate in the presence of CaM (5 μM) and free Ca2+ (50 μM). At various times, the reaction was quenched for 1 min by diluting an aliquot of autophosphorylated eEF-2K 5-fold in a buffer containing EGTA (final concentration of 5 mM), following which kinase activity was assessed against a peptide substrate. Western blotting was used to detect phosphorylation on Ser-500, and both eEF-2K activity and phosphate incorporation at Ser-500 were recorded as the percentage of their maximal values (at 180 min). Inset, expansion of the data for 0–20 min. B, eEF-2K activity following autophosphorylation for 1 h in the presence of CaM (5 μM) and free Ca2+ (50 μM), and a 1-min quench by EGTA (5 mM). Kinase activity in the absence or presence of CaM, Ca2+, or EGTA, as indicated, is reported as a percentage of the maximal activity of eEF-2K obtained in the presence of saturating Ca2+ and CaM. C, Ca2+/CaM-independent kinase activity of autophosphorylated eEF-2K against 150 μM wheat germ eEF-2. Upper panel, autoradiograph. Lower panel, Coomassie-stained gel. D, scheme for the analysis of Ca2+/CaM-independent activity of eEF-2K with or without separation of CaM. 1, autophosphorylation of eEF-2K in the presence of Ca2+ and CaM. 2, autophosphorylation is quenched in a buffer containing EGTA (final concentration of 5 mM). 3a, without separation of CaM, the effect of autophosphorylation on Ca2+/CaM-independent eEF-2K activity against 150 μM peptide substrate is determined by assaying the autophosphorylated enzyme (50 nM) in a buffer containing 5 mM EGTA (lacking CaCl2 and CaM), resulting in a calculated [Ca2+]free of ~0.2 mM, 500 nM CaM, and 7.5 mM EGTA. 3b, alternatively, to separate CaM, the autophosphorylated quenched sample is run over a HiPrepTM 26/60 SephacrylTM S-200 HR gel filtration column with a buffer containing 5 mM EGTA. Inset, upper panel, chromatogram (peak (i), eEF-2K; and peak (ii), CaM). Lower panel, corresponding peaks from chromatogram resolved by SDS-PAGE. 4b, autophosphorylated eEF-2K separated from CaM is assayed for Ca2+/CaM-independent activity against 150 μM peptide substrate in a buffer containing 5 mM EGTA (lacking CaCl2 and CaM). E, activity of autophosphorylated eEF-2K following separation from CaM (separation scheme depicted in D and described under "Experimental Procedures"). Where indicated, CaM, Ca2+, or EGTA was added to the assay. F, kcat,app versus [CaM] for eEF-2K WT (solid line) and eEF-2K S500D (dashed line) (monophosphorylated on Thr-348) in the absence of Ca2+. The lines correspond to the best fit through the data according to Equation 2 with the following parameters: kcat,app = 11 ± 0.13 s⁻¹ and EC50 = 37 ± 1.1 μM (WT); and kcat,app = 25.7 ± 0.14 s⁻¹ and EC50 = 1.5 ± 0.03 μM (S500D). G, eEF-2K (2 mM) (WT, S500A, and S500D) were assayed using 1 mM [γ-32P]ATP and 4 μM wheat germ eEF-2 in the presence and absence of CaM or Ca2+ as indicated. EGTA (1 mM) was added to all assays conducted in the absence of Ca2+. Upper panel, autoradiograph. Lower panel, Coomassie-stained gel.
Ca\(^{2+}\), CaM, and MgATP. Fig. 4D shows that no phosphorylation is detectable, suggesting that the autophosphorylation of Ser-500 is intramolecular.

As Ca\(^{2+}\) regulates the activity of eEF-2K, we tested whether it regulates Ser-500 autophosphorylation. When eEF-2K was extensively incubated with 5 μM CaM in the absence of Ca\(^{2+}\), no phosphorylation of Ser-500 was detectable, despite significant phosphorylation at other sites within eEF-2K (Fig. 4E). Notably, even a supersaturating concentration of CaM (i.e. 100 μM), which is capable of robustly promoting the activity of eEF-2K toward a peptide substrate in the absence of Ca\(^{2+}\) (Fig. 3F), cannot overcome the requirement of Ca\(^{2+}\) for Ser-500 autophosphorylation. Together, these studies suggest that autophosphorylation of Ser-500 requires Ca\(^{2+}\), the phosphorylation of Thr-348, and a functional phosphate-binding pocket. Thus, calcium ions (50 μM) can transduce a signal in vitro to the apo-CaM·eEF-2K complex to autophosphorylate on Ser-500. It remains to be determined whether this is through the binding of Ca\(^{2+}\) to apo-CaM or another site within the complex.

As the rate of Ser-500 autophosphorylation of bacterially expressed recombinant eEF-2K is quite slow in vitro, with a half-life of 10–20 min, we compared its rate of autophosphorylation with enzyme expressed in MDA-MB-231 cells. Fig. 4F shows that the mammalian enzyme autophosphorylates on Ser-500 at a similar rate, suggesting that relatively slow autophosphorylation of Ser-500 is indeed a characteristic feature of the enzyme in vitro.

Regulation of Thr-348 Phosphorylation by Phosphorylation of Ser-500, Calmodulin, and Calcium—As there is substantial cellular evidence to suggest that PKA activates eEF-2K (40, 41, 55), we wondered whether it could promote activation of eEF-2K directly under conditions of low Ca\(^{2+}\). As phosphorylation of Thr-348 is essential for the activity of eEF-2K (32), we examined whether phosphorylation of Ser-500 facilitated the phosphorylation of Thr-348 by using the S500D mutant as a mimic. To do

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**FIGURE 4.** Autophosphorylation of eEF-2K on Ser-500 is intramolecular, occurs in mammalian cells, and requires Ca\(^{2+}\), CaM, and prior phosphorylation of Thr-348. A, MDA-MB-231 cells were transfected with vectors encoding either FLAG-tagged eEF-2K WT or S500A. After 48 h, cells were treated with ionomycin (5 μM, 5 min). Lysates or eEF-2K immunoprecipitated using a FLAG antibody were analyzed by Western blotting using a phospho-specific antibody for Ser-500. B, MDA-MB-231 cells were transfected with vectors encoding eEF-2K WT, S500A or D284A. After 48 h, cells were treated with ionomycin and calyculin A, and lysates then analyzed by Western blotting for phosphorylation of Ser-500. C, HEK 293T cells were transfected with vectors encoding eEF-2K WT, S500A, or D284A. After 48 h, cells were treated with forskolin for 30 min, and the media were then supplemented with calyculin A for an additional 5 min. Lysates were analyzed by Western blotting with a phospho-specific antibody for Ser-500. D, various eEF-2K mutants were allowed to autophosphorylate in the presence of saturating CaM and Ca\(^{2+}\). The reactions were quenched after 1 h, and the samples were analyzed by Western blotting with a phospho-specific antibody for Ser-500. E, eEF-2K (500 nM) was allowed to autophosphorylate in the presence or absence of Ca\(^{2+}\) and/or CaM as indicated. Reactions were quenched after 1 h and analyzed by Western blotting for the various phosphorylated forms of eEF-2K. F, comparison between the rate of autophosphorylation at Ser-500 on eEF-2K expressed in mammalian cells and the recombinant kinase expressed in bacteria. MDA-MB-231 cells were transfected with a vector encoding eEF-2K WT and lysed after 48 h, and the lysate was incubated with Ca\(^{2+}\), CaM, and MgATP to allow for eEF-2K autophosphorylation. For comparison, recombinant human eEF-2K expressed in bacteria was allowed to autophosphorylate in the presence of Ca\(^{2+}\), CaM, and MgATP. Phosphorylation at Ser-500 was monitored by Western blotting. Con, control.
this, eEF-2K (which had been previously treated with λ-phosphatase) was pre-incubated with EGTA in the absence of CaM and the reaction (autophosphorylation) initiated by mixing the solution with MgATP. The reaction was quenched at various time points, and the level of Thr-348 phosphorylation was analyzed in each quenched sample via Western blotting with a phosho-specific antibody for Thr-348. We found that in the absence of both Ca\(^{2+}\) and CaM, phosphorylation of eEF-2K on Thr-348 was detectable only after 20 min. In contrast, phosphate was detected in the S500D mutant earlier (after 5 min, see Fig. 5A). A similar rate enhancement was observed when eEF-2K or S500D was assayed in the presence of 0.5 or 1.5 μM CaM (Fig. 5A).

We also wondered whether Ser-500 phosphorylation could directly promote the activation of eEF-2K under conditions of elevated Ca\(^{2+}\). Thus, eEF-2K (co-expressed and treated with λ-phosphatase) was pre-incubated with saturating Ca\(^{2+}\) and CaM and the reaction (autophosphorylation) initiated by rapidly mixing the solution with MgATP using a rapid chemical quench apparatus. The reaction was quenched at various time points over the course of 1 min, and the level of Thr-348 phosphorylation was analyzed by Western blotting (Fig. 5B). The signal for phospho-Thr-348 was normalized to that of total eEF-2K and plotted as a percentage of the final phosphorylation signal for phospho-Thr-348 was normalized to that of total eEF-2K WT and S500D were equivalent (Fig. 5B), suggesting that the phosphorylation of Ser-500 has no effect on the ability of a phosphatase to dephosphorylate Thr-348. However, when the lysates were supplemented with Ca\(^{2+}\)/CaM and MgATP (to stimulate Thr-348 autophosphorylation), the net rate of Thr-348 dephosphorylation was significantly slower for the S500D sample compared with the WT control (Fig. 5E). Taken together, these data indicate that phosphorylation of Ser-500 induces a 4–6-fold enhanced ability of eEF-2K to autophosphorylate on Thr-348, which may sustain Thr-348 phosphorylation in cells.

Phosphorylation of Thr-348 and Ser-500 Regulates the Ca\(^{2+}\)/CaM-dependent Activity of eEF-2K—As S500D increased the efficiency of the concentration response of activated eEF-2K (previously phosphorylated on Thr-348) to apo-CaM, we tested whether it also increased the efficiency of the concentration response of activated eEF-2K to Ca\(^{2+}\)/CaM. Therefore, we determined \(k_{\text{obs}}\) versus [CaM] for eEF-2K WT and S500D (both phosphorylated on Thr-348) using a sub–saturating concentration of 150 μM Pep-S as substrate, in the presence of 50 μM free Ca\(^{2+}\) (Fig. 5F). The best fits through the data according to Equation 2 yielded values of \(E_{50}\) and \(k_{\text{cat}}^{\text{app}}\) for both enzyme forms corresponding to \(E_{50} = 42 ± 1.7 \text{ nm}\) and \(k_{\text{cat}}^{\text{app}} = 24.5 ± 0.5 \text{ s}^{-1}\) for the WT enzyme, and \(E_{50} = 2.3 ± 0.2 \text{ nm}\) and \(k_{\text{cat}}^{\text{app}} = 24.9 ± 0.04 \text{ s}^{-1}\) for S500D.

\[
k_{\text{app}} = k_{\text{cat}}^{\text{app}} \frac{[E] + [C] + E_{50}}{\sqrt{[E] + [C] + E_{50}^2 - 4[E][C]}}
\]

(Eq. 2)

The parameters are defined as follows: \(k_{\text{app}}^{\text{cat}}\) is the apparent rate constant; \(k_{\text{cat}}^{\text{app}}\) is the apparent catalytic constant; \([E]\) is the concentration of eEF-2K; \([C]\) is the concentration of CaM; \(E_{50}\) is the apparent concentration of CaM required to achieve half-maximal activity. Notably, the maximal activity of eEF-2K WT and eEF-2K S500D at saturating CaM were similar, suggesting that S500D had little impact on the activity of eEF-2K toward the peptide substrate under these conditions. A striking characteristic of the S500D mutant, however, is its ability to be activated by a concentration of CaM some 18-fold lower than that required to activate WT eEF-2K to a similar level. Taken together, the data suggest that in the presence of Pep-S, the dual phosphorylated form of eEF-2K (Thr-348 and Ser-500) exhibits an enhanced concentration response to CaM both in the presence (Fig. 5F) as well as the absence (Fig. 3F) of Ca\(^{2+}\).

S500D Stabilizes an Active Conformation of eEF-2K That Is Induced by CaM Binding—Our data suggest that the phosphorylation of Ser-500 induces a conformational transition in eEF-2K. In the unactivated enzyme, the S500D mutation promotes autophosphorylation on Thr-348, but as noted above, this mutation has no effect on the affinity of Ca\(^{2+}\)/CaM for unphosphorylated eEF-2K. To examine the ability of the S500D phosphomimetic to promote Thr-348 phosphorylation in a more physiological setting, we tested this reaction in cell lysates in the presence of endogenous protein phosphatases. Thus, eEF-2K S500D (pre-autophosphorylated at Thr-348) was added to MDA-MB-231 lysates (supplemented with protease but not phosphatase inhibitors), and the level of Thr-348 phosphorylation was monitored by Western blotting. In the absence of supplemental Ca\(^{2+}\)/CaM and MgATP, the rates of dephosphorylation of eEF-2K WT and S500D were equivalent (Fig. 5D), suggesting that S500D had little influence on the ability of a phosphatase to dephosphorylate Thr-348. However, when the lysates were supplemented with Ca\(^{2+}\)/CaM and MgATP (to stimulate Thr-348 autophosphorylation), the net rate of Thr-348 dephosphorylation was significantly slower for the S500D sample compared with the WT control (Fig. 5E).
whereas in Fig. 6B the rate constant for Pep-S phosphorylation and product release (which is unaffected by the S500D mutation) is depicted as $k^{(sub)}_c$.

Accordingly, $\text{Ca}^{2+}/\text{CaM}$ binding and autophosphorylation follow simple hyperbolic curves, which, to a first approximation, are described by the same $\text{EC}_{50}$, which is given by Equation

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\text{FIGURE 5. S500D promotes phosphorylation of eEF-2K on Thr-348.} \ A, \text{ phosphorylation of Thr-348 versus time for eEF-2K WT and S500D in the presence of varying non-saturating concentrations of CaM in the absence of Ca}^{2+}. \ B, \text{ phosphorylation of Thr-348 versus time for eEF-2K WT and S500D in the presence of saturating concentrations of Ca}^{2+} \text{ and CaM. A solution containing eEF-2K, Ca}^{2+}, \text{ and CaM was mixed with saturating MgATP. The reaction was quenched at various time points, and the samples were analyzed by Western blotting with a phospho-specific antibody for Thr-348. Blots were quantified and data plotted as the percent of Thr-348 phosphorylation as a function of autophosphorylation time and fit to Equation 1 to give } k_{\text{auto}} = 2.1 \pm 0.4 \text{ s}^{-1} \ (t_{1/2} = 330 \text{ ms}) \text{ and } k_{\text{auto}} = 12 \pm 0.5 \text{ s}^{-1} \ (t_{1/2} = 58 \text{ ms}) \text{ for eEF-2K WT and S500D, respectively. Results are the average of three independent experiments, and error bars represent S.D.} \ C, \text{ binding of eEF-2K to dansyl-CaM. The fraction of fluorescent dansyl-labeled CaM (dansyl-CaM) bound } (F_b) \text{ to eEF-2K was measured and used to determine the affinity of eEF-2K for CaM } (K_D), \text{ as described previously (35). Error bars represent S.D. of duplicate (WT) and quadruplicate (S500D) measurements. Data shown for eEF-2K WT were previously reported (35). The calculated } K_D \text{ values of eEF-2K WT and S500D are } 23 \pm 7 \text{ and } 16 \pm 7 \text{ nM, respectively.} \ D \text{ and } E, \text{ dephosphorylation of Thr-348 on recombinant eEF-2K WT or S500D (pre-autophosphorylated at Thr-348) in MDA-MB-231 cell lysates in the absence } (D) \text{ or presence } (E) \text{ of supplemented Ca}^{2+}, \text{ CaM, and MgATP. Blots were quantified, and data were plotted as the percent of Thr-348 phosphorylation versus time. Results are the average of three independent experiments, and error bars represent S.E.} \ F, \text{ } k_{\text{obs}} \text{ versus [CaM] for eEF-2K WT (solid line) and eEF-2K S500D (dashed line) (monophosphorylated on Thr-348) in the presence of } 50 \mu \text{M Ca}^{2+}. \text{ The lines correspond to the best fit through the data according to Equation 2, and } k_{\text{cat}} = 24.5 \pm 0.5 \text{ s}^{-1}, \text{EC}_{50} = 42 \pm 1.7 \text{ nM (WT); and } k_{\text{cat}} = 24.9 \pm 0.04 \text{ s}^{-1}, \text{EC}_{50} = 2.3 \pm 0.02 \text{ nm (S500D).}
Phosphorylated Ser-500 Regulates eEF-2K Activation

A Activation

I $\xrightleftharpoons{K_d}^{(l)}$ I-CaM $\xrightarrow{k_{(IA)}}$ A-CaM $\xleftarrow{k_{(auto)}}$ F*-CaM

B Substrate phosphorylation

I* S $\xrightarrow{K_d^{(IA)}}$ I*-CaM*S $\xrightarrow{k_{(sub)}^{(IA)}}$ F*-CaM*S $\xrightarrow{K_d^{(sub)}}$ F*-CaM

E*Cam*S

EC_{50} = \frac{K_d^{(l)}}{1 + K_{eq}^{(IA)}} \tag{Eq. 3}

k_{cat}^{(IA)} = \frac{k_{auto}^{(IA)}}{1 + K_{eq}^{(IA)}} \tag{Eq. 4}

EC_{50} = \frac{K_d^{(sub)}}{1 + K_{eq}^{(sub)}} \tag{Eq. 5}

k_{cat}^{(sub)} = \frac{k_{auto}^{(sub)}}{1 + K_{eq}^{(IA)}} \tag{Eq. 6}

The parameters are defined as follows: EC_{50} is the concentration of CaM that promotes half-maximal activity. $K_d^{(l)}$ is the dissociation constant of CaM from I-CaM to A-CaM. $K_{eq}^{(IA)}$ is the equilibrium constant describing the conversion of I-CaM to A-CaM. $k_{cat}^{(IA)}$ is the catalytic constant. $k_{auto}^{(IA)}$ is the first-order rate constant for autophosphorylation of A-CaM. If we assume that following Ca^{2+}/CaM binding the active conformation is relatively high in energy (i.e. $K_{eq}^{(IA)} << 1$), then it follows that $EC_{50} \equiv K_d^{(l)}$ and $k_{cat}^{(IA)} \equiv k_{auto}^{(IA)}$. Here, any influence of S500D on $K_{eq}^{(IA)}$ is predicted to have no effect on the observed EC_{50} but will result in a higher $k_{cat}^{(IA)}$, as we observe.

In contrast, for substrate phosphorylation (Fig. 6B) where EC_{50} and $k_{cat}^{(IA)}$ are described by Equations 5 and 6, respectively, and we assume that Ca^{2+}/CaM binding to the auto-activated kinase fully stabilizes the active conformation relative to the inactive conformation in the presence of ATP and a peptide substrate (i.e. $K_{eq}^{(sub)} >> 1$), it follows that $EC_{50} = K_d^{(sub)}$ and $k_{cat}^{(sub)} = k_{auto}^{(sub)}$. The parameters are defined as follows: EC_{50} is the concentration of CaM that promotes half-maximal activity. $k_{cat}$ is the catalytic constant. $K_d^{(sub)}$ is the dissociation constant of CaM from I-CaM*S to F*-CaM*S. $K_{eq}^{(sub)}$ is the first-order rate constant for substrate phosphorylation within the F*-CaM*S complex. In this case, EC_{50} is predicted to be sensitive to changes in $K_{eq}$ induced by the S500D mutation, whereas $k_{cat}$ is predicted to be independent, as observed. Interestingly, this model may also explain why apo-CaM does not induce full activation of the WT enzyme (2-fold lower apparent $k_{cat}$), as apo-CaM may not fully stabilize the active conformation of the ternary complex (i.e. $K_{eq}^{(sub)} \sim 1$). However, the introduction of the S500D mutation is sufficient to allow apo-CaM to do so, resulting in the maximal apparent $k_{cat}$ (Fig. 3F).
Phosphorylated Ser-500 Regulates eEF-2K Activation

**A**

| Control (Cont) | WT | W85S | D284A | S500D | S500E | S500D/D284A | S500E/D284A |
|---------------|----|------|-------|-------|-------|-------------|-------------|
| eEF-2K        |    |      |       |       |       |             |             |
| p-eEF-2Th56   |    |      |       |       |       |             |             |
| Aktm          |    |      |       |       |       |             |             |

**B**

![Graph showing eEF-2K S500D exhibits high activity in unstimulated cells.](image)

eEF-2K S500D Is Highly Active in Mammalian Cells—Next, we assessed whether S500D (or S500E, an alternative phosphomimetic) could promote eEF-2K activity in mammalian cells. Previous studies had shown that the S500E mutant is poorly expressed in mammalian cells (39), and thus its activity was difficult to evaluate due to the presence of endogenous eEF-2K. To overcome this limitation, we transfected MCF10A eEF-2K−/− cells, which lack endogenous eEF-2K, with either eEF-2K S500D or eEF-2K S500E and examined the activity of the exogenously expressed enzyme under basal conditions. As expected, the mutations severely compromised the amount of eEF-2K in cell lysates compared with wild type-transfected control cells (Fig. 7A). However, despite the reduced amount of eEF-2K in these lysates, phosphorylation of eEF-2 at Thr-56 was still detected. When corrected for the level of eEF-2K expression, the S500D or S500E mutants promote eEF-2 phosphorylation some 15-fold more than wild type (Fig. 7B). Additionally, to test the importance of CaM binding for the activity of the S500D or S500E mutants in cells, double mutants, which included a Trp-85 to Ser mutation in the CaM-binding site, were assessed. The W85S mutation significantly compromises the ability of eEF-2K to bind CaM as well as the activation of eEF-2K by various stimuli in cells (35). The S500D/W85S and S500E/W85S double mutants show a significantly decreased ability to phosphorylate eEF-2 compared with the S500D or S500E single mutants. This result corroborates our finding that, in vitro, the binding of CaM is still required for the activity of eEF-2K phosphorylated at Ser-500. Interestingly, the S500D/W85S and S500E/W85S mutants are 10- and 5-fold more active than the W85S single mutant, respectively. Although not definitive, this may reflect our in vitro experiments demonstrating that Ser-500 phosphorylation greatly enhances the ability of CaM to activate eEF-2K, enough so as to partially rescue the activity of eEF-2K W85S, which binds CaM very weakly (35).

Because phosphorylation at Ser-445 has been reported to promote ubiquitin-mediated proteasomal degradation of eEF-2K (43), we tested whether the site is involved in the instability of the S500D or S500E mutants by mutation of Ser-445 to alanine. The stability of the S500D/S445A and S500E/S445A eEF-2K double mutants was not significantly different from that of the single S500D or S500E mutants (Fig. 7A). However, when the S500D or S500E mutants are converted to their kinase-dead forms by introduction of the D284A mutation (i.e. S500D/D284A and S500E/D284A), steady-state levels of eEF-2K are increased but are still less than that of both eEF-2K WT and D284A (Fig. 7A). Thus, the high level of eEF-2K activity induced by the S500D or S500E mutation cannot by itself account for the reduced expression of these mutants. Furthermore, the S500D/W85S and S500E/W85S double mutants are expressed at higher levels than that of the S500D or S500E mutants, suggesting that the ability of CaM to bind the phospho-Ser-500 form of eEF-2K may be involved in promoting its degradation. These data suggest that phosphorylation of Ser-500 may regulate steady-state levels of eEF-2K through a presently undescribed mechanism. Taken together, these studies provide unequivocal evidence that phosphorylation of Ser-500 has a profound effect on the activity of eEF-2K under basal (resting Ca2+) conditions in mammalian cells.

**Discussion**

Although eEF-2K has been known for some time to be activated by Ca2+/CaM, it was only recently that we discovered that its binding to eEF-2K induces autophosphorylation of Thr-348, which contributes to its activation, according to the mechanism in Fig. 2 (27, 32). Although a number of protein kinases phosphorylate eEF-2K, the underlying mechanisms by which these various phosphorylation events integrate into a single coherent output is unknown. This study represents the first report of how phosphorylation of a regulatory site (Ser-500) integrates with Ca2+ and CaM to influence the activity of eEF-2K.

*Apo-CaM and Ca2+/CaM Adopt a Common Mode of Binding at the C-lobe to Promote eEF-2K Activity toward eEF-2—We found that saturating apo-CaM induces a maximal eEF-2K activity against Pep-S to within 2-fold of Ca2+/CaM (Fig. 3F), suggesting that the final active conformations of auto-activated eEF-2K do not discriminate between these two structurally distinct forms of CaM when phosphorylating Pep-S. Our recent structure of the CBD·Ca2+-CaM complex (Protein Data Bank code 5J8H) suggests this may be due to a common binding mode of the Ca2+-loaded and Ca2+-free forms of CaM, where the CBD engages CaM mainly through the C-lobe, with engagement of the N-lobe (35) only occurring in the presence of Ca2+, thereby increasing the overall affinity.

*Autophosphorylation of Ser-500 Requires Ca2+ Ions and Phosphorylation of Thr-348—Our data indicate that phosphorylation of Ser-500 occurs autocatalytically in MDA-MB-231
FIGURE 8. Regulation of eEF-2K. The scheme shows a proposed mechanism for how Ca$^{2+}$, CaM, and phosphorylation of Ser-500 regulate eEF-2K. Nucleotide binding (ATP) is assumed. Steps predicted to be influenced by Ca$^{2+}$ or Ser-500 phosphorylation are indicated as circles containing Ca or S, respectively. According to the mechanism, CaM binds eEF-2K, $K_{cam}$, to induce its autophosphorylation on Thr-348, $k_{auto}$, to give the fully activated complex, $F_{*}$, CaM. This complex binds the substrate eEF-2 to give $F_{*}$, CaM-$S$, which catalyzes the phosphorylation of eEF-2, which then dissociates, $k_{sub}$, to give the inactive complexes $I_{*}$ and $I_{*}$-$S$, respectively. Ca$^{2+}$ enhances the fraction of active eEF-2K by promoting the binding of Ca$^{2+}$/CaM to $I_{*}$, $I_{*}$-$S$, and $I_{*}$-$S$ as indicated. Phosphorylation of Ser-500 promotes the fraction of active eEF-2K, as indicated, by enhancing the rate of Thr-348 autophosphorylation, $k_{auto}$, to elevate the concentration of activated complexes (e.g., $F_{*}$, CaM and $F_{*}$, CaM-$S$), and by enhancing the association of Ca$^{2+}$/CaM to $F_{*}$.

cells treated with the Ca$^{2+}$ ionophore ionomycin (Fig. 4B). Although our structure suggests that a common mode of binding at the C-lobe of CaM supports substrate phosphorylation by eEF-2K (35), we found that the autophosphorylation of Ser-500 absolutely requires Ca$^{2+}$ (Fig. 4E). This suggests that Ca$^{2+}$ stabilizes a conformation of eEF-2K that promotes the phosphorylation of Ser-500. Whether this is due to the binding of Ca$^{2+}$ to the N-lobe of CaM or to an additional Ca$^{2+}$-binding site remains to be determined. Furthermore, as low pH is reported to induce a Ca$^{2+}$-bound-like state in CaM (56), it will be important to determine whether the requirement of Ca$^{2+}$ for Ser-500 autophosphorylation is mitigated at a pH lower than was tested here (pH 7.5). We also found that autophosphorylation of Ser-500 requires prior phosphorylation of Thr-348, an intact phosphate-binding pocket and Ca$^{2+}$/CaM, further supporting the notion that it is regulated through a conformational change in eEF-2K. Interestingly, Pigott et al. (45) have reported that there is an association between the N- and C-terminal regions of eEF-2K, which has led them to propose a model involving interplay between the various domains of the kinase.

Phosphomimetic S500D Promotes eEF-2K Activity through Two Mechanisms—Our data suggest that phosphorylation of Ser-500 enhances the activity of eEF-2K in two ways. First, it enhances the rate of Thr-348 autophosphorylation, which as noted above occurs through a mechanism where the binding of Ca$^{2+}$/CaM induces the autophosphorylation of eEF-2K on Thr-348 to fully activate the enzyme (Fig. 2). Substitution of Ser-500 for the phosphomimetic residue aspartic acid (S500D) results in an increase in the rate of Thr-348 autophosphorylation in the presence or absence of CaM (Fig. 5, A and B), and it also results in a greater resistance to Thr-348 dephosphorylation in cell lysates supplemented with Ca$^{2+}$ and CaM (Fig. 5, D and E). This is consistent with the phosphorylation of Ser-500 stabilizing a conformation of eEF-2K that is also favored by Ca$^{2+}$/CaM binding to promote phosphorylation of Thr-348 ($K_{eq^{(LA)}}$) in Fig. 6A). The overall enhanced ability of the S500D mutant to autophosphorylate appears to be due entirely to its higher intrinsic rate of phosphorylation (step 2 in Fig. 2), as supported by its higher rate of Thr-348 phosphorylation in the absence of CaM (Fig. 5A), the 6-fold higher rate constant for autophosphorylation of the Ca$^{2+}$/CaM bound species ($k_{auto} = 12 \pm 0.5$ s$^{-1}$ for S500D versus $2.1 \pm 0.4$ s$^{-1}$ for WT) (Fig. 5B), and the similarity in the affinity of the wild type enzyme and S500D mutant (both lacking Thr-348 phosphorylation) to Ca$^{2+}$/CaM (Fig. 5C).

Second, the S500D mutant enhances the substrate phosphorylation response curve of eEF-2K (autophosphorylated on Thr-348) to Ca$^{2+}$/CaM. It does not influence the apparent $k_{cat}$ of the Ca$^{2+}$/CaM complex of eEF-2K (Fig. 5F). This is consistent with the notion that phosphorylation of Ser-500 stabilizes a conformation of eEF-2K that is favored strongly by Ca$^{2+}$/CaM binding ($K_{eq^{(FP)}} \gg 1$ in Fig. 6B), which contributes to the stability of the Thr-348-autophosphorylated enzyme when bound to substrates, $F_{*}$, CaM.$S$.

Cellular Regulation—Our in vitro data suggest that phosphorylation of Ser-500 enhances the Ca$^{2+}$/CaM-mediated activation of eEF-2K in cells through two mechanisms (see proposed mechanism in Fig. 8). First, Ser-500 phosphorylation enhances the rate of Thr-348 autophosphorylation and increases the cellular fraction of eEF-2K phosphorylated on Thr-348. This is supported by studies where the S500D mutation promotes a higher steady-state level of the active (i.e. phosphorylated on Thr-348) kinase in cell lysates supplemented with Ca$^{2+}$ and CaM (Fig. 5E). Second, Ser-500 phosphorylation enhances the response of eEF-2K to Ca$^{2+}$/CaM for substrate phosphorylation.

The primary role of Ca$^{2+}$ is to promote CaM binding to eEF-2K, as neither Ca$^{2+}$ nor the phosphorylation of Ser-500 enhance the $k_{cat}$ value of eEF-2K to a comparable fold increase (see Fig. 3, E and F). However, Ca$^{2+}$ promotes the autophosphorylation of eEF-2K on Ser-500, thereby providing a potential Ca$^{2+}$-regulated feed-forward control system for regulating
Phosphorylated Ser-500 Regulates eEF-2K Activation

protein synthesis (Fig. 4E). Feed-forward systems involving Ca^{2+} have been shown to be important for driving biological phenotypes, such as cancer cell invasion (57) and the innate immune response (58), and are important regulatory components of cell signaling. Under basal conditions (i.e. resting Ca^{2+}/CaM concentrations), Ca^{2+} levels are sufficient to promote some binding of CaM to eEF-2K, as evidenced by significant eEF-2 phosphorylation in unstimulated cells. Accordingly, phosphorylation of Ser-500 is predicted from our in vitro data to further enhance the active fraction of eEF-2K, even under basal concentrations of Ca^{2+}. Consistent with this model, the activity of the S500D mutant under basal conditions in MCF10A cells is sufficient to promote the steady-state level of phospho-eEF-2 around 15-fold more efficiently than the wild type enzyme (Fig. 7).

Conclusions—This study investigates how Ca^{2+} and CaM regulate the activity of eEF-2K and how the phosphorylation of Ser-500 modulates this regulation. Interestingly, our analysis reveals CaM as an essential ligand for eEF-2K activity. Several protein kinases phosphorylate eEF-2K to either activate or regulate the activity of eEF-2K and how the phosphorylation of Ser-500 modulates this regulation. Interestingly, our analysis reveals CaM as an essential ligand for eEF-2K activity. Several protein kinases phosphorylate eEF-2K to either activate or inhibit it (21–25). In no case has the biochemical basis for the regulation of eEF-2K by upstream kinases clearly been determined. We find that Ser-500 phosphorylation, occurring either autokinetically or by a forskolin-sensitive kinase, enhances the response of eEF-2K to both Ca^{2+}-loaded and apo-CaM and enhances the rate of eEF-2K autophosphorylation at Thr-348. The net effect of Ser-500 phosphorylation is to enhance the fraction of active (i.e. CaM-bound and phosphorylated on Thr-348) eEF-2K within the cell, leading to significantly enhanced eEF-2 phosphorylation.

Experimental Procedures

Reagents, Equipment, Strains, Plasmids, and Molecular Biology

Reagents, plasmids, strains and equipment were obtained and used as described previously (27, 31, 32). Site-directed mutagenesis of eEF-2K and cloning of the eEF-2K cDNA into mammalian expression vectors were performed as described earlier (27, 32).

Expression and Purification of Proteins

Tobacco etch virus protease (60, 61) and CaM (59, 62) were purified, and Pep-S (31) was synthesized as described earlier. Recombinant human eEF-2K and the various mutants were co-expressed with λ-phosphatase in Escherichia coli and purified (including λ-phosphatase treatment) as described earlier (27, 32, 31). However, the eEF-2K W855 mutant was purified by anion-exchange chromatography (Mono Q) instead of CaM-agarose affinity chromatography prior to the final gel filtration step.

Analytical Methods

General Kinetic Assays—eEF-2K activity was assayed at 30 °C in Buffer A (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 100 μM EGTA, 150 μM CaCl\textsubscript{2}, 2 μM CaM, and 10 mM MgCl\textsubscript{2}), containing 150 μM Pep-S, 2 nM eEF-2K, and 1 mM [γ-\textsuperscript{32}P]ATP (100–1000 cpm/pmol) as described earlier (27, 32).

Autophosphorylation Assay—As described earlier (32), autophosphorylation of eEF-2K was carried out in Buffer B (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 100 μM EGTA, and 10 mM MgCl\textsubscript{2}), using phosphate-free eEF-2K enzyme, CaCl\textsubscript{2}, and CaM as indicated, and initiated with 1 mM ATP. (a) For analysis of Thr-348 and Ser-500 autophosphorylation by immunoblotting, the reactions were carried out with 1 mM ATP, quenched at the various indicated times, and then analyzed for the incorporation of phosphate at Thr-348 or Ser-500 by Western blotting with specific antibodies. (b) For specificity of autophosphorylation, the reaction was carried out at 30 °C for 1 h using 500 nM phosphate-free eEF-2K WT or various mutants, ± 5 or 100 μM CaM, ± 50 μM free Ca\textsuperscript{2+}, and ± 2.5 mM EGTA as indicated. Samples were then analyzed by Western blotting. (c) For separation of calmodulin after autophosphorylation, the reaction was carried out at 30 °C for 1 h using 2 μM phosphate-free eEF-2K WT or S500D, 20 μM CaM, 200 μM free Ca\textsuperscript{2+}, and 1 mM ATP. The reaction was quenched with 5 mM EGTA for 30 min at 4 °C and then applied to a HiPrep\textsuperscript{TM} 26/60 Sephacryl\textsuperscript{TM} S-200 HR gel filtration column pre-equilibrated with Buffer C (20 mM Tris-HCl (pH 8.0), 0.15 mM NaCl, 5 mM EGTA, and 0.1% 2-mercaptoethanol (v/v)) to separate CaM. Chromatography was performed over 1 column volume (320 ml) at a flow rate of 1 ml/min. Fractions were collected and analyzed for purity by resolving the samples by SDS-PAGE. Fractions that contained the eluted monomeric kinase were pooled and dialyzed against storage buffer (Buffer D) (25 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, and 10% glycerol) and stored at −80 °C. Activity assays using the autophosphorylated kinase were carried out using the general kinetic assay ± 2 μM CaM, ± 50 μM free Ca\textsuperscript{2+}, and ± 5 mM EGTA as indicated.

Effect of Autophosphorylation on Enzyme Activity—eEF-2K (500 nM) was pre-incubated in Buffer B (with 5 μM CaM and 150 μM CaCl\textsubscript{2}) for 10 min at 30 °C, and autophosphorylation then initiated by the addition of 1 mM ATP as described above. To quench the reaction, at the various indicated times (0–180 min), an aliquot of autophosphorylated eEF-2K was diluted 5-fold in Buffer E (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA and 6.25 mM EGTA), to a final concentration of 5 mM EGTA. After quenching for 1 min, the effect of autophosphorylation on Ca\textsuperscript{2+}-independent kinase activity was determined against the peptide substrate or wheat germ eEF-2. (a) For the assay against peptide substrate, the autophosphorylated enzyme (50 nM) was assayed at 30 °C in Buffer F (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 5 mM EGTA, and 10 mM MgCl\textsubscript{2}) containing 150 μM peptide substrate and 1 mM [γ-\textsuperscript{32}P]ATP (100–1000 cpm/pm mol). Because of dilutions, this results in a calculated [Ca\textsuperscript{2+}]_{free} of ~0.2 mM, 500 nM CaM, and 7.5 mM EGTA in the assay. The rate of phosphorylation of the peptide (μM · s\textsuperscript{−1}) was determined using the general kinetic assay described earlier. In a similar manner, Ca\textsuperscript{2+}-independent kinase activity of the unautophosphorylated control (incubated in the absence of ATP) was also determined. Comparisons were made to autophosphorylated and unautophosphorylated eEF-2K (5 nM) assayed against the peptide substrate in the presence of 2 μM CaM and 50 μM free Ca\textsuperscript{2+}. All the assays were performed in triplicate. (b) For assay against wheat germ eEF-2, the assays were performed using 10 nM autophosphorylated eEF-2K in Buffer F containing 10 μM wheat germ eEF-2 and 1
Response assays in the absence of Ca\textsuperscript{2+} assays conducted in the absence of Ca\textsuperscript{2+}

Equation 1 to approximate the rate of autophosphorylation at phosphate at Thr-348 by Western blotting. The data were fit to samples loading buffer, and analyzed using a PhosphorImager.

Pre-steady-state Enzymatic Activity Assays—Experiments were performed with eEF-2K WT and S500D on a KinTek RQF-3 rapid quench-flow apparatus as described previously (32). The samples were then analyzed for the incorporation of phosphate at Thr-348 by Western blotting. The data were fit to Equation 1 to approximate the rate of autophosphorylation at Thr-348 in eEF-2K.

Characterization of eEF-2K Calmodulin Dependence—Dose-response assays in the presence of Ca\textsuperscript{2+} were performed in Buffer H (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 mM BSA, 100 mM EGTA, 150 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}), and several concentrations of CaM (0–1 mM). Reactions were performed with 0.5 nM eEF-2K WT or S500D. Dose-response assays in the absence of Ca\textsuperscript{2+} were performed in Buffer J (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 mM BSA, 2.5 mM EGTA, and 10 mM MgCl\textsubscript{2}), and 150 mM peptide substrate, with 1 mM [γ-\textsuperscript{32}P]ATP (100–1000 cpm/pmol) and several concentrations of CaM (0–1 mM). Reactions were performed with 5 nM eEF-2K WT or S500D. Kinase activity was determined in a similar manner to the general kinetic assay described earlier, and the data were fit to Equation 2.

**Cell Work**

**Cell Lines, Culture Conditions, Transfections, and Treatment of Cells with Stimuli—MDA-MB-231, HEK 293T, MCF10A, and MCF10A eEF-2K\textsuperscript{-/-}** were obtained and maintained as described earlier (32). MDA-MB-231 and HEK 293T cells were seeded in 6-well plates (1 × 10\textsuperscript{6} cells/well), and after 24 h (~80% confluency), the cells were transfected with the eEF-2K pcDNA3 HA-FLAG vector (4 μg/well) using Lipofectamine\textsuperscript{®} 2000 (Life Technologies, Inc.), according to the manufacturer’s protocol. MCF10A and MCF10A eEF-2K\textsuperscript{-/-} cells were transfected as described earlier (32). Cells were incubated for a further 48 h to allow for expression and then either lysed or treated with various stimuli before being lysed. Cells were treated with 50 μM forskolin for 30 min, 5 μM ionomycin for 5 min, and/or 100 mM calyculin A for 5 or 10 min as described earlier (32).

**Cell Lysis—**Cells were lysed as described earlier (32); however, for experiments analyzing the dephosphorylation of Thr-348 on eEF-2K by phosphatases, ice-cold Buffer K (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 1% Triton X-100, and 10% glycerol), supplemented with complete EDTA-free Protease Inhibitor Mixture, lacking phosphatase inhibitors was used.

**Analysis of Thr-348 Dephosphorylation in Cell Lysates—**MDA-MB-231 cell lysate (80 μg) lacking phosphatase inhibitors was used for the analysis. The lysate was supplemented with 1 mM CaCl\textsubscript{2}, 0.2 mM MnCl\textsubscript{2}, and 100 μM ATP in a final reaction volume of 100 μL. The reaction mixture was incubated at 30 °C for 3 min before the reaction was initiated by the addition of 25 nM pre-autophosphorylated eEF-2K WT or S500D (CaM separated by size exclusion chromatography). Aliquots of eEF-2K (50 ng) were removed at the various indicated times, and the reaction was then quenched by addition of hot SDS-PAGE sample loading buffer followed by heating for 10 min at 95 °C. Samples were then analyzed for phosphorylation at Thr-348 by Western blotting with specific antibodies.

**Immunoprecipitation of FLAG-tagged eEF-2K from Cell Lysates—**Equal amounts of protein (250 μg) from cell lysate samples were made up to a final volume of 500 μL in lysis buffer (containing protease and phosphatase inhibitors). Samples were then incubated with 50 μL of anti-FLAG\textsuperscript{®} M2 affinity gel (Sigma) at 4 °C overnight. Beads were washed three times with lysis buffer (containing protease and phosphatase inhibitors). The bound sample was eluted by heating the beads with SDS-PAGE sample loading buffer and then analyzed by Western blotting. Alternatively, the bound sample was allowed to autophosphorylate in the presence of Ca\textsuperscript{2+} and CaM for various times before being eluted and analyzed.

**Western Blot Analysis and Quantification of Blots—**For detection by chemiluminescence, Western blotting and quantification of blots were performed as described previously (27, 32). (b) For detection by fluorescence, Immobilon-FL PVDF membrane (Millipore) was used for transfer, and membranes were processed with Odyssey\textsuperscript{®} Blocking Buffer (LI-COR, Lincoln, NE), primary antibodies in 5% BSA or nonfat dry milk in TBST, and the appropriate secondary antibody conjugated to a fluorescent dye. Fluorescence detection was performed on an Odyssey\textsuperscript{®} Sa Imaging System (LI-COR).

**Commercial Antibodies—**Most antibodies were used as described earlier (32). The following antibodies were purchased: Cell Signaling Technology (Danvers, MA), phospho-eEF2k (Ser-366) antibody (catalog no. 3691, 1:2000); Santa Cruz Biotechnology, Inc. (Dallas, TX), p-eEF2K antibody (Ser-78) (catalog no. sc-33051, 1:1000); ECM Biosciences (Versailles, KY), eEF2K (Thr-348), phospho-specific (catalog no. EP4411, 1:1000); eEF2K (Ser-359), phospho-specific (catalog no. EP4431, 1:1000); eEF2K (Ser-500), phospho-specific (catalog no. EP4451, 1:1000); LI-COR Biosciences, IRDye\textsuperscript{®} 680RD goat anti-mouse IgG (H+L) (catalog no. 926-68170, 1:15,000); IRDye 800CW goat anti-rabbit IgG (H+L) (catalog no. 827-08365, 1:15,000).

**Author Contributions—**C. D. J. T. and K. N. D. conceived the project and designed the research strategy and experimental approach. C. D. J. T. performed the experiments with assistance from D. H. G., G. S., C. A. C., S. B. F., R. M. W., and T. S. K. The data were analyzed by C. D. J. T. and K. N. D. The manuscript was written by C. D. J. T., D. H. G., R. G., and K. N. D. The studies were supervised by K. N. D.
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Phosphorylated Ser-500 Regulates eEF-2K Activation

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