The Molecular Mechanism of Eukaryotic Elongation Factor 2 Kinase Activation*  

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Clint D. J. Tavares1,2,3, Scarlett B. Ferguson1,4, David H. Giles5, Qiantao Wang6, Rebecca M. Wellmann5, John P. O’Brien1,2, Mangalika Warthaka7, Jennifer S. Brodbelt3,5, Pengyu Ren4, and Kevin N. Dalby3,4,5

From the 1Graduate Program in Cell and Molecular Biology, the 2Division of Medicinal Chemistry, College of Pharmacy, and the 3Department of Biomedical Engineering, Cockrell School of Engineering, and the 4Department of Chemistry and Biochemistry, College of Natural Sciences, University of Texas, Austin, Texas 78712

Background: Eukaryotic elongation factor 2 kinase (eEF-2K) regulates protein translation elongation rates. 

Results: eEF-2K activation involves a two-step process of calmodulin binding and rapid Thr-348 autophosphorylation. 

Conclusion: Activation of eEF-2K involves two distinct allosteric steps, both of which potentially induce a conformational change. 

Significance: This mechanism provides a framework for understanding how eEF-2K integrates inputs from multiple upstream signaling pathways.

Calmodulin (CaM)-dependent eukaryotic elongation factor 2 kinase (eEF-2K) impedes protein synthesis through phosphorylation of eukaryotic elongation factor 2 (eEF-2). It is subject to complex regulation by multiple upstream signaling pathways, through poorly described mechanisms. Precise integration of these signals is critical for eEF-2K to appropriately regulate protein translation rates. Here, an allosteric mechanism comprising two sequential conformations is described for eEF-2K activation. First, Ca2+/CaM binds eEF-2K with high affinity (Kapp/ CaM = 24 ± 5 nM) to enhance its ability to autophosphorylate Thr-348 in the regulatory loop (R-loop) by > 104-fold (kcat = 2.6 ± 0.3 s−1). Subsequent binding of phospho-Thr-348 to a conserved basic pocket in the kinase domain potentially drives a conformational transition of the R-loop, which is essential for efficient substrate phosphorylation. Ca2+/CaM binding activates autophosphorylated eEF-2K by allosterically enhancing kcat for peptide substrate phosphorylation by 103-fold. Thr-348 autophosphorylation results in a 25-fold increase in the specificity constant (Kcat/Km for Pep-S), with equal contributions from Kapp and Kcat for Pep-S, suggesting that peptide substrate binding is partly impeded in the unphosphorylated enzyme. In cells, Thr-348 autophosphorylation appears to control the catalytic output of active eEF-2K, contributing more than 5-fold to the ability to promote eEF-2 phosphorylation. Fundamentally, eEF-2K activation appears to be analogous to an amplifier, where output volume may be controlled by either toggling the power switch (switching on the kinase) or altering the volume control (modulating stability of the active R-loop conformation). Because upstream signaling events have the potential to modulate either allosteric step, this mechanism allows for exquisite control of eEF-2K output.

Eukaryotic elongation factor 2 kinase (eEF-2K) is a ubiquitously expressed eukaryotic calcium/calmodulin (Ca2+/CaM)-regulated protein kinase (1–4), which lacks sequence homology with typical protein kinases. It is therefore classified as an atypical kinase, belonging to the same family as myosin II heavy chain kinase (MHCK A) (5, 6). Its only known substrate is eukaryotic elongation factor 2 (eEF-2), which it phosphorylates on Thr-56, disrupting the elongation phase of protein synthesis by impeding the interaction between eEF-2 and the ribosome (1, 2, 7–9).

eEF-2K expression (mRNA) correlates poorly with outcome for at least three types of cancer, including hormone-sensitive breast cancer and glioblastoma (10–17), and has been shown to promote the survival of various cancer cell lines in response to a range of stresses (13, 14, 17–20). Most notably, it has been shown to mediate an adaptive response to severe nutrient deprivation in transformed cells, suggesting that it confers protection to the hypoxic and nutrient-deprived environment of the tumor (17). Consistent with this idea, we found that siRNA-mediated knockdown of eEF-2K reduced the growth of an MDA-MB-231 xenograft in nude mice (16). eEF-2K is also critical for the appropriate functioning of neurons (21), where it regulates local protein synthesis associated with changes in synaptic structure and spine morphology that are required for learning and memory. It has been shown that eEF-2K associates directly with group I metabotropic glutamate receptors (mGluRs) in a

* The abbreviations used are: eEF-2K, eukaryotic elongation factor 2 kinase; eEF-2, eukaryotic elongation factor 2; MHCK A, myosin II heavy chain kinase; mGluR, group I metabotropic glutamate receptor; IAE, IAEDANS (5-((2-iodoacetyl)amino)ethyl)aminomethan sulfonic acid); CaM, calmodulin; CaMK, CaM kinase; R-loop, regulatory loop; Pep-S, peptide substrate; 2-DOG, 2-deoxy-D-glucose; AMPK, 5′-adenosine monophosphate-activated protein kinase; mTOR, mammalian target of rapamycin.

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1 Both authors contributed equally to this work.

2 To whom correspondence may be addressed: Division of Medicinal Chemistry, College of Pharmacy, 107 W. Dean Keeton, Biomedical Engineering Bldg., University of Texas, Austin, TX 78712. Tel.: 512-232-8585; Fax: 512-232-2606; E-mail: clinittvares@utexas.edu.

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5 To whom correspondence may be addressed: Division of Medicinal Chemistry, College of Pharmacy, 107 W. Dean Keeton, Biomedical Engineering Bldg., University of Texas, Austin, TX 78712. Tel.: 512-471-9267; Fax: 512-232-2606; E-mail: dalby@Austin.utexas.edu.
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Ca\(^2+\)-dependent and mGluR activity-dependent manner, which may allow the kinase to act as a rapid and transient switch controlling the translation of proteins required for mGluR-dependent long term synaptic depression (22).

A three-dimensional structure has not been reported for eEF-2K, but several studies have identified the overall layout of the protein and location of the various domains (23–25). Based on these studies, we generated a model utilizing the programs SMART and PSIPRED, which describes the protein and location of the various domains (23–25). Highlighted are a putative Ca\(^2+\)-regulatory domain (CRD (28), a CaM-binding domain (CBD), an atypical kinase catalytic domain (KD), and a CaM-binding domain containing three potential SEL1-like helical repeats (starting at 576, 610, and 665) (SHR). The C terminus is essential for eEF-2 binding to eEF-2K (25). Several phosphorylation sites associated with eEF-2K regulation are indicated: Ser-78 (which is phosphorylated by an unknown mTOR-regulated kinase (25)), Thr-348 (an autophosphorylation site (31, 57)), Ser-359 (phosphorylated by p38 (58) and the Cdc2-cyclin B complex (53)), Ser-366 (phosphorylated by p70 S6 kinase (59) and p90\(^{RSK1}\) (59)), Ser-441 (phosphorylated by an unknown kinase (30, 60)), Ser-445 (phosphorylated by an unknown kinase (30, 60)), Ser-445 (an autophosphorylation site (30, 31, 57)), and Ser-500 (phosphorylated by PKA (32) and an autophosphorylation site (31)). Binding of phospho-Thr-348 to ABP facilitates full activation of eEF-2K. Sites that promote or inhibit eEF-2K activity are colored green and red, respectively.

FIGURE 1. Proposed domains and major sites of phosphorylation of eEF-2K. Domain boundaries of eEF-2K (residues 1–725) based on biochemical experiments and primary and secondary sequence prediction software (PSIPRED) (26, 27, 56). Highlighted are a putative Ca\(^2+\)-regulatory domain (DXDXDG; CRD (28), a CaM-binding domain (CBD), an atypical kinase catalytic domain (KD), a basic allosteric binding pocket (ABP), a degron motif (DSGXXS; DM), and a C-terminal helical domain containing three potential SEL1-like helical repeats (starting at 576, 610, and 665) (SHR). The C terminus is essential for eEF-2 binding to eEF-2K (25). Several phosphorylation sites associated with eEF-2K regulation are indicated: Ser-78 (which is phosphorylated by an unknown mTOR-regulated kinase (25)), Thr-348 (an autophosphorylation site (31, 57)), Ser-359 (phosphorylated by p38 (58) and the Cdc2-cyclin B complex (53)), Ser-366 (phosphorylated by p70 S6 kinase (59) and p90\(^{RSK1}\) (59)), Ser-398 (phosphorylated by AMPK (55)), Ser-441 (phosphorylated by an unknown kinase (30, 60)), Ser-445 (an autophosphorylation site (30, 31, 57)), and Ser-500 (phosphorylated by PKA (32) and an autophosphorylation site (31)). Binding of phospho-Thr-348 to ABP facilitates full activation of eEF-2K. Sites that promote or inhibit eEF-2K activity are colored green and red, respectively.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, Strains, and Equipment

Reagents, plasmids, strains, and equipment were obtained and used as described previously (31, 33). The pcDNA3 FLAG HA vector (Plasmid 10792) was obtained from Addgene (Cambridge, MA). Rapid quench experiments were performed on a KinTek RQF-3 rapid quench-flow apparatus. Fluorescence measurements were taken on a Jobin-Yvon Spex Fluorolog-3 model FL3–11 fluorometer using a SpetraAcq controller and FluorEssence\textsuperscript{TM} software.
**Molecular Biology**

Site-directed eEF-2K mutants were generated as described earlier (31). Wild type and mutant eEF-2K cDNA were cloned into the mammalian expression vector pcDNA3 FLAG HA (Addgene) using specific primers, *PfuUltra*™ II Fusion HS DNA polymerase, restriction enzymes, and the Quick Ligation™ kit (New England Biolabs), according to the manufacturer’s protocol.

**Expression and Purification of Proteins**

Tobacco etch virus protease (35, 36) and CaM (37, 38) were purified, and peptide substrate (Pep-S) (33) was synthesized as described earlier.

Recombinant human eEF-2K was co-expressed with λ-phosphatase in *E. coli*, and purified as described earlier (31, 33). However, to further decrease the possibility of having phosphates on eEF-2K, an additional step of treatment with λ-phosphatase was performed between the tobacco etch virus protease cleavage of the Trx–His8 tag and CaM-agarose affinity chromatography steps. After the addition of MnCl2 to a final concentration of 1 mM, the cleaved sample was incubated with 5000 units of λ-phosphatase (New England Biolabs) for 30 min at 30 °C, as per the manufacturer’s protocol. To prepare eEF-2K phosphorylated on Thr-348, the kinase was neither co-expressed nor treated with λ-phosphatase. Unpublished7 and previous (31) mass spectrometric and immunoblotting analyses indicate that this kinase sample has no significant phosphorylation at the known (auto)phosphorylation sites (including Ser-78, Ser-359, Ser-366, Ser-377, Ser-396, Ser-398, Ser-445, Ser-474, and Ser-500) while having 80–90% of Thr-348 phosphorylated.

**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 mM BSA, 100 mM EGTA, 150 mM CaCl2, 2 mM CaM, and 10 mM MgCl2), containing 150 mM Pep-S, 2 nM eEF-2K enzyme and 1 mM [γ-32P]ATP (100–1000 cpm/pmol) as described earlier (31).

**Autophosphorylation assay**

Buffer B (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 mM BSA, 100 mM EGTA, and 10 mM MgCl2) was used to carry out eEF-2K autophosphorylation, using 100 mM phosphate-free eEF-2K, with or without 150 mM CaCl2 or with or without 2 mM CaM, as indicated, and 1 mM ATP. EGTA (2.5 mM) was added to all assays conducted in the absence of Ca2+. The reaction was quenched at the various indicated times and then analyzed for the incorporation of phosphate at Thr-348 by Western blotting with specific antibodies, as described previously (31).

**Pre-steady-state enzymatic activity assays**

Experiments were performed on a KinTek RQF-3 rapid quench-flow apparatus at 30 °C in Buffer A. Experiments were initiated by the rapid mixing of Solution A (200 nM eEF-2K in Buffer A), with an equal volume of Solution B (2 mM ATP in Buffer A). Reactions were quenched at the various indicated times (0–1 s) with 4 volumes of Buffer C (200 mM KCl, 50 mM EDTA, and 10 mM EGTA), followed by the immediate addition of hot SDS-PAGE sample loading buffer and further heating for 10 min at 95 °C. The samples were then analyzed for the incorporation of phosphate at Thr-348 by Western blotting. To approximate the rate of autophosphorylation at Thr-348 in eEF-2K, the data were fit to Equation 1,

\[
\% \text{Phosphorylation} = \frac{\text{Maximum % phosphorylation} \times (1 - e^{-kt_{\text{auto}}})}{(1 - e^{-kt_{\text{auto}}})}
\]

where \(k_{\text{auto}}\) represents the rate of Thr-348 autophosphorylation, and \(t\) is time in seconds.

**Characterization of enzymatic activity**

Kinase activity in each case was determined by calculating the rate of phosphorylation of the peptide (μM s⁻¹) in a similar manner to the general kinetic assay described above. EGTA (2.5 mM) was added to all assays conducted in the absence of Ca2+.

**Calmodulin Dependence**—Dose-response assays were performed in Buffer D (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 mM BSA, 100 mM EGTA, 150 mM CaCl2, and 10 mM MgCl2), against 150 μM peptide substrate, with 1 mM [γ-32P]ATP and several CaM concentrations. Reactions were performed with 0.5 nM eEF-2K WT and 0–5 μM CaM, 2 nM eEF-2K WT and 0–2 μM CaM(C75)IAE, or 20 nM eEF-2K T348A and 0–10 μM CaM WT. Data were fit to Equation 2,

\[
k_{\text{app}} = k_{\text{cat}} \frac{[E] + [C]}{K_{C}} - \frac{([E] + [C] + K_{C}^{\text{app}})^2 - 4[E][C]}{2[E]}
\]

where \(k_{\text{app}}^{\text{obs}}\) 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 varied substrate (MgATP or peptide substrate, with several concentrations of \([\gamma-32P]\)ATP), and \(K_{C}^{\text{app}}\) is the apparent CaM concentration required to achieve half-maximal activity (\(K_{C}^{\text{app}}(\text{CaM})\)).

**MgATP Dependence**—Assays were performed in Buffer B, in the presence or absence of 2 μM CaM and 150 μM CaCl2, against 150 μM peptide substrate, with several concentrations of [γ-32P]ATP (0–1 mM). Reactions were performed with 1 nM eEF-2K WT in the presence of Ca2+/CaM, 200 nM eEF-2K WT in the absence of Ca2+/CaM, or 20 nM eEF-2K T348A in the presence of Ca2+/CaM. The data were fit to Equation 3,

\[
k_{\text{app}}^{\text{obs}} = k_{\text{cat}}^{\text{app}} \frac{[S]}{K_{m}^{\text{app}} + [S]}
\]

where \([S]\) is the concentration of varied substrate (MgATP or Pep-S), and \(K_{m}^{\text{app}}\) is the apparent substrate concentration required to achieve half-maximal activity (\(K_{m}^{\text{app}}(\text{MgATP})\) or \(K_{m}^{\text{app}}(\text{Pep-S})\)).

**Pep-S Dependence**—Assays were performed in Buffer B, in the presence or absence of 2 μM CaM and 150 μM CaCl2, against several concentrations of peptide substrate (0–800 μM), with

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Fold change in activity fit to Equation 4, ing measurements at 590 nm (a wavelength where the emission fluctuation in the signal due to noise of the instrument, by tak- of eEF-2K, both using blank subtraction. The intensity at 470 nm to determine dose dependence for spectral data and at 470 nm. The data were converted to fold change in activity (compared with kinase activity at 0 mM KH₂PO₄) and fit to Equation 4,

Fold change in activity

\[
\frac{1}{1 + \frac{\text{Maximum -fold change in activity} [C]}{K^{app} + [C]}} \quad (\text{Eq. 4})
\]

where \([C]\) is the concentration of varied KH₂PO₄, and \(K^{app}\) is the apparent KH₂PO₄ concentration required to achieve half-maximal activity (\(K^{app}_{\text{eEF-2K}}\)).

Analysis of calmodulin binding by fluorescence

Binding of CaM to various forms of eEF-2K was measured using 25 nM CaM(C75)IAE, that was labeled using a protocol described earlier (39). Assays were performed at 30 °C in Buffer E (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 mM EGTA, and 150 mM CaCl₂) and titrated with eEF-2K at a starting volume of 350 µl. CaM(C75)IAE was excited at 345 nm using 3-mm slits, and the emission was measured from 400 to 600 nm for spectral data and at 470 nm to determine dose dependence of eEF-2K, both using blank subtraction. The intensity at 470 nm was corrected for changes in the assay volume as well as fluctuation in the signal due to noise of the instrument, by taking measurements at 590 nm (a wavelength where the emission of CaM(C75)IAE is known to be unaffected by eEF-2K binding) and subtracting that from the intensity at 470 nm. The fraction of CaM bound was then determined using Equation 5,

\[
\frac{\text{Fraction of CaM bound}}{\text{max}} = \frac{I_{470} - I_{470}^{max}}{I_{470}^{max} - I_{470}^{max}} \quad (\text{Eq. 5})
\]

where \(I_{470}\) is the intensity at 470 nm when [eEF-2K] = x, \(I_{470}^{max}\) is the intensity at 470 nm when [eEF-2K] = 0, and \(I_{470}^{max}\) is the intensity at 470 nm at maximum [eEF-2K]. The fraction of CaM bound was then plotted as a function of eEF-2K concentration. The data were fit to Equation 6,

Fraction of CaM bound

\[
\frac{([C] + [E] + K^{app}_{\text{CaM}}) - \sqrt{([C] + [E] + K^{app}_{\text{CaM}})^2 - 4[C][E]}}{2[C]} \quad (\text{Eq. 6})
\]

where \(K^{app}_{\text{CaM}}\) is the apparent CaM dissociation constant. When \(K^{app}_{\text{CaM}}\) is converted to fractional activity, \(K^{app}_{\text{CaM}}\) is taken as 1, and when it is converted to percentage of maximal activity, \(K^{app}_{\text{CaM}}\) is taken as 100.

Homology model and sequence alignments

COBALT was used to perform eEF-2K (Homo sapiens) and MHCK A (Dictyostelium discoideum) sequence alignments (40). PSIPRED, a primary and secondary sequence prediction software, was used to estimate potential regulatory regions and domains in eEF-2K (26). A computationally generated three-dimensional homology model of the catalytic subunit of eEF-2K was derived based on the catalytic domains of MHCK A and TRPM7. PyMOL was used for highlighting the various indicated regions in the homology model.

Cell Work

Cell lines and culture conditions

Isogenic non-tumorigenic breast epithelial cell lines MCF-10A and KO MCF-10A (eEF-2K⁻/⁻) (MCF-10A with both alleles of the eEF-2K gene knocked out) were obtained from Sigma-Aldrich. MDA-MB-231 (breast adenocarcinoma), MIA PaCa-2 (pancreatic carcinoma), and HEK 293T cell lines were obtained from American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were cultured in DMEM/F-12 supplemented with 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. MIA PaCa-2 and HEK 293T cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. The MCF-10A cell lines were cultured in DMEM/F-12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 100 ng/ml cholera toxin, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cell cultures were maintained at 37 °C in a humidified incubator containing 5% CO₂. All cell culture reagents were from Invitrogen or Sigma-Aldrich.

Transfections

MCF-10A and KO MCF-10A (eEF-2K⁻/⁻) cells were seeded in 6-well plates, and after 24 h (~50% confluence), cells were transfected with the eEF-2K pcDNA3 FLAG HA vector using Lipofectamine® LTX with Plus™ reagent (Invitrogen), according to the manufacturer’s protocol. Cells were incubated for a further 48 h to allow for expression and then either lysed or treated with various stimuli before being lysed. Controls included transfection with the empty pcDNA3 FLAG HA vector.

Treatment of cells with stimuli

Cells were treated with 400 µM H₂O₂ for 1 h or with 5 µM ionomycin for 5 min in the normal growth media described earlier. For treatment with 2-deoxy-D-glucose (2-DOG), cells were preincubated in medium with low glucose (5.6 mM) for 6 h, before being treated with 25 mM 2-DOG for 30 min in the same low glucose medium. For starvation experiments, cells were incubated in Dulbecco’s PBS, without serum supplementation, for 6 h. For analysis of the specificity of the anti-phospho-Thr-348 antibody, cells were treated for 10 min with 100 nM calyculin A (to inhibit cellular phosphatases). Additionally, some cells were treated for 30 min with 10 nM bradykinin and 1 µM thapsigargin (to increase cytoplasmic Ca²⁺ levels) prior to calyculin A treatment.

Cell lysis and western blot analysis

Following treatments, cells were washed twice in ice-cold PBS (pH 7.4) (Invitrogen) and lysed in ice-cold Buffer F (50 mM

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FIGURE 2. Thr-348 autophosphorylation is rapid and CaM-dependent. A, activity of 1 nM WT and 25 nM T348A eEF-2K was determined against peptide substrate in the presence of Ca\(^{2+}\) and CaM and is reported as the percentage of the WT activity. B, rapid quench analysis was utilized to measure the rate of Thr-348 autophosphorylation on the millisecond time scale. eEF-2K was preincubated with saturating Ca\(^{2+}\) and CaM before rapid mixing 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. Data were plotted as the percentage of Thr-348 phosphorylation as a function of autophosphorylation time and were fit to Equation 1, where \(k_{\text{auto}} = 2.6 \pm 0.3\) s\(^{-1}\) and \(t_{1/2} = 279\) ms gives the best fit. Results are the average of three independent experiments, and error bars represent S.D. C, the rate of phosphate incorporation at Thr-348 for eEF-2K WT under varying CaM and Ca\(^{2+}\) conditions (as indicated) was determined. At the indicated times, the reaction was quenched, and samples were then analyzed by Western blotting with a phospho-specific antibody for Thr-348.

HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl\(_2\), 1% Triton X-100, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na\(_2\)VO\(_4\), and 10% glycerol), supplemented with PhosSTOP phosphatase inhibitor mixture (Roche Applied Science), complete EDTA-free protease inhibitor mixture (Roche Applied Science), Halt protease and phosphatase inhibitor mixture (Thermo Fisher Scientific), 1 μM microcystin-LR, and 100 mM calyculin A. Lysates were subjected to one freeze-thaw cycle and then clarified by centrifugation at 15,000 × g for 15 min. Total protein concentration for each sample was determined by a Bradford assay (Bio-Rad). Equal amounts of protein from samples were resolved by SDS-PAGE and then analyzed by Western blotting as described previously (31).

Commercial antibodies

The following antibodies were purchased from the following suppliers: phospho-eEF2 (Thr-56) (catalog no. 2331) (Cell Signaling Technology (Danvers, MA)); anti-actin, clone C4 (catalog no. MAB1501) and anti-eEF2 (C-terminal) (catalog no. 07-1382) (Millipore); eEF-2K (C-terminal) (catalog no. 1754-1) and eEF2 phospho (Thr-56/58) (catalog no. 1853-1) (Epitomics (Burlingame, CA)); eEF2K (Thr-348), phospho-specific (catalog no. EP4411) (ECM Biosciences (Versailles, KY)); and goat anti-rabbit IgG (H+L)-HRP conjugate (catalog no. 172-1019) and goat anti-mouse IgG (H+L)-HRP conjugate (catalog no. 172-1011) (Bio-Rad).

Quantification and normalization of blots

Blots were quantified either using Image Studio version 3.1 (LI-COR) or ImageJ software. For analysis of lysates from KO MCF-10A (eEF-2K\(^{-/-}\)) cells transfected with eEF-2K, the following normalization was performed. First, all samples were normalized to pan-actin to account for any error in gel loading. Despite using an eEF-2K knock-out cell line, blotting results indicate very low levels of phospho-eEF-2 (Thr-56) in samples transfected with the control vector, possibly due to nonspecific binding of the antibody. To account for this low background, the quantified value was subtracted across all of the samples in the second step of normalization. In the final normalization step, to take into account differences in eEF-2K expression levels either due to variations in transfection or mutant expression, phospho-eEF-2 values were further normalized to eEF-2K levels.

RESULTS

Calcium/Calmodulin Induces Rapid Thr-348 Autophosphorylation—We recently identified Thr-348 as the first site phosphorylated in vitro following incubation of eEF-2K with Ca\(^{2+}\)/CaM and MgATP and found it to be important for the activation of eEF-2K (Fig. 2A) (31). To evaluate the magnitude of the allosteric activation of eEF-2K by Ca\(^{2+}\)/CaM and to provide insight into the mechanism, we took advantage of our ability to prepare eEF-2K free of phosphate and utilized rapid quench kinetics to determine the rate of autophosphorylation of Thr-348. Therefore, to measure autophosphorylation of eEF-2K, the reaction was initiated by mixing the kinase (preincubated with saturating amounts of CaM and Ca\(^{2+}\)) with saturating MgATP. Following rapid mixing (~2 ms), the reaction was allowed to proceed for various times before being quenched. To quantify progress of the reaction, quenched samples were recovered and analyzed by SDS-PAGE and Western blotting using a phospho-specific antibody for phosphorylated Thr-348 (31). The best fit to the data (appearance of an immunoreactive species) according to Equation 1, which describes a first-order reaction and assumes rapid binding of MgATP, furnished a rate constant of \(k_{\text{auto}} = 2.6 \pm 0.3\) s\(^{-1}\) (\(t_{1/2} = 0.28\) s) (Fig. 2B). When autophosphorylation was assessed over 600 s in the absence of Ca\(^{2+}\) and CaM, an immunoreactive band appeared at a greatly reduced rate. Estimates based on the relative intensity of the bands at 600 s in Fig. 2C indicate that the reaction (autophosphorylation of Thr-348) has a half-life of at least 6000 s in the absence of Ca\(^{2+}\) and CaM. Interestingly, Ca\(^{2+}\) appears to enhance the basal rate of autophosphorylation in the absence of CaM (compare the intensity of the bottom two panels of Fig. 2C). These results establish that Ca\(^{2+}\)/...
CaM enhances the ability of eEF-2K to autophosphorylate on Thr-348 by \( \approx \times 10^{3} \)-fold.

**Calcium/Calmodulin Regulates the Intrinsic Activity of eEF-2K**—Although our studies suggest that Ca\(^{2+}\)/CaM binding and autophosphorylation contribute to the activity of eEF-2K, little is known about the underlying mechanisms. To further understand the contribution of Ca\(^{2+}\)/CaM to the activation of eEF-2K, we evaluated its effect on the ability of eEF-2K to phosphorylate the peptide substrate, Ac-RKKYKFNEDT*ERRRFL-NH\(_{2}\) (Pep-S) (33). This peptide contains a basic residue at position 3, which is reported to be important for efficient peptide phosphorylation by eEF-2K (41), and corresponds to the Arg residue relative to Thr-348 of eEF-2K, as well as Thr-56, the major phosphorylation site in eEF-2 (42). We have previously shown that eEF-2K phosphorylates Pep-S through a sequential mechanism (43, 44). In contrast to these kinases, we find that Ca\(^{2+}\)/CaM controls the intrinsic activity of eEF-2K without apparently promoting the access of substrates to its active site.

**Autophosphorylation at Thr-348 Regulates Substrate Access but Not Calcium/Calmodulin Binding**—Because Ca\(^{2+}\)/CaM stimulates the phosphorylation of Thr-348, we considered the possibility that the C-terminal regulatory loop containing Thr-348 impairs substrate binding. To test this possibility, we purified a mutant of eEF-2K that cannot autophosphorylate on residue 348 (eEF-2K T348A) and examined its ability to phosphorylate Pep-S. We found that the ability of the mutant to phosphorylate Pep-S is severely compromised, as evidenced by a 5-fold increase in \( K_{\text{m}} \) (Pep-S) and a 5-fold...
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**TABLE 1**

| eEF-2K<sup>WT</sup> | Ca<sup>2+</sup>/CaM<sup>WT</sup> | K<sub>app</sub> | Km<sub>(MgATP)</sub> | Km<sub>(Pep-S)</sub> |
|-------------------|-----------------------------|--------------|----------------|------------------|
| WTThr-348–P       | 0.0075 ± 0.0007             | 81 ± 0.5     | 154 ± 30        |
| WTThr-348–P       | 18.1 ± 0.3                  | 66 ± 1       | 66 ± 3          |
| T348A             | 3.1 ± 0.1                   | 59 ± 2       | 327 ± 15        |

*WT and all eEF-2K mutants were co-expressed with λ-phosphatase and subsequently treated with λ-phosphatase during purification to obtain the eEF-2K catalytic domain. Cys-75 with an IAEDANS fluorophore (39). Ca<sup>2+</sup>/CaM and Ca<sup>2+</sup>/CaMNAE exhibit a similar ability to activate eEF-2K (Fig. 4A) and Ca<sup>2+</sup>/CaMNAE exhibit a blue shift in a similar manner at 78 ± 8 nm respectively (Fig. 4D and Table 2). Interestingly, the T348A mutant binds Ca<sup>2+</sup>/CaM with a lower affinity (K<sub>app</sub>(CaM<sub>WT</sub>) = 225 ± 49 nm) and also exhibits a 10-fold decrease in sensitivity to Ca<sup>2+</sup>/CaM (compared with WT eEF-2K) when assayed for its ability to phosphorylate Pep-S (Fig. 4, D and E, and Table 2). This suggests that Thr-348 may communicate to the Ca<sup>2+</sup>/CaM binding site prior to phosphorylation. Taken together, these data suggest that rapid autophosphorylation of Thr-348 contributes to the activation of eEF-2K and does not significantly modulate the binding of Ca<sup>2+</sup>/CaM to eEF-2K. 

**Phospho-Thr-348 Appears to Bind a Basic Pocket in the Catalytic Domain to Regulate Substrate Access**—MHCK A, whose catalytic domain is ~36% identical to that of eEF-2K, requires autophosphorylation of Thr-825 for activity (45). Phosphorylation of Thr-825 in MHCK A allosterically activates the kinase by providing a ligand for a phosphate-binding pocket present within the catalytic domain (45). When the sequences of MHCK A and eEF-2K are aligned, three residues required for activation in MHCK A are found to be conserved in eEF-2K (Lys-205, Arg-252, and Thr-254) (Fig. 5, A and B) and are highlighted in magenta in the three-dimensional homology model shown in Fig. 5C. To investigate whether they constitute a functional site in eEF-2K, each residue was separately mutated to alanine, and the ability of the kinase to autophosphorylate at Thr-348 and phosphorylate Pep-S was examined. Although CaM binding and the rate of Thr-348 autophosphorylation are unaffected by each mutation (Fig. 6, A and B, and Table 2), the mutants’ ability to phosphorylate Pep-S is compromised (Fig. 6C). However, it must be noted that the earliest time point in the Thr-348 autophosphorylation assay is 15 s. Because our rapid quench data indicate that the WT kinase undergoes autophosphorylation at Thr-348 with a t<sub>1/2</sub> = 279 ms, it is possible that for the mutants, a decrease in the rate of Thr-348 autophosphorylation may not be detected at the time points assayed. To further test the idea that phosphorylated Thr-348 binds the basic pocket to activate eEF-2K, we examined whether phosphate (KH<sub>2</sub>PO<sub>4</sub>) activates the eEF-2K T348A mutant. Fig. 6D reveals a 2.4-fold increase in the activity of T348A in a dose-dependent manner, consistent with a mechanism of phosphate-mediated activation.

**Thr-348 Is Phosphorylated in MCF-10A Cells and Correlates with eEF-2K Activity**—Previous large scale proteomic mass spectrometry studies have provided evidence for the phosphorylation of eEF-2K on Thr-348 in mammalian cells (46–49). To ascertain whether Thr-348 is a regulatory site in vivo, we first confirmed that the phosphorylation of Thr-348 could be detected by Western blotting using a custom-made phospho-specific antibody. Fig. 7A shows that phosphorylation of Thr-348 increases in MCF-10A cells following treatments known to induce phosphorylation of eEF-2 through eEF-2K activation (e.g., by inhibition of glycolysis (25 mM 2-DOG, low glucose, 30 min), oxidative stress (400 μM H<sub>2</sub>O<sub>2</sub>, 30 min), and 10-fold increase in the activity of T348A in a dose-dependent manner, consistent with a mechanism of phosphate-mediated activation. To confirm the mechanism of activation by phosphate, we made several double mutants (K205A/T348A, R252A/T348A, and T254A/T348A), which could not autophosphorylate at Thr-348, and were predicted to have a compromised interaction with phospho-Thr-348 based on the experiments above. We found that none of the double mutants could be rescued by the addition of phosphate (Fig. 6E). These results taken together suggest that autophosphorylation of Thr-348 favors a conformational change of the R-loop associated with the binding of phospho-Thr-348 to a basic pocket on the catalytic domain, which drives the CaM-bound kinase into a more active conformation.
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**TABLE 2**

*In vitro* kinetic parameters for various forms of eEF-2K

| eEF-2K* | $k_{app}^{15}$ | $K_{app}^{15}$ | $k_{app}^{16}$ | $K_{app}^{16}$ | $K_{cat}^{16}$ | $K_{cat}^{16}$ |
|---------|----------------|---------------|---------------|---------------|---------------|---------------|
| WT      | ND             | ND            | ND            | ND            | ND            | ND            |
| WTThr-348-P | 19.0 ± 1.0 | 66 ± 2       | 78 ± 8       | 225 ± 49    |               |               |
| T348A   | 1.0 ± 0.2     | 491 ± 13     | 225 ± 49     |               |               |               |
| K205A   | 3.3 ± 0.1     | ND           | 10 ± 3       |               |               |               |
| R252A   | 6.5 ± 0.5     | ND           | 12 ± 1       |               |               |               |
| T254A   | 1.8 ± 0.2     | ND           | 77 ± 9       |               |               |               |

*See Table 1.

* $k_{app}^{15}$ determined against 150 μM peptide substrate in the presence of 2 μM CaM, 50 μM free Ca$^{2+}$, and 1 mM MgATP.

* $k_{app}^{16}$ determined against peptide substrate.

* $K_{cat}^{16}$ determined by fluorescence following binding of CaM(C75)IAE to various forms of eEF-2K.

* ND, not determined.

T348A mutant and allowed to express for 48 h. Using Western blotting, cell lysates were probed for phosphorylation of eEF-2 on Thr-56 (Fig. 7F). Levels of phospho-eEF-2 (Thr-56) were normalized to controls (Fig. 7F) to account for variability in the level of eEF-2K expression as well as nonspecific binding of the antibody. Based on these experiments, expression of eEF-2K T348A results in ~20% of the phosphorylated eEF-2 seen by exogenous expression of WT eEF-2K in the same cell line. To further evaluate whether the potential conformational change induced by Thr-348 autophosphorylation is important, KO MCF-10A (eEF-2K−/−) cells were transfected with eEF-2K K205A, R252A, or T254A, and the phosphorylation of eEF-2 on Thr-56 was monitored (Fig. 7H). Under basal conditions, each of the mutants exhibited compromised ability to phosphorylate eEF-2 by 2–3-fold (Fig. 7I). These experiments establish that Thr-348 is autophosphorylated in cells and suggest that the probable conformation driven by the binding of phospho-Thr-348 to the basic pocket promotes the basal activity of eEF-2K toward eEF-2 in cells.

**Autophosphorylation of Thr-348 Is Not Rate-limiting following eEF-2K Stimulation**—We then sought to determine whether autophosphorylation of Thr-348 contributes to the
regulation of eEF-2K in cells. To examine this, KO MCF-10A (eEF-2K/H11002/H11002) cells were transfected with eEF-2K WT or T348A, and treated as in Fig. 7A. The cell lysates were then probed for phosphorylation of eEF-2 on Thr-56 by Western blotting (Fig. 8, A–C). Data were plotted as -fold change in the normalized levels of phospho-eEF-2 (Thr-56) compared with the untreated wild-type transfected cells (Fig. 8, D–F). These experiments demonstrate that the magnitude of the stimulation of eEF-2K (7–9-fold) is independent of phosphorylation at Thr-348 (Fig. 8G). Taken together, these experiments suggest that autophosphorylation at Thr-348 is rapid and contributes about 5-fold to the activity of eEF-2K in cells under both basal and stimulated conditions (Fig. 8H).

**DISCUSSION**

Although eEF-2K was first discovered more than 20 years ago and shown to be a Ca²⁺/CaM-dependent protein kinase, its mechanism of activation has remained elusive. In this study, we

![Figure 5](image-url)

**Disruption of the potential phosphate-binding pocket in eEF-2K.** A, sequence alignment between eEF-2K from *H. sapiens* and MHCK A from *D. discoideum*. Residues located within the proposed phosphate-binding pocket are highlighted in red. B, primary sequence alignment of eEF-2K with residues in the phosphate-binding pocket, catalytic loop, and C-tail of MHCK A. Through x-ray crystallography studies, Crawley et al. (45) have shown that the residues Lys-684, Arg-734, Thr-736, and Asp-762 form electrostatic interactions with the phosphate molecule, and are critical for the activity of MHCK A. The corresponding conserved residues in eEF-2K have been highlighted in red (Lys-205, Arg-252, Thr-254, and Asp-280). C, a computationally generated three-dimensional homology model of the catalytic subunit of eEF-2K based on the catalytic domains of the atypical kinases MHCK A and TRPM7. The phosphate-binding pocket resides, Lys-205, Arg-252, and Thr-254, are in magenta. The glycine-rich loop (G-loop) (GXGXXG), which is involved in localization of ATP in the cleft, is likely present between residues 296 and 301 (in dark blue) (24, 61). The hinge region of the ATP binding site is shown in yellow (57, 61).
take advantage of recent advances in the preparation of eEF-2K (31, 33) to identify the molecular basis for its activation in vitro and in cells (see Fig. 9).

Allosteric Activation of eEF-2K by Calcium/Calmodulin Binding—Sequence alignment analysis suggests that residues 79–96 conform to a Ca\(^{2+}\)/H\(_{11001}\)/CaM binding site. This is supported by mutagenesis studies, which indicate that Trp-85 may correspond to a hydrophobic anchor for binding to the C-terminal domain of CaM (24). Our previous studies showed that the binding of Ca\(^{2+}\)/CaM to eEF-2K stimulates its autophosphorylation through an intramolecular mechanism (31). An early event was shown to be the autophosphorylation of Thr-348, and both Ca\(^{2+}\)/CaM binding and autophosphorylation were shown to support the overall activity of eEF-2K (31). However, little is known about how these events are coupled and how they each contribute to the overall activation of eEF-2K. We measured the rate of eEF-2K autophosphorylation in the presence of Ca\(^{2+}\)/CaM using a custom-made antibody to quantify phospho-Thr-348 by Western blotting. We found that in the absence of Ca\(^{2+}\)/CaM, autophosphorylation of eEF-2K on Thr-348 occurs slowly with a rate constant of \(k_{\text{auto}} \approx 10^{-4} \text{s}^{-1}\) (Fig. 2C).

To evaluate the allosteric effect of Ca\(^{2+}\)/CaM binding, we used a rapid chemical quench apparatus to determine the rate of autophosphorylation within the Ca\(^{2+}\)/CaM/eEF-2K complex. This allowed us to establish, for the first time, that the Ca\(^{2+}\)/CaM/eEF-2K complex autophosphorylates \(10^4\)-fold faster...
than apo-eEF-2K, with a rate constant of $k_{\text{auto}} \approx 2.6 \pm 0.3 \text{s}^{-1}$ (Fig. 2B).

To further understand how Ca$^{2+}$/CaM contributes to the activation of eEF-2K, we evaluated its effect on the ability of eEF-2K to phosphorylate a peptide substrate (33). We took advantage of being able to prepare eEF-2K stoichiometrically phosphorylated on Thr-348 (Fig. 4C). This allowed us to identify the contribution of Ca$^{2+}$/CaM binding without complications due to the induction of phosphorylation. We reasoned that if Ca$^{2+}$/CaM regulates an intrasteric step to remove an autoinhibitory domain from the active site, this would be reflected in a significant decrease in $K_{\text{m(app)}}$ for peptide phosphorylation upon Ca$^{2+}$/CaM binding. Our study revealed that Ca$^{2+}$/CaM induced an increase in the steady-state rate of Pep-S phosphorylation, which is similar in magnitude to the rate enhancement seen for autophosphorylation, suggesting that
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despite significant differences in the mechanism, they are probably regulated by the same allosteric process. The minimal effect of Ca\(^{2+}\)/CaM binding on \(K_m\) for both peptide and MgATP strongly suggests that it has little influence on the access of either substrate to the active site. In fact, the observed 7-fold increase in \(K_m\) suggests that it may destabilize a non-productive binding mode of ATP (25). Taken together, our kinetic data support a mechanism where Ca\(^{2+}\)/CaM binding restructures the active site of eEF-2K, to enhance its ability to catalyze phosphoryl-transfer by more than 3 orders of magnitude. Notably, Ca\(^{2+}\)/CaM appears to increase the intrinsic activity of the kinase domain through a mechanism fundamentally different from other CaMKs, where Ca\(^{2+}\)/CaM displaces an inhibitory region of the protein from the active site (43, 44).

A Potential Conformational Transition of the R-loop Promotes Substrate Phosphorylation—Thr-348 lies in a regulatory region C-terminal to the catalytic domain (R-loop) (Fig. 1) in an analogous position to Thr-825, a major autophosphorylation site of the atypical kinase MHCK A, which is known to regulate its activity (45). To understand how autophosphorylation of Thr-348 contributes to the regulation of eEF-2K, we turned to the proposed mechanism of activation of MHCK A (45). Upon Thr-825 autophosphorylation, MHCK A is activated through a conformational change induced by the binding of phosphorylated Thr-825 to a positively charged pocket composed of several basic residues. Sequence alignment and a three-dimensional homology model of eEF-2K indicate that the residues that comprise the proposed phosphate-binding pocket of

FIGURE 8. Thr-348 autophosphorylation is required for maximal activation of eEF-2K in cells. A–F, knock-out MCF-10A (eEF-2K\(^{-/-}\)) cells were transfected with a vector encoding either eEF-2K WT or T348A. After 48 h, cells were treated with 25 mM 2-DOG (30 min) (A), 400 \(\mu\)M \(\text{H}_2\text{O}_2\) (1 h) (B), or 5 \(\mu\)M ionomycin (5 min) or starvation with Dulbecco’s PBS (6 h) (C), along with the appropriate controls. A–C, lysates were analyzed by Western blotting using the indicated antibodies. D–F, graphical representation of A–C. Data are reported as the fold change in normalized levels of phospho-eEF-2 (Thr-56) as compared with the untreated WT transfected cells. The experiments were performed in triplicate, and error bars represent S.D. G, graphical summary comparing the fold change in eEF-2K WT and T348A mutant activity upon treatment with the various stimuli. H, graphical summary of the fold difference between eEF-2K WT and T348A activities under basal conditions as well as upon treatment with the various indicated stimuli. For G and H, normalized phospho-eEF-2 (Thr-56) was considered as an estimate for cellular eEF-2K activity.
MHCK A are conserved in eEF-2K (Fig. 5). Several experiments in our study also support the notion that a conserved mechanism contributes to the activation of the two kinases. For example, as in MHCK A, mutageneses of basic residues in the putative phosphate-binding pocket of eEF-2K diminish its ability to phosphorylate a peptide substrate. Furthermore, the addition of inorganic phosphate partially rescues the activity of the eEF-2K T348A mutant, with a concentration for half-maximal activation of \( K_{\text{cat}} (\text{KH}_2\text{PO}_4) \approx 0.2 \text{ mM} \). Similarly, MHCK A is activated 4-fold with \( K_{\text{cat}} (\text{KH}_2\text{PO}_4) \approx 0.44 \pm 0.15 \text{ mM} \) (45).

The dose-dependent activation of the eEF-2K T348A mutant by phosphate suggests that neutralization of the proposed positively charged pocket may contribute to the activation of eEF-2K. A computationally generated three-dimensional homology model of eEF-2K provides further support for this idea, because Asp-280 and Tyr-282, which are located on the same loop as Asp-284 (a conserved residue thought to be important for catalysis in eEF-2K (50)), are within hydrogen bonding distance of Lys-205 and Arg-252, respectively. Thus, occupancy of the proposed phosphate-binding pocket by phospho-Thr-348 could influence the conformation of Asp-284 and consequently affect \( k_{\text{cat}} \). Because our results suggest that the phosphorylation of Thr-348 may induce a conformational change of the regulatory domain, we wondered whether the domain might also inhibit substrate binding in the absence of phosphorylation. To assess this possibility, we compared the kinetic parameters of WT eEF-2K and the T348A mutant for the phosphorylation of Pep-S. We found that both \( k_{\text{cat}}^{\text{app}} \) and \( K_{\text{m}}^{\text{app}}(\text{Pep-S}) \) contribute to the 25-fold increase in specificity (\( k_{\text{cat}}^{\text{app}}/K_{\text{m}}^{\text{app}}(\text{Pep-S}) \)) induced by autophosphorylation of Thr-348. It is interesting to note that in the presence of Ca\(^{2+}/\text{CaM}, k_{\text{cat}}^{\text{app}} \) for phosphorylation of Pep-S is \( \sim 6 \)-fold faster than the observed rate of autophosphorylation of Thr-348. This may be because Thr-348 is not optimally aligned for phosphoryl-transfer within the active site, or this may indicate that phosphorylation of Thr-348 contributes to the catalytic enhancement of the active site, as is suggested by the ability of phosphate to rescue the activity of the T348A mutant. Interestingly, we have not found any evidence to support a mechanism where autophosphorylation of Thr-348 regulates the affinity of eEF-2K for Ca\(^{2+}/\text{CaM} as has been noted for the autophosphorylation of CaMK-II on Thr-286 (51).

**A Molecular Model for the Regulation of eEF-2K in Vivo**—Given the large dependence on Ca\(^{2+}/\text{CaM} binding for activation of eEF-2K in vitro and the absence of any biochemical evidence to support a mechanism where eEF-2K is active in the absence of CaM, we propose that eEF-2K is regulated through a
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general mechanism that involves two sequential conformational transitions (Figs. 9 and 10). We propose that, according to this mechanism, activation of eEF-2K is initiated by CaM binding, which induces a conformational change to drive the initial 10-fold activation of the kinase domain (step 1 in Fig. 10). Several mechanisms may promote this process, including an elevation in cellular Ca\(^{2+}\) levels, the dephosphorylation of Ser-78 (52), or the phosphorylation of Ser-500 on eEF-2K by PKA (31, 32). The activation of the kinase by the binding of CaM then induces autophosphorylation of Thr-348, which drives eEF-2K into a conformation capable of phosphorylating eEF-2 (step 2 in Fig. 10). We established that Thr-348 is phosphorylated in MCF-10A cells using a custom-made anti-phospho-Thr-348 antibody and found that Thr-348 phosphorylation positively correlates with eEF-2 phosphorylation in response to various stimuli known to activate eEF-2K (Fig. 7A). Utilizing an MCF-10A cell line lacking endogenous eEF-2K, we were able to evaluate the role of the second allosteric step by expressing mutants of eEF-2K whose ability to undergo this step is compromised and then monitoring eEF-2 phosphorylation both before and after cell stimulation. Our data suggest that mutants that cannot attain the active conformation (step 2 in Fig. 10) exhibit a 2.5–5-fold lower ability to promote phosphorylation of endogenous eEF-2 in KO MCF-10A (eEF-2K\(^{-/-}\)) cells. Our cellular studies also reveal that the degree of stimulation of eEF-2K is independent of its ability to autophosphorylate at Thr-348. This suggests that the various cell stresses known to elevate cellular Ca\(^{2+}\) levels or regulate upstream pathways (e.g. AMPK or mTOR) do not appear to significantly regulate the second allosteric step of eEF-2K activation under the conditions tested. Although these studies suggest that, in these cases, the second allosteric step is not rate-limiting and is not significantly regulated by the cell stresses, they do not rule out the possibility of modulation of this step by other signals, such as those mediated by Cdc2/cyclin B and p38\(^{\text{\alpha}}\), which phosphorylate Ser-359.

We made the surprising observation that the T348A mutant has relatively high activity compared with the wild type enzyme when assessed in cells. Although the mutant exhibits a 10-fold lower sensitivity to Ca\(^{2+}/\text{CaM}\) in vitro and exhibits a 25-fold lower \(K_{\text{cat}}^{\text{app}}/K_{m}^{\text{app}}\) toward the peptide substrate as noted above, its ability to promote phosphorylation of eEF-2 is only suppressed 5-fold. The lower affinity of the T348A mutant for Ca\(^{2+}/\text{CaM}\) does not appear to be significant because the K205A, R252A, and T254A mutants, which also cannot undergo the second allosteric step but are able to bind Ca\(^{2+}/\text{CaM}\) as tightly as eEF-2K WT, show a similar level of cellular activity as the T348A mutant. It has been suggested that phosphorylation of Ser-78 regulates eEF-2K activity by impeding its ability to bind Ca\(^{2+}/\text{CaM}\) (25). Taken together, these results suggest that a careful quantification of the cellular concentration of CaM\(\cdot\)eEF-2K complexes is warranted to test the proposed mechanism in Fig. 10 as well as to assess its possible regulation by the phosphorylation of eEF-2K at Ser-78 and/or other sites.

In addition to demonstrating a 25-fold decrease in \(K_{\text{cat}}^{\text{app}}/K_{m}^{\text{app}}\) for peptide phosphorylation, we observed a 20-fold decrease in the ability of the T348A mutant to phosphorylate 2 \(\mu\text{M eEF-2} \text{in vitro}\) (31). Although it is possible that the extent of eEF-2 phosphorylation in cells is not linearly dependent on eEF-2K activity, another possibility is that an additional modification(s) of eEF-2K in cells renders it less dependent on Thr-348 autophosphorylation. Preliminary unpublished studies\(^{9}\) suggest that the S500D mutant shows less sensitivity to the T348A mutation in steady-state kinase assays, supporting the notion that other modifications may also contribute to the underlying mechanism of activation, potentially by stabilizing an active conformation of the R-loop. Thus, although our data suggest that Thr-348 autophosphorylation favors a conformational transition of the R-loop, we cannot rule out the possibility that it is also influenced by other post-translational modifications. In fact, suppression of the proposed R-loop conformational transition might allow for the inhibition of eEF-2K under conditions where Ca\(^{2+}/\text{CaM}\) binding is strongly favored. Interestingly, such a situation has been reported by Smith and Proud (53), where during mitosis, eEF-2K is inhibited by phosphorylation at Ser-359 despite an expected rise in intracellular Ca\(^{2+}\) levels.

At its fundamental level, the mechanism of eEF-2K activation appears to be analogous to an amplifier, where the output volume may be controlled by either toggling the power switch (step 1 in Fig. 10, switching on the kinase) or altering the volume control (step 2 in Fig. 10, modulating the stability of the active conformation). Because upstream signaling events have the potential to modulate either allosteric step as well as the intrinsic activity of the kinase domain (i.e. input signal), this mechanism provides for exquisite control of eEF-2K output. To date, the molecular mechanisms underlying the activation of eEF-2K by PKA (32, 54) and AMPK (55), which phosphorylate Ser-500 and Ser-398, respectively, are unknown. We believe this study provides a mechanistic foundation for the elucidation of these mechanisms.

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