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Calcium/Calmodulin Stimulates the Autophosphorylation of Elongation Factor 2 Kinase on Thr-348 and Ser-500 To Regulate Its Activity and Calcium Dependence

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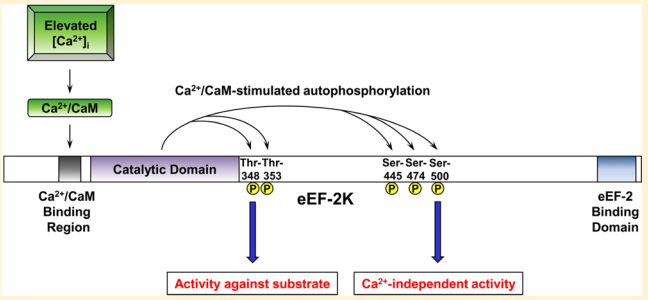
Supporting Information

ABSTRACT: Eukaryotic elongation factor 2 kinase (eEF-2K) is an atypical protein kinase regulated by Ca^{2+} and calmodulin (CaM). Its only known substrate is eukaryotic elongation factor 2 (eEF-2), whose phosphorylation by eEF-2K impedes global protein synthesis. To date, the mechanism of eEF-2K autophosphorylation has not been fully elucidated. To investigate the mechanism of autophosphorylation, human eEF-2K was coexpressed with λ-phosphatase and purified from bacteria in a three-step protocol using a CaM affinity column. Purified eEF-2K was induced to autophosphorylate by incubation with Ca^{2+}/CaM in the presence of MgATP. Analyzing tryptic or chymotryptic peptides by mass spectrometry monitored the autophosphorylation over 0−180 min. The following five major autophosphorylation sites were identified: Thr-348, Thr-353, Ser-445, Ser-474, and Ser-500. In the presence of Ca^{2+}/CaM, robust phosphorylation of Thr-348 occurs within seconds of addition of MgATP. Mutagenesis studies suggest that phosphorylation of Thr-348 is required for substrate (eEF-2 or a peptide substrate) phosphorylation, but not self-phosphorylation. Phosphorylation of Ser-500 lags behind the phosphorylation of Thr-348 and is associated with the Ca^{2+}-independent activity of eEF-2K. Mutation of Ser-500 to Asp, but not Ala, renders eEF-2K Ca^{2+}-independent. Surprisingly, this Ca^{2+}-independent activity requires the presence of CaM.



Cellular homeostasis demands a controlled balance between protein synthesis and protein degradation. Eukaryotes regulate their rate of protein synthesis through a variety of pathways, several of which include phosphorylation of translation initiation and elongation factors.[3−6] An important component of this regulatory process is the eukaryotic elongation factor 2 kinase (eEF-2K). eEF-2K generally functions to impede the elongation phase of translation, thereby disrupting global protein synthesis.[7−11] eEF-2K inhibits translation by phosphorylating and thereby blocking the ability of elongation factor 2 (eEF-2) to bind the ribosome.[7−11] eEF-2 is responsible for the ribosomal translocation of the nascent peptide chain from the A-site to the P-site during translation.[12−14] Additionally, eEF-2K may also induce the translation of specific transcripts.[15]

eEF-2K was first identified as a Ca^{2+}/CaM-dependent protein kinase (CaMK-III),[7,10,16,17] because it requires Ca^{2+} and calmodulin (CaM) for autophosphorylation. Redpath and Proud demonstrated that autophosphorylation increased kinase activity by 2−3-fold.[17] In contrast, Mitsui et al. suggested that this is not the case.[16] Both groups suggested that autophosphorylation imparts significant Ca^{2+}-independent activity to the kinase;[16,17] however, the autophosphorylation site(s) responsible for inducing this activity remains to be determined. The mechanism of regulation of eEF-2K activity by Ca^{2+}/CaM-stimulated autophosphorylation also remains to be detailed.

Because of its lack of sequence homology with conventional protein kinases, eEF-2K is classified as an atypical protein kinase,[18] a group that includes myosin II heavy chain kinase A

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(MHCK A).[19] Recently, Crawley et al. reported that in *Dictyostelium*, autophosphorylation activates MHCK A.[20] Autophosphorylated Thr-825 is proposed to act as an intramolecular ligand for a phosphothreonine-binding pocket on the surface of the kinase, whose occupancy allosterically induces a conformational change in the enzyme, which results in its activation. On the basis of sequence similarity, the group has also suggested that eEF-2K may be regulated in a similar manner.[20]

In addition to its activation by Ca^{2+}/CaM, other factors that influence the functioning of eEF-2K have been determined. Two central signaling pathways, the mTOR and the MAPK (MEK/ERK) cascades, are involved in inhibiting the activity of the kinase via phosphorylation.[21−25] On the other hand, two kinases have been shown to activate eEF-2K: the cAMP-dependent PKA[26−28] and the energy-supply regulator AMPK.[29] Phosphorylation of Ser-500 by PKA additionally imparts Ca^{2+}-independent activity to eEF-2K.[26,28]

A compelling factor behind deciphering the mechanism of activation and regulation of eEF-2K is its association with enhancing tumor survival. eEF-2K is upregulated in glioblastoma and breast cancer, where it is suggested to promote proliferation, migration, and survival of cancer cells.[30−35] eEF-2K has also recently been implicated in depression,[36] and hence, an understanding of the regulation of kinase activity is crucial for detailing its contribution to these various disease states. We have recently purified recombinant human eEF-2K expressed in bacteria,[2] which allows us to assess its regulation by autophosphorylation.

Despite eEF-2K being known to undergo rapid autophosphorylation upon activation by Ca^{2+} and CaM, the autophosphorylation sites on the kinase have not been reported.[a] In this study, we identify five major eEF-2K autophosphorylation sites: Thr-348, Thr-353, Ser-445, Ser-474, and Ser-500. We show that the phosphorylation of Thr-348 occurs within seconds and is required for substrate phosphorylation (eEF-2 or a peptide substrate) but not self-phosphorylation. Evidence that the phosphorylation of Ser-500, which occurs within a few minutes, is associated with Ca^{2+}-independent activity of eEF-2K is presented. Mutagenesis studies did not reveal a function for the phosphorylation of Thr-353, Ser-445, and Ser-474.

EXPERIMENTAL PROCEDURES

Reagents, Strains, Plasmids, and Equipment. Yeast extract, tryptone, and agar were purchased from USB Corp. (Cleveland, OH). Restriction enzymes and reagents for site-directed mutagenesis were obtained from New England BioLabs (Ipswich, MA). Oligonucleotides for mutagenesis were from Integrated DNA Technologies, Inc. (Coralville, IA). The Stratagene *PfuUltra* II Fusion HS DNA Polymerase kit was purchased from Agilent Technologies, Inc. (Santa Clara, CA). Qiagen (Valencia, CA) supplied the QIAprep Spin Miniprep Kit and Ni-NTA agarose. Affi-Gel 15 activated affinity medium for the generation of CaM-agarose beads was obtained from Bio-Rad Laboratories (Hercules, CA). The BenchMark Protein Ladder was from Invitrogen Corp. (Carlsbad, CA). Ultrapure grade Tris-HCl and HEPES were from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT) was obtained either from US Biological (Swampscott, MA) or Gold Biotechnology (St. Louis, MO). Trypsin and chymotrypsin were from Promega (Madison, WI) and Sigma-Aldrich, respectively. PerkinElmer (Waltham, MA) or MP Biomedicals (Solon, OH) supplied [γ-^{32}P]ATP. All other buffer components or chemicals were purchased from Sigma-Aldrich, Fisher Scientific (Pittsburgh, PA), or MP Biomedicals.

Escherichia coli strains NovaBlue, for cloning, and BL21-(DE3) and Rosetta-gami 2(DE3), for recombinant protein expression, were from Novagen, EMD4Biosciences (Gibbstown, NJ). The pET-32a vector was obtained from Novagen.

A Techne Genius Thermal Cycler purchased from Techne, Inc. (Burlington, NJ), was used for site-directed mutagenesis. The ÄKTA FPLC System and the HiPrep 26/60 Sephacryl S-200 HR gel filtration column were from Amersham Biosciences/GE Healthcare Life Sciences (Piscataway, NJ). Absorbance readings were performed on a Cary 50 UV−Vis spectrophotometer. Proteins were resolved using the Mini-PROTEAN 3 vertical gel electrophoresis apparatus from Bio-Rad Laboratories. Amicon Ultra Centrifugal Filter Units were from Millipore (Billerica, MA). P81 cellulose filters were from Whatman/GE Healthcare Life Sciences (Florham Park, NJ). Radioactivity measurements were performed on a Packard 1500 Lab TriCarb liquid scintillation analyzer from PerkinElmer. The Phosphorimager cassette and the Typhoon Phosphorimager were from GE Healthcare Life Sciences (Piscataway, NJ).

Molecular Biology. A modified pET-32a vector (p32TeEF-2K[2]) containing cDNA encoding human eEF-2K (GenBank entry NM_013302) was used for the expression of Trx-His_6-tagged eEF-2K. Alanine and aspartate autophosphorylation site mutants were generated by site-directed mutagenesis using the *PfuUltra* II Fusion HS DNA Polymerase kit from Stratagene, specific primers, and the p32TeEF-2K vector as a template.

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 EDTA, 100 μM EGTA, 250 μM $CaCl_2$, 2 μM CaM, and 10 mM $MgCl_2$], containing 150 μM (acetyl-RKKYKFNEDTERRRFL-amide) peptide substrate (Pep-S), 2 nM eEF-2K enzyme, and 0.5 mM [γ-^{32}P]ATP (100−1000 cpm/pmol) in a final reaction volume of 100 μL. The reaction mixture was incubated at 30 °C for 10 min before the reaction was initiated by addition of 0.5 mM [γ-^{32}P]ATP. At set time points, 10 μL aliquots were taken and spotted onto P81 cellulose filters (Whatman, 2 cm × 2 cm). The filter papers were then washed thrice in 50 mM phosphoric acid (15 min each wash) and once in acetone (15 min) and finally dried. The amount of labeled peptide associated with each paper was determined by measuring the counts per minute on a Packard 1500 scintillation counter.

Characterization of Enzymatic Activity. Buffer B [25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 50 mM KAcO, 100 μM EGTA, 1 mM [γ-^{32}P]ATP (100−1000 cpm/pmol), and 150 μM peptide substrate] was used for the assay of dependence on Ca^{2+}, CaM, enzyme, and magnesium concentrations. Kinase activity in each case was determined by calculating the rate of phosphorylation of the peptide (μM s^{-1}) in a manner similar to the general kinetic assay described above. The assays were performed in duplicate.

a. Calcium Dependence. Dose−response Ca^{2+} dependence assays were performed using 0.5 nM eEF-2K, 10 mM $MgCl_2$, 2 μM CaM, and several concentrations of free Ca^{2+} (0−3 μM), and the data were fit to eq 1. Free Ca^{2+} concentrations were calculated using an EGTA calculator (http://www.stanford.edu/~cpatton/CaEGTA-TS.htm), which allows input of EGTA and Ca^{2+} concentrations, as well as temperature, pH, and ionic conditions.

b. CaM Dependence. Assays were performed using 0.5 nM eEF-2K, 10 mM $MgCl_2$, 50 μM free Ca^{2+}, and several concentrations of CaM (0−1 μM), and the data were fit to eq 2.

c. Enzyme Concentration Dependence. Assays were performed using 10 mM $MgCl_2$, 2 μM CaM, 50 μM free Ca^{2+}, and several concentrations of eEF-2K (0−10 nM), and the data were fit with linear regression.

d. Magnesium Dependence. Assays were performed using 2 nM eEF-2K, 2 μM CaM, 50 μM free Ca^{2+}, and several concentrations of free magnesium (0−10 mM), and the data were fit to eq 2. Free magnesium concentrations were determined on the basis of the known amount of ATP added.

e. Salt Dependence. Assays were performed using 2 nM eEF-2K, 10 mM $MgCl_2$, 2 μM CaM, and 150 μM $CaCl_2$, in a buffer containing 25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 100 μM EGTA, 150 μM peptide substrate, 1 mM [γ-^{32}P]ATP (100−1000 cpm/pmol), and several concentrations of either NaCl, KCl, or KAcO (0−500 mM).

$$k_{obs}^{app} = \frac{k_{cat}^{app}[C]^n}{K_c^{app\,n} + [C]^n} \quad (1)$$

$$k_{obs}^{app} = \frac{k_{cat}^{app}[C]}{K_c^{app} + [C]} \quad (2)$$

where k_{obs}^{app} is the apparent rate constant, k_{cat}^{app} is the apparent catalytic constant, [C] is the concentration of varied coactivator (Ca^{2+}, CaM, or Mg^{2+}), K_c^{app} is the apparent coactivator concentration required to achieve half-maximal activity, and n is the Hill coefficient.

Autophosphorylation Assay. Autophosphorylation of eEF-2K was conducted in buffer C [25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 50 mM KAcO, 100 μM EGTA, 150 μM $CaCl_2$, 5 μM CaM, and 10 mM $MgCl_2$] containing 500 nM eEF-2K and 1 mM [γ-^{32}P]ATP (100−1000 cpm/pmol) in a final volume of 250 μL. The reaction mixture was incubated at 30 °C for 10 min before the reaction was initiated by addition of 1 mM [γ-^{32}P]ATP. Aliquots (10 pmol) of eEF-2K were removed at intervals over a 3 h time period, and the reaction was quenched by the addition of SDS−PAGE sample loading buffer [125 mM Tris-HCl (pH 6.75), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 4% SDS, and 0.02% bromophenol blue] and the mixture heated for 10 min at 95 °C. The samples were resolved by SDS−PAGE and stained with Coomassie Brilliant Blue. Gels were exposed for 3 h in a Phosphorimager cassette that was then scanned in a Typhoon Phosphorimager and analyzed using ImageQuant TL. To determine the stoichiometry of the autophosphorylation, the gels were dried, the pieces containing eEF-2K were excised, and the associated radioactivity was measured with a Packard 1500 liquid scintillation analyzer. The mechanism of autophosphorylation was analyzed using buffer D [25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 50 mM KAcO, 100 μM EGTA, 150 μM $CaCl_2$, 2 μM CaM, and 10 mM $MgCl_2$] containing 1 mM [γ-^{32}P]ATP and varying concentrations of the purified enzyme (0−500 nM). The reaction was conducted under conditions in which linear incorporation of ^{32}P was achieved (1 min incubation) and quenched by addition of hot SDS−PAGE sample loading buffer. The extent of phosphate incorporation for each sample was determined as described above and then plotted as a function of enzyme concentration.

Effect of Autophosphorylation on Enzyme Activity. eEF-2K (20 nM) was preincubated in buffer D for 10 min at 30 °C, and autophosphorylation was then initiated by the addition of 1 mM ATP as described above. At predetermined intervals of time (0−180 min), the autophosphorylated enzyme (2 nM) was assayed at 30 °C in buffer E [25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 50 mM KAcO, 100 μM EGTA, 150 μM $CaCl_2$, 2 μM CaM, and 10 mM $MgCl_2$] containing 150 μM peptide substrate and 1 mM [γ-^{32}P]ATP (100−1000 cpm/pmol). The rate of phosphorylation of the peptide (μM s^{-1}) was determined using the general kinetic assay described earlier, and a graph of k_{obs}^{app} (s^{-1}) as a function of autophosphorylation time (min) was plotted. The activity of the unautophosphorylated control (incubated in the absence of ATP) was also determined. The assays were performed in duplicate.

Analysis of the Autophosphorylation Site Mutants. a. Assay against Peptide Substrate. Assays were performed in buffer A using 2 nM eEF-2K, 150 μM peptide substrate, and 0.5 mM [γ-^{32}P]ATP (100−1000 cpm/pmol) in a final reaction volume of 100 μL. Kinase activity in each case was determined by calculating the rate of phosphorylation of the peptide (μM s^{-1}) in a manner similar to the general kinetic assay described earlier. The assays were performed in triplicate.

b. Assay against Wheat Germ eEF-2. Assays were performed in buffer D using 2 nM eEF-2K, 4 μM wheat germ eEF-2, and 1 mM [γ-^{32}P]ATP (100−1000 cpm/pmol) in a final reaction volume of 50 μL. The reaction mixture was incubated at 30 °C for 10 min before the reaction was initiated by addition of 1 mM [γ-^{32}P]ATP. The reaction was conducted for 1 min and quenched by addition of hot SDS−PAGE sample loading buffer. The samples were resolved by SDS−PAGE and stained with Coomassie Brilliant Blue. Gels were exposed for 2 h in a Phosphorimager cassette that was then scanned in a Typhoon Phosphorimager.

c. Autophosphorylation of eEF-2K Mutants. Assays were performed in buffer C using 1 μM eEF-2K and 1 mM [γ-^{32}P]ATP (100−1000 cpm/pmol) in a final reaction volume of 50 μL. The assay was performed, and samples were analyzed as described above for the wheat germ eEF-2 assay, except that the reaction proceeded for 10 min before being quenched.

Analysis of Autophosphorylation of Thr-348 and Ser-500 by Immunoblotting. Autophosphorylation of eEF-2K (500 nM) was conducted in buffer C containing 1 mM ATP as described earlier. Aliquots (50 ng) of eEF-2K were removed at intervals over a 3 h time period, and the reaction was quenched by addition of hot SDS−PAGE sample loading buffer and the mixture heated for 10 min at 95 °C. Samples were analyzed by immunoblotting as described below. Blots were quantified using ImageJ, and data were plotted as the percent phosphorylation of Thr-348 or Ser-500 against autophosphorylation time. The experiments were performed in duplicate.

a. Commercial Antibodies. Anti-eEF-2K antibody (catalog no. 3692, 1:2000) was purchased from Cell Signaling Technology (Danvers, MA), and goat anti-rabbit IgG (H+L)-HRP conjugate (catalog no. 172-1019, 1:2000) was from Bio-Rad.

b. Phospho-Specific Antibodies for Thr-348 and Ser-500 of eEF-2K. ECM Biosciences (Versailles, KY) generated affinity-purified rabbit polyclonal antibodies against Thr-348 (catalog no. EP4411) and Ser-500 (catalog no. EP4451) of eEF-2K. To characterize the anti-eEF-2K (Thr-348 and Ser-500), phospho-specific antibodies, using the Western blotting technique

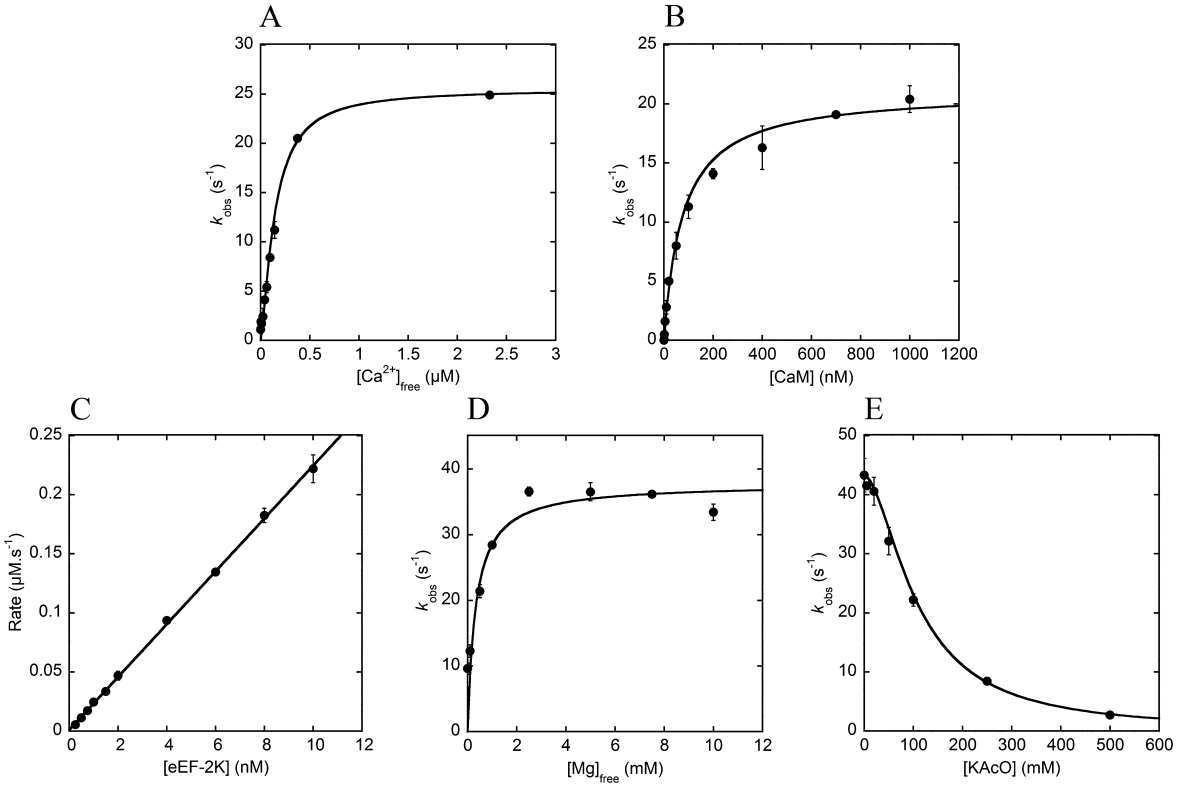

Figure 1. Characterization of enzymatic activity. The buffers are described in Experimental Procedures. Kinase activity was determined by measuring the rate of phosphorylation of the peptide (μM s^{-1}). (A) Ca^{2+} dependence assays were conducted with 0.5 nM eEF-2K and 0−3 μM free Ca^{2+}. Data were fit to eq 1, where n = 1.4 ± 0.03, K_c^{app} = 0.14 ± 0.003 μM, and k_{cat}^{app} = 25.5 ± 0.2 s^{-1}. (B) CaM dependence assays were conducted with 0.5 nM eEF-2K and 0−1 μM CaM. The data were fit to eq 2, where K_c^{app} = 76 ± 5 nM and k_{cat}^{app} = 21.1 ± 0.2 s^{-1}. (C) Enzyme concentration dependence assays were conducted with 0−10 nM eEF-2K. (D) Magnesium dependence assays were conducted with 2 nM eEF-2K and 0−10 mM free magnesium. The data were fit to eq 2, where K_c^{app} = 0.33 ± 0.01 mM and k_{cat}^{app} = 37.8 ± 0.3 s^{-1}. (E) Salt dependence assays were conducted with 2 nM eEF-2K and several concentrations (0−500 mM) of KAcO.

mentioned below, we tested it against recombinant human eEF-2K coexpressed in bacteria with λ-phosphatase. The samples probed for phosphorylation at Thr-348 included untreated or autophosphorylated eEF-2K wild type (WT), T348A, and T348D, as well as λ-phosphatase-treated eEF-2K WT. The samples probed for phosphorylation at Ser-500 included untreated or autophosphorylated eEF-2K WT, S500A, and S500D. Autophosphorylated samples were incubated in the presence of CaM, Ca^{2+}, and MgATP for 1 h. λ-phosphatase (New England BioLabs) treatment was conducted as per the manufacturer's protocol.

c. Western Blot Analysis. Samples (50 ng of eEF-2K) were resolved by 10% SDS−PAGE and then transferred to Amersham Hybond-P PVDF membranes (GE Healthcare). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and Tween 20 (TBST) and incubated with primary antibodies eEF-2K (Thr-348 or Ser-500) phospho-specific or anti-eEF-2K, followed by the secondary antibody goat anti-rabbit IgG (H+L)-HRP conjugate. To determine the total levels and Thr-348 or Ser-500 phosphorylation status of eEF-2K, chemiluminescent detection was performed with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare).

Analysis of the Calcium-Independent Activity of eEF-2K. eEF-2K activity (WT, S500A, and S500D) was assayed at 30 °C in buffer F [25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 μM BSA, 100 μM EGTA, and 10 mM $MgCl_2$] containing 150 μM peptide substrate, with or without 150 μM $CaCl_2$, with or without 2 μM CaM, and with 0.5 mM [γ-^{32}P]ATP (100−1000 cpm/pmol) in a final reaction volume of 100 μL. EGTA (1 mM) was added to all assays conducted in the absence of Ca^{2+}. For eEF-2K WT, S500A, and S500D assayed in the presence of both Ca^{2+} and CaM, and eEF-2K S500D assayed in the presence of only CaM, activities were much higher than the basal level of kinase activity, and hence, only 5 nM of kinase was used. For all the other assays, 50 nM eEF-2K was used to detect an increase in kinase activity over the basal level. Kinase activity in each case was determined by calculating the rate of phosphorylation of the peptide (μM s^{-1}) in a manner similar to the general kinetic assay described earlier.

RESULTS

Purification and Characterization of eEF-2K. *Coexpression of eEF-2K with λ-Phosphatase and Purification Using a Calmodulin Affinity Column.* Bacteria, being prokaryotic, are not known to express CaM. Despite eEF-2K being a Ca^{2+}/CaM-dependent protein kinase, to reduce the likelihood of autophosphorylation in *E. coli* even further, the enzyme was coexpressed with λ-phosphatase. Taking advantage of the absence of CaM kinases in bacteria, we employed a CaM affinity column in the purification of recombinant eEF-2K. A three-step protocol using a Ni-NTA affinity column followed by a CaM affinity column and finally a size-exclusion column yielded milligram amounts of >98% pure kinase (data not shown).

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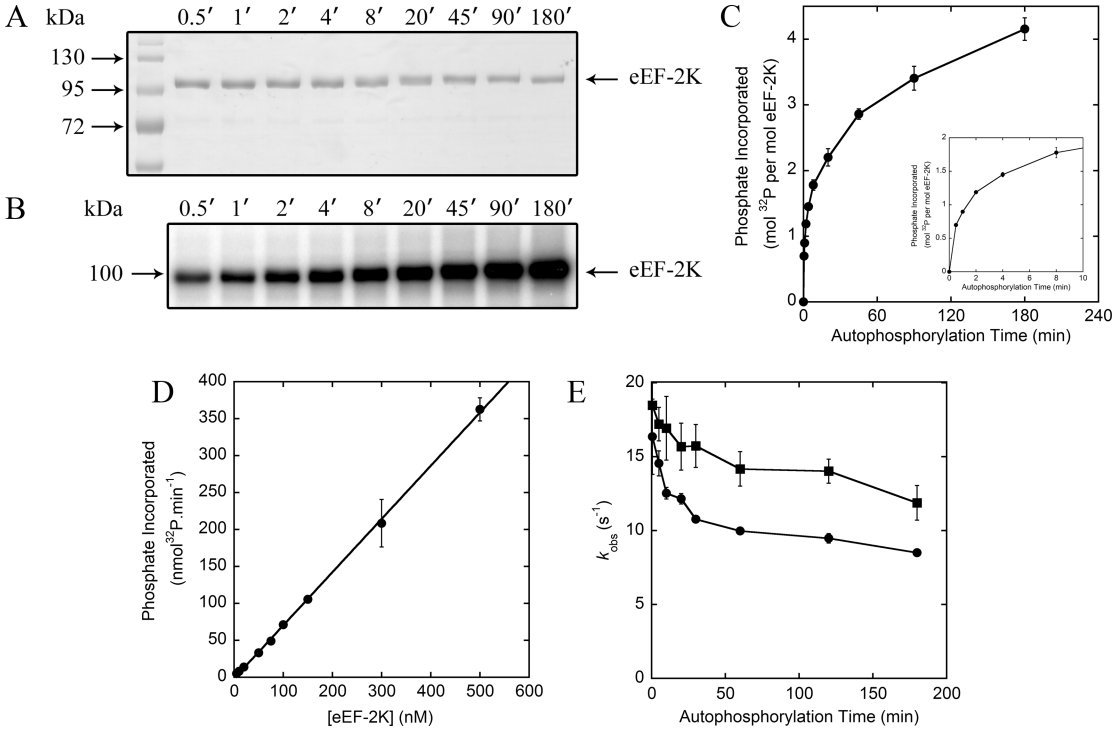

Figure 2. Autophosphorylation of eEF-2K. (A–C) eEF-2K (500 nM) was allowed to autophosphorylate in the presence of 5 μM CaM and 50 μM free Ca^{2+}. At the indicated times, 10 pmol of eEF-2K was removed and the reaction quenched with hot SDS–PAGE sample loading buffer. The samples were then analyzed as described in Experimental Procedures. (A) Coomassie-stained gel. (B) Autoradiograph. (C) Phosphate incorporation as a function of autophosphorylation time – stoichiometry of the autophosphorylation of eEF-2K. The inset shows an expansion of the data from 0 to 10 min. (D) Rate of phosphate incorporation (nanomoles of ^{32}P incorporated per minute) as a function of enzyme concentration. To analyze the mechanism of autophosphorylation of recombinant human eEF-2K, varying concentrations of the purified enzyme (0–500 nM) were allowed to autophosphorylate in the presence of 2 μM CaM and 50 μM free Ca^{2+}. The reaction was conducted under conditions in which linear incorporation of ^{32}P was achieved (1 min incubation) and quenched by addition of hot SDS–PAGE sample loading buffer. The samples were then analyzed as described in Experimental Procedures. The experiment was duplicated with similar results. (E) Effect of autophosphorylation on kinase activity. eEF-2K (20 nM) was allowed to autophosphorylate in the presence of 2 μM CaM and 50 μM free Ca^{2+}. At the indicated times (0–180 min), the effect of autophosphorylation on kinase activity against the peptide substrate was determined by assaying the autophosphorylated enzyme (2 nM) in the presence of 55 μM free Ca^{2+} and 2.2 μM CaM (●) as described in Experimental Procedures. The rate of phosphorylation of the peptide (μM s^{-1}) was determined using the general kinetic assay, and a graph of k_{obs}^{app} (s^{-1}) as a function of the autophosphorylation time (min) was plotted. The activity of the unautophosphorylated control (without ATP) was also determined (■).

Enzymatic Characterization. As the eEF-2K used in this study has not previously been described in detail, it was important to establish its kinetic properties. Thus, characterization of the enzymatic activity of autophosphorylated eEF-2K was performed using a peptide substrate as described in Experimental Procedures.

a. Calcium Dependence. Assays were performed using 0.5 nM eEF-2K, 2 μM CaM, and several concentrations of free Ca^{2+} (0–3 μM). Data were fit using eq 1, where $n = 1.4 \pm 0.03$, $K_c^{app} = 0.14 \pm 0.003$ μM, and $k_{cat}^{app} = 25.5 \pm 0.2$ s^{-1} (Figure 1A). The concentration of Ca^{2+} required to achieve half-maximal activity is 140 ± 3 nM. Maximal activity is observed up to around 3 μM free Ca^{2+}. Once the free Ca^{2+} concentration exceeds this limit, a concentration-dependent inhibitory effect of Ca^{2+} is observed (data not shown). The kinase possesses low enzymatic activity ($k_{obs}^{app} = 1.1 \pm 0.4$ s^{-1}) in the presence of 2 μM CaM alone (100 μM EGTA and no added Ca^{2+}). Thus, the increase in the free Ca^{2+} concentration from 0 to 3 μM enhances this kinase activity by ~25-fold ($k_{cat}^{app} = 25.5 \pm 0.2$ s^{-1}). A Hill coefficient of 1.4 ± 0.03 suggests that no significant cooperativity is involved in the enzyme activation by the Ca^{2+}/CaM complex.

b. CaM Dependence. Assays were performed using 0.5 nM eEF-2K, 50 μM free Ca^{2+}, and several concentrations of CaM (0–1 μM). The data were fit to eq 2, where $K_c^{app} = 76 \pm 5$ nM and $k_{cat}^{app} = 21.1 \pm 0.2$ s^{-1} (Figure 1B). Earlier studies of the native kinase indicate a half-maximal activation of <1 nM CaM.[16] Further work is necessary to test the native enzyme under buffer conditions identical to those used here.

c. Enzyme Concentration Dependence. Assays used 0 to 10 nM eEF-2K. The linear plot of rate versus enzyme concentration (Figure 1C) is consistent with the notion that eEF-2K is monomeric over the concentration range employed, in agreement with the light scattering data obtained by Abramczyk et al.[2]

d. Magnesium Dependence. Assays used 2 nM eEF-2K and 0–10 mM free magnesium. The data were fit to eq 2, where $K_c^{app} = 0.33 \pm 0.01$ mM and $k_{cat}^{app} = 37.8 \pm 0.3$ s^{-1}. The concentration of free magnesium required for half-maximal activation of eEF-2K is 330 ± 10 μM (Figure 1D).

e. Salt Dependence. Studies on the presence of salt in the buffer indicate an inhibitory effect by KAcO on the activity of the kinase. The kinase has half-maximal activity at a KAcO concentration of ~110 mM (Figure 1E). NaCl and KCl also inhibit the kinase, however to a slightly greater extent, with half-maximal activity being observed at a salt concentration of ~80 mM (Figure 2A,B of the Supporting Information).

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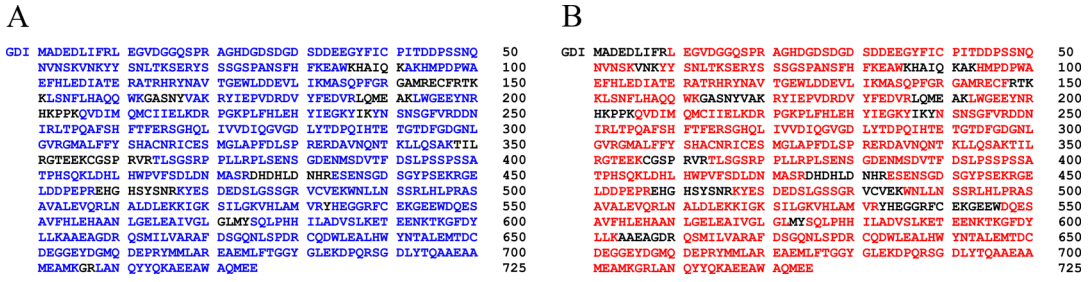

Figure 3. Mass spectrometry analysis sequence coverage of eEF-2K. (A) Sequence coverage of the purified recombinant eEF-2K from *E. coli* is ~90%, indicated by the blue residues. (B) Sequence coverage of the autophosphorylated enzyme (incubation for 3 h with CaM, Ca^{2+}, and MgATP) is ~86%, indicated by the red residues. Both samples were resolved by SDS−PAGE and subjected to tryptic and chymotryptic in-gel digestion, and the peptide digests were then used for mass spectrometry analysis as described in the Experimental Procedures of the Supporting Information.

Because our data indicate that autophosphorylation of eEF-2K is required for activity, it was important to determine whether autoactivation was rate-limiting under conditions of low observed activity (e.g., low concentrations of Ca^{2+}, CaM, and Mg^{2+} or a high salt concentration). To assess this, eEF-2K was preincubated in the presence of ATP for either 0 or 30 min under the reaction conditions. No significant difference in activity was observed for eEF-2K incubated for 0 or 30 min (data not shown), suggesting that autophosphorylation is not rate-limiting under any of the reaction conditions tested. Furthermore, support for this conclusion was obtained by noting that under all assay conditions, the appearance of product with time was linear.

Autophosphorylation of Recombinant eEF-2K. *Stoichiometry of Autophosphorylation.* Upon incubation with Ca^{2+} and CaM, the enzyme undergoes rapid autophosphorylation (Figure 2A,B). The stoichiometry of phosphate incorporation was measured by incubating the kinase (500 nM) with 5 μM CaM, 50 μM free Ca^{2+}, 10 mM $MgCl_2$, and 1 mM ATP, for various amounts of time (0.5−180 min) before the amount of incorporated phosphate was measured. Aliquots of the reaction mixture were fractionated by SDS−PAGE, and the radioactivity of each sample was measured with a liquid scintillation counter as described in Experimental Procedures. Results revealed the incorporation of approximately 4 mol of phosphate/mol of enzyme over the 3 h incubation time (Figure 2C). The rate of autophosphorylation of the enzyme appears to be quite complex, with an initial incorporation of ~1 mol of phosphate occurring within the first minute, followed by a progressively slower incorporation of a further 3 mol of phosphate. SDS−PAGE analysis indicated that the phosphorylation was accompanied by a slight shift in the protein band (Figure 2A), as noted previously.[17] Prior studies of the enzyme, however, show varied results with regard to the rate and extent of phosphate incorporation upon autophosphorylation. Analysis of eEF-2K from rat pancreas and rabbit reticulocytes indicated the maximal incorporation of ~1 mol of phosphate/mol of enzyme,[16] whereas other reports on the kinase from rabbit reticulocytes showed the incorporation of ~3.5 and 5 mol of phosphate/mol of enzyme over a 1 h incubation period.[17,26]

Mechanism of Autophosphorylation. Autophosphorylation of eEF-2K was previously proposed to occur through an intramolecular mechanism.[16,17] To assess the mechanism of autophosphorylation of recombinant eEF-2K, the initial rate of phosphate incorporation was determined for various concentrations of the kinase. The previous autophosphorylation assay with 500 nM eEF-2K showed the reaction to be approximately linear over the first minute. Hence, varying concentrations of the enzyme (0−500 nM) were incubated with 2 μM CaM, 50 μM free Ca^{2+}, 10 mM $MgCl_2$, and 1 mM ATP, and the enzyme was allowed to autophosphorylate for 1 min, after which the rate of incorporation of phosphate for each of the reactions was determined as described in Experimental Procedures. The rate of phosphate incorporation was found to be proportional to the concentration of eEF-2K over the entire range of concentrations examined (Figure 2D). As eEF-2K shows no propensity to self-associate over this concentration range, a mechanism corresponding to more than one eEF-2K molecule in the rate-limiting transition state may be excluded. Thus, following binding of Ca^{2+}/CaM and MgATP, eEF-2K is presumed to autophosphorylate in an intramolecular manner (within the same polypeptide) rather than within an eEF-2K dimer, with regard to the initial rapid incorporation of the first mole of phosphate. However, the possibility of the subsequent incorporation of phosphate occurring in an intermolecular manner cannot be ruled out.

Effect of Autophosphorylation on Kinase Activity. To examine the effects of autophosphorylation on kinase activity, the enzyme was allowed to autophosphorylate in the presence of CaM, Ca^{2+}, and MgATP for various intervals of time (0.5−180 min) before being assayed for activity against the peptide substrate in the presence of 55 μM free Ca^{2+} and 2.2 μM CaM as described in Experimental Procedures. Over the 3 h period of autophosphorylation, the kinase activity gradually decreases to approximately 50% of its initial value (Figure 2E). A similar trend is also observed with the unautophosphorylated control (no ATP), albeit to a lesser degree, suggesting that the decrease in activity is due to the loss of stability of the enzyme over time at 30 °C. Earlier characterization of eEF-2K from a mammalian source by Redpath and Proud suggested that kinase activation was partially dependent on autophosphorylation,[17] while Mitsui et al. report no significant effect of autophosphorylation on the activity of the enzyme.[16] It should be noted that because of the nature of the assay, which is conducted over 2 min, any rapid effect of autophosphorylation on kinase activity is unlikely to have been detected. As noted below, we have identified Thr-348 as a major early site of phosphorylation; we believe its phosphorylation leads to the activation of the kinase toward substrates. Pre-steady state studies are underway to investigate this further.

Mapping the Autophosphorylation Sites on eEF-2K. *Examining the Post-Translational Phosphorylation of eEF-2K in the Absence of CaM.* To examine the phosphorylation status of the protein purified from *E. coli*, the recombinant enzyme was resolved by SDS−PAGE and then

Table 1. Summary of Phosphopeptides Indicating Autophosphorylation Sites[a]

phosphopeptide ID (tryptic digest)	Mascot score[b] [37]	peptide[c]	p-residue[d]
K.YYSNLTK.S + phospho (ST)	11	59−65	Thr-64
K.LLQSAKTILR.G + phospho (ST)	57, 62	342−351	Thr-348
K.LLQSAKTILRGTEEK.C + 2 phospho (ST)	58	342−356	Thr-348, Thr-353
K.TILRGTEEK.C + 2 phospho (ST)	22	348−356	Thr-348, Thr-353
R.ESENSGDSGYPSEK.R + phospho (ST)	32, 42, 61, 34	434−447	Ser-445
R.KYESDEDSLGSSGR.V + phospho (ST)	93, 86, 90, 18	467−480	Ser-474
K.WNLLNSSR.L + phospho (ST)	15	486−493	Ser-491
R.ASAVALEVQR.L + phospho (ST)	55, 42, 51, 43	499−508	Ser-500
phosphopeptide ID (chymotryptic digest)	**Mascot score**	**peptide**	**p-residue**
F.DLSPRERDAVNQNTKLLQSAKTIL.R + phospho (ST)	47	327−350	Thr-348
L.HLPRASAVAL.E + phospho (ST)	37	487−504	Ser-500
W.NLLNSSRLHLPRASAVAL.E + phospho (ST)	44	495−504	Ser-491

[a]Autophosphorylation sites on eEF-2K were detected by mass spectrometry analysis of the in-gel tryptic and chymotryptic digests. eEF-2K (5 μM) was allowed to autophosphorylate in the presence of CaM, Ca^{2+}, and MgATP for 3 h, resolved by SDS−PAGE, and subjected to digestion with trypsin or chymotrypsin as described in the Experimental Procedures of the Supporting Information. The in-gel peptide digests were then screened for phosphorylated peptides by mass spectrometry. [b]Peptide ion scores equal to or greater than 45−49 represent identifications with at least 95% confidence. Multiple Mascot scores indicate a particular peptide being identified in multiple trials of the experiment. [c]Range of residues of the detected peptide. [d]Phosphorylated Ser or Thr residue detected that corresponds to the amino acid that is underlined in the phosphopeptide ID sequence.

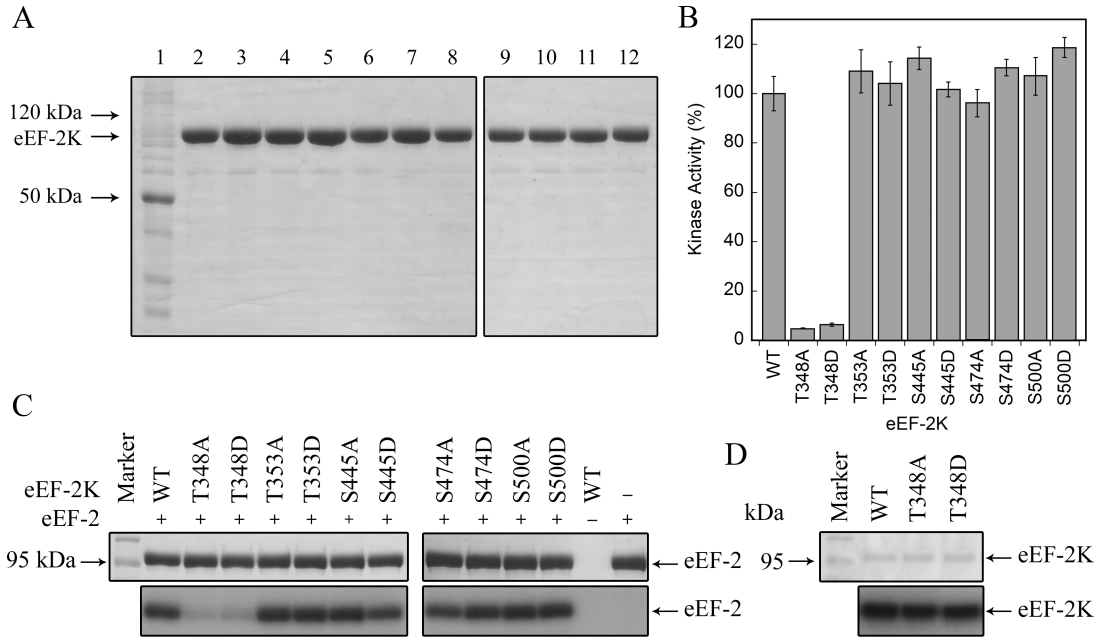

Figure 4. Purification and kinetic analysis of WT eEF-2K and autophosphorylation site mutants expressed in *E. coli*. (A) Samples purified by Ni-NTA affinity, CaM-agarose affinity, and gel filtration chromatography were resolved by SDS−PAGE. (B) Kinase activity of the autophosphorylation site mutants. The buffers are described in Experimental Procedures. Assays were performed with 2 nM eEF-2K enzyme, 2 μM CaM, and 50 μM free Ca^{2+}. The kinase activity of the autophosphorylation site mutants was determined by measuring the rate of phosphorylation of the peptide ($\mu M\ s^{-1}$). Activities of the mutants are reported as the percentage of the wild-type activity. The assays were performed in triplicate, and error bars represent the standard deviation. (C) Activity of the autophosphorylation site mutants against 4 μM wheat germ eEF-2, using 2 nM eEF-2K enzyme, 2 μM CaM, and 50 μM free Ca^{2+}, over an incubation time of 1 min: (top) Coomassie-stained gel and (bottom) autoradiograph. (D) Autophosphorylation of eEF-2K WT, T348A, and T348D using 1 μM eEF-2K enzyme in the presence of 5 μM CaM and 50 μM free Ca^{2+}, over an incubation time of 10 min: (top) Coomassie-stained gel and (bottom) autoradiograph.

subjected to in-gel digestion with trypsin or chymotrypsin as described in the Experimental Procedures of the Supporting Information. Digests were then analyzed by tandem mass spectrometry using a Q-TOF Premier mass spectrometer (Waters Corp.), and MS/MS spectra from the analysis were searched against the modified Swiss-Prot all-species database using Mascot (http://www.matrixscience.com).[37] Peptide identifications with Mascot scores equal to or above ~48 typically represent an assignment with ≥95% confidence (<5% chance that the peptide ID is a random event). No peptides were found to be significantly phosphorylated in the analysis of the eEF-2K purified from *E. coli*, and the tryptic and chymotryptic peptide digest study accounted for ~90% (649 of 725) of the eEF-2K sequence, with ~92% (76 of 83) of the threonine and serine residues covered (Figure 3A). This is consistent with the notion that Ca^{2+}/CaM is required for autophosphorylation.[16,17] Some trace phosphorylation was

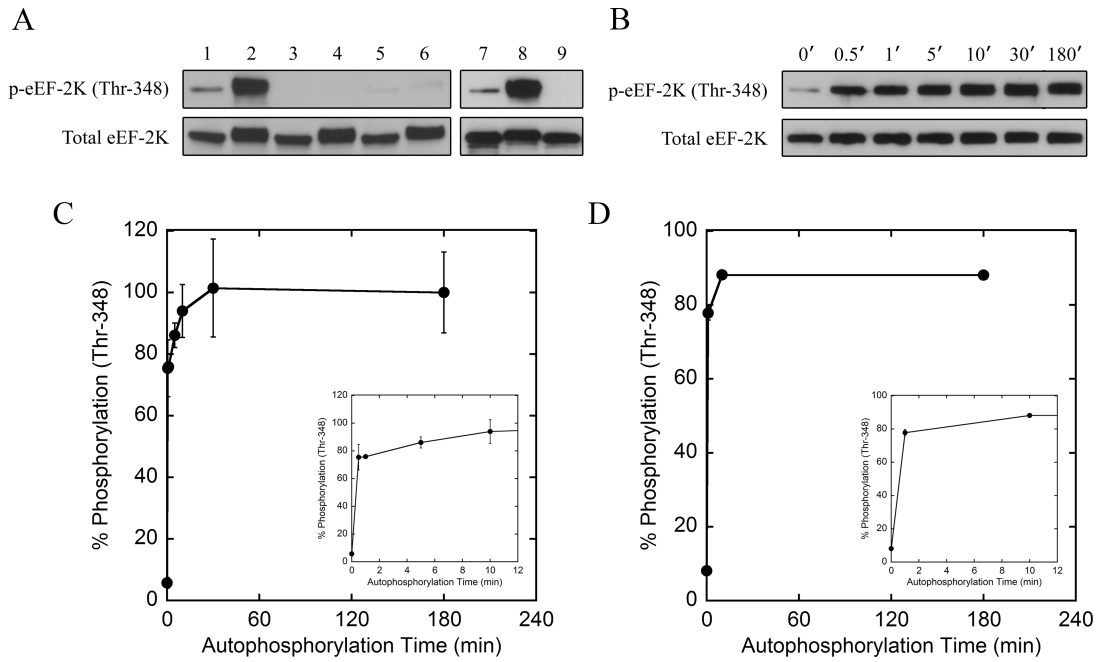

Figure 5. Analysis of incorporation of phosphate at Thr-348. (A) Characterization of the anti-phospho-eEF-2K (Thr-348) antibody. The antibody was characterized against 50 ng of recombinant eEF-2K by immunoblotting as described in Experimental Procedures: lane 1, untreated eEF-2K WT; lane 2, autophosphorylated eEF-2K WT; lane 3, untreated eEF-2K T348A; lane 4, autophosphorylated eEF-2K T348A; lane 5, untreated eEF-2K T348D; lane 6, autophosphorylated eEF-2K T348D; lane 7, untreated eEF-2K WT; lane 8, autophosphorylated eEF-2K WT; lane 9, λ-phosphatase-treated eEF-2K WT. (B) Time course of incorporation of phosphate at Thr-348. eEF-2K (500 nM) was allowed to autophosphorylate in the presence of 5 μM CaM and 50 μM free Ca^{2+}. At the indicated times, 50 ng of eEF-2K was removed and the reaction quenched with hot SDS−PAGE sample loading buffer. The samples were then analyzed by Western blotting using the anti-phospho-eEF-2K (Thr-348) antibody as described in Experimental Procedures. (C) Graphical representation of panel B. Western blots were quantified using ImageJ, and data were plotted as the percent phosphorylation of Thr-348 against autophosphorylation time. The inset shows an expansion of the data from 0 to 12 min. Experiments were performed in duplicate, and error bars represent the standard deviation. (D) Average percent phosphorylation of Thr-348 of eEF-2K based on monitoring the ^{348}TILR^{351} peptide by LC−MS/MS. eEF-2K was allowed to autophosphorylate in the presence of CaM, Ca^{2+}, and MgATP. After 0 min (no ATP added), 1 min, 10 min, and 3 h, the reaction was quenched and the sample subjected to tryptic in-gel digestion followed by analysis by mass spectrometry as described in the Experimental Procedures of the Supporting Information. The inset shows an expansion of the data from 0 to 12 min. Runs for each sample were performed in triplicate, and error bars represent the standard deviation.

however detected, in agreement with the kinase possessing a low level of Ca^{2+}-independent kinase activity.[27]

Autophosphorylation Sites on eEF-2K. To determine the possible autophosphorylation sites on eEF-2K, the recombinant enzyme was allowed to autophosphorylate in the presence of CaM, Ca^{2+}, and MgATP for 3 h. The sample was resolved by SDS−PAGE and then subjected to in-gel digestion with trypsin or chymotrypsin as described in the Experimental Procedures of the Supporting Information. Tryptic and chymotryptic digests were then analyzed by tandem mass spectrometry, and MS/MS spectra from the analysis were searched against the modified Swiss-Prot all-species database using Mascot. Peptide identifications with Mascot scores equal to or above 45 (tryptic digest) or 49 (chymotryptic digest) represent an identification with ≥95% confidence and were considered for protein identification and phosphorylation site determination. Combined data from the analysis of both digests gave coverage of ~86% (624 of 725) of the eEF-2K sequence, with ~94% (78 of 83) of the threonine and serine residues covered (Figure 3B). Mass spectrometric analysis of the autophosphorylated sample, the results of which are summarized in Table 1, revealed five sites of autophosphorylation in recombinant human eEF-2K: Thr-348, Thr-353, Ser-445, Ser-474, and Ser-500. MS data also indicated other residues (Thr-64 and Ser-491) as being phosphorylated, but these peptides did not have significant Mascot scores and hence could not be confidently identified as autophosphorylation sites.

Analysis of the eEF-2K Autophosphorylation Site Mutants. To analyze the significance of autophosphorylation for activity, alanine and aspartate autophosphorylation site mutants were generated by site-directed mutagenesis, and the mutant proteins were purified to homogeneity as described in the Experimental Procedures of the Supporting Information (Figure 4A). Activity assays were performed using 2 nM eEF-2K in a buffer containing 2 μM CaM, 250 μM $CaCl_2$, 100 μM EDTA, 100 μM EGTA, 10 mM $MgCl_2$, 0.5 mM ATP, and 150 μM peptide substrate. The results are displayed in Figure 4B as the percentage of kinase activity of each of the mutant enzymes compared to that of WT eEF-2K. Alanine and aspartate mutants of four of the autophosphorylation sites (Thr-353, Ser-445, Ser-474, and Ser-500) do not show any significant difference in activity compared to WT eEF-2K with respect to their ability to phosphorylate a peptide substrate. This suggests that the autophosphorylation of these sites may not be essential for the kinase to be active. In contrast, autophosphorylation of Thr-348 appears to be critical for activity of the kinase. Mutation of this site to alanine results in a loss of ~95% of the kinase activity. An attempt to rescue the loss of activity in the form of a mutation of Thr-348 to aspartate was unsuccessful. The T348D mutant exhibited only ~7% of the kinase activity,

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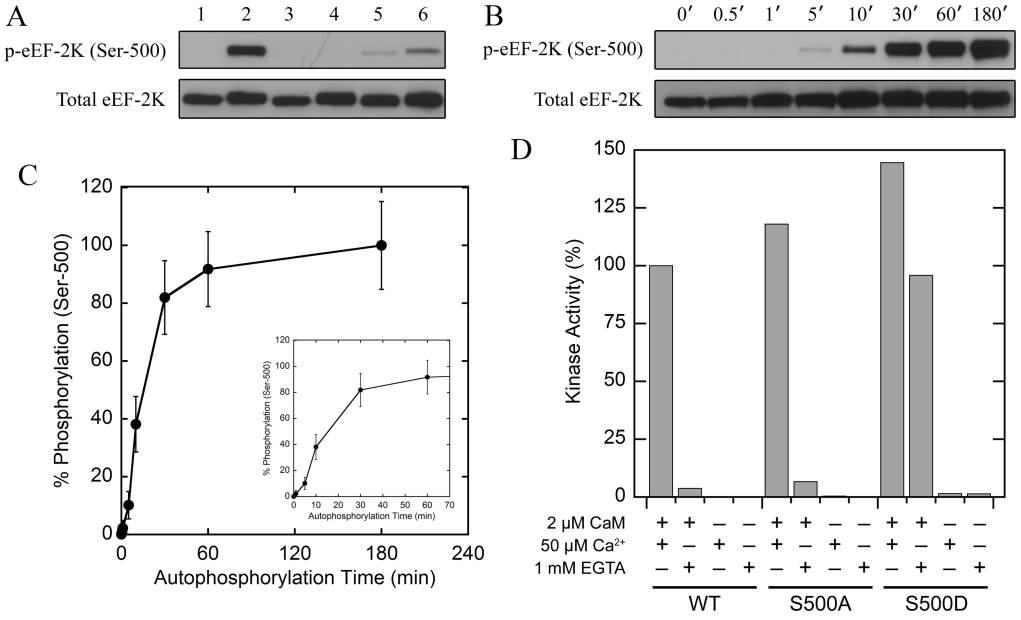

Figure 6. Analysis of phosphorylation at Ser-500. (A) Characterization of the anti-phospho-eEF-2K (Ser-500) antibody. The antibody was characterized against 50 ng of recombinant eEF-2K by immunoblotting as described in Experimental Procedures: lane 1, untreated eEF-2K WT; lane 2, autophosphorylated eEF-2K WT; lane 3, untreated eEF-2K S500A; lane 4, autophosphorylated eEF-2K S500A; lane 5, untreated eEF-2K S500D; lane 6, autophosphorylated eEF-2K S500D. (B) Time course of incorporation of phosphate at Ser-500. eEF-2K (500 nM) was allowed to autophosphorylate in the presence of 5 μM CaM and 50 μM free Ca^{2+}. At the indicated times, 50 ng of eEF-2K was removed and the reaction quenched with hot SDS−PAGE sample loading buffer. The samples were then analyzed by Western blotting using the anti-phospho-eEF-2K (Ser-500) antibody as described in Experimental Procedures. (C) Graphical representation of panel B. Western blots were quantified using ImageJ, and data were plotted as the percent phosphorylation of Ser-500 against autophosphorylation time. The inset shows an expansion of the data from 0 to 70 min. Experiments were performed in duplicate, and error bars represent the standard deviation. (D) Buffers are described in Experimental Procedures. Assays were performed with eEF-2K enzyme with or without 50 μM free Ca^{2+} and with or without 2 μM CaM. EGTA (1 mM) was added to all assays conducted in the absence of Ca^{2+}. For eEF-2K WT, S500A, and S500D assayed in the presence of both Ca^{2+} and CaM, and eEF-2K S500D assayed in the presence of only CaM, activities were much higher than the basal level of kinase activity, and hence, only 5 nM kinase was used. For all the other assays, 50 nM eEF-2K was used to detect an increase in kinase activity over the basal level. Kinase activity was determined by measuring the rate of phosphorylation of the peptide ($\mu M\ s^{-1}$). Activities of the mutants are reported as the percentage of the wild-type activity.

indicating that a negative charge at this position was unable to compensate for the loss of a phosphate at Thr-348.

To confirm that the effects observed were not an artifact of using a peptide substrate, 2 nM kinase was assayed against 4 μM wheat germ eEF-2 for 1 min, in the presence of 2 μM CaM and 50 μM free Ca^{2+} as described in Experimental Procedures. Results indicate that phosphorylation of Thr-348 is crucial for its ability to phosphorylate its substrate eEF-2 (Figure 4C). Because the T348D mutant could not rescue kinase activity, it is possible that Thr-348 is important for the structural integrity of the kinase, and the inactivity of the mutants is related to structure rather than a result of the lack of Thr-348 phosphorylation. However, both the T348A and T348D mutants are able to autophosphorylate (Figure 4D), suggesting that Thr-348 phosphorylation is required for the activity of eEF-2K against its substrate.

Analysis of Incorporation of Phosphate at Thr-348.

Monitoring Incorporation of Phosphate at Thr-348 by Immunoblotting. It is highly likely that the rapid initial incorporation of phosphate (Figure 2C) occurs at Thr-348 and is required for activation of the kinase. To detect incorporation of phosphate at this site, ECM Biosciences generated affinity-purified rabbit polyclonal anti-eEF-2K (Thr-348) phospho-specific antibodies, which were used in Western blotting. Interestingly, results obtained from characterization of the phospho-specific antibody revealed that despite coexpression of eEF-2K with λ-phosphatase, the kinase purified from bacteria displays a low level of phosphorylation at Thr-348 (Figure 5A). A loss of phosphorylation at this site is observed when the kinase is treated with λ-phosphatase in vitro (Figure 5A). Additionally, the antibody showed a high specificity for the form of the enzyme autophosphorylated at Thr-348, as assessed by probing the autophosphorylated eEF-2K mutants T348A and T348D (Figure 5A). Upon incubation with CaM, Ca^{2+}, and MgATP, eEF-2K undergoes rapid autophosphorylation at Thr-348 in the first minute (Figure 5B,C) with phosphorylated levels increasing to ~80%. The percentage of phosphate incorporated at Thr-348 levels out after approximately 10 min, after which there is no significant increase in the level of autophosphorylation at this site.

Monitoring Incorporation of Phosphate at Thr-348 by Mass Spectrometry. To verify the rapid incorporation of phosphate at Thr-348 upon incubation with Ca^{2+} and CaM, the percent phosphorylation was determined on the basis of monitoring the ^{348}TILR^{351} peptide by LC−MS/MS using a ThermoFisher LTQ XL linear ion trap mass spectrometer. The recombinant enzyme was allowed to autophosphorylate in the presence of CaM, Ca^{2+}, and MgATP, and after 0 min (no ATP added), 1 min, 10 min, and 3 h, the reaction was quenched. The sample was resolved by SDS−PAGE and subjected to in-gel digestion with trypsin, and the tryptic digest was then analyzed by mass spectrometry. The average percent phosphorylation of residue Thr-348 was calculated on the basis of monitoring the abundance of ^{348}TILR^{351} and ^{348}pTILR^{351} by LC−MS/MS for each of the digests as

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described in the Experimental Procedures of the Supporting Information. Representative CID mass spectra for ^{348}TILR^{351} and ^{348}pTILR^{351} are shown in Figure 1 of the Supporting Information. The results, which indicate the reproducibility of the mass spectrometry data, are summarized in Table 1 of the Supporting Information and shown graphically in Figure 5D. The mass spectrometry data mirrored the results from immunoblotting, with detection of a small amount of phosphate (~8%) at Thr-348 in recombinant eEF-2K purified from bacteria (Figure 5D). As expected, the level of autophosphorylation at Thr-348 rapidly increases in the presence of CaM, Ca^{2+}, and MgATP, with ~78% of this site being phosphorylated within the first minute, thus validating the results obtained by Western blotting. The percentage of phosphate incorporated at Thr-348 levels out at approximately 88% after 10 min, after which there is no notable increase in the level of autophosphorylation at this site.

Analysis of Incorporation of Phosphate at Ser-500. *Monitoring Incorporation of Phosphate at Ser-500 by Immunoblotting.* To analyze the time course of incorporation of phosphate at Ser-500, ECM Biosciences generated affinity-purified rabbit polyclonal anti-eEF-2K (Ser-500) phospho-specific antibodies, which were used in Western blotting. Results obtained from characterization of the phospho-specific antibody indicated that it showed a high specificity for the form of the enzyme autophosphorylated at Ser-500, as assessed by probing autophosphorylated eEF-2K mutants S500A and S500D (Figure 6A). Upon incubation with CaM, Ca^{2+}, and MgATP, phosphate is incorporated at Ser-500 within the first 5 min (~7%), with phosphorylation levels increasing to >80% after 30 min, when compared to the maximal level of phosphorylation (100%) detected after 3 h (Figure 6B,C).

Analysis of the Calcium-Independent Activity of eEF-2K. Two groups in 1993 demonstrated that eEF-2K gains Ca^{2+}-independent activity with autophosphorylation.[16,17] However, the autophosphorylation site responsible for imparting this Ca^{2+}-independent activity has not yet been reported. Additional studies of eEF-2K have shown that PKA also induces Ca^{2+}-independent activity of eEF-2K by phosphorylation of Ser-500.[28] Interestingly, we identified Ser-500 as an autophosphorylation site (Table 1), which suggests that it could potentially be the site responsible for autophosphorylation-induced Ca^{2+}-independent activity. Substitution of alanine completely abrogated Ca^{2+}-independent activity following autophosphorylation, supporting this notion (data not shown).

To examine the mechanism further, we assayed S500D against the peptide substrate in the presence or absence of Ca^{2+} or CaM, as described in Experimental Procedures, and compared S500D to WT eEF-2K and the S500A mutant. It should be noted that under the experimental conditions employed, eEF-2K is not expected to undergo extensive phosphorylation at Ser-500 during the course of the experiment. Both mutants were active in the presence of Ca^{2+} and CaM, indicating that the mutations do not compromise structural integrity (Figure 6D). On the basis of previous reports about the kinase, the S500D mutant (if able to mimic phosphorylation at Ser-500) was expected to be active in the absence of Ca^{2+} and CaM. However, as indicated in Figure 6D, the mutant did not display a considerable gain in activity when compared to the basal level of activity of the wild-type enzyme in the absence of both Ca^{2+} and CaM. Intriguingly, however, eEF-2K S500D did display a significant increase in Ca^{2+}-independent activity in the presence of CaM alone (~95% of the maximal wild-type activity); this suggests that autophosphorylation of eEF-2K at Ser-500 induces a Ca^{2+}-independent activity that is dependent on CaM.

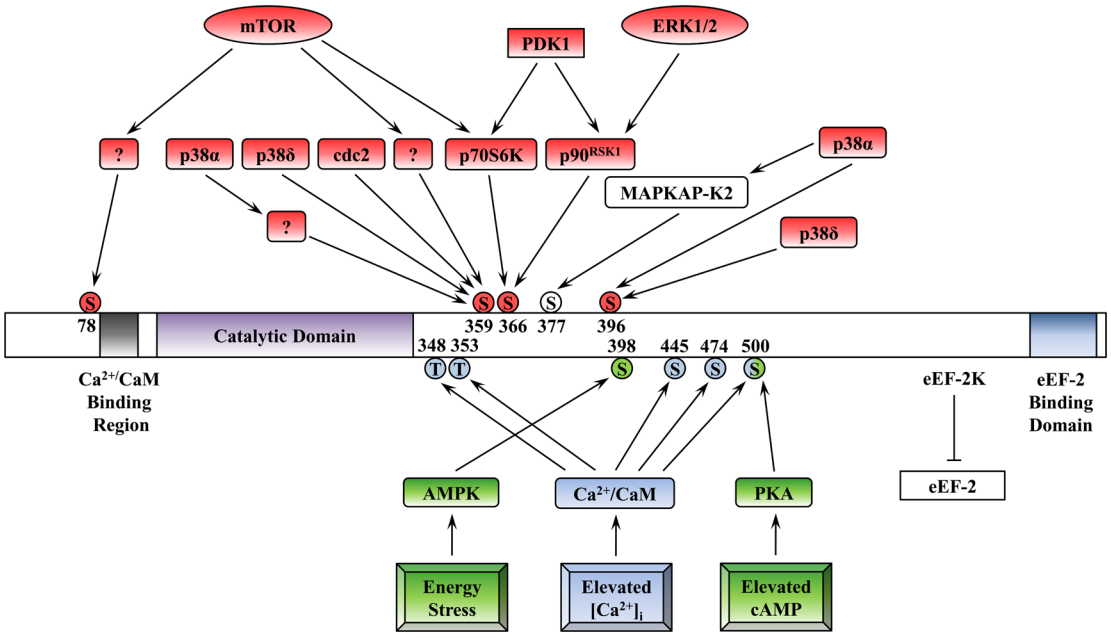
DISCUSSION

Regulation of eEF-2K by Calcium and Calmodulin. The primary Ca^{2+}-signaling protein in eukaryotes is CaM. Activation of CaM kinases is generally dependent on the binding of Ca^{2+}/CaM, and in some cases, the enzyme is reported to become Ca^{2+}-independent following activation or requires additional phosphorylations by other protein kinases to achieve full activity.[38] Our in vitro data suggest that at a CaM concentration of 2 μM, eEF-2K exhibits significant activity (k_{cat}^{app} = 3 s^{-1}) when the concentration of Ca^{2+}, $[Ca^{2+}]_{free}$, is low (e.g., 25 nM, calculated from known concentrations of Ca^{2+} and EGTA). This compares to a 10-fold higher apparent k_{cat} (k_{cat}^{app} = 26 s^{-1}) when $[Ca^{2+}]_{free}$ is increased to 3 μM (Figure 1A). Free CaM concentrations within cells are suggested to vary from around 10^{-12} to 10^{-5} M in different tissues and at different stages of the cell cycle.[39] Our unpublished studies suggest that activation of eEF-2K by Ca^{2+}/CaM has little effect on substrate binding, suggesting that Ca^{2+} influx, which increases $[Ca^{2+}]_i$ from basal levels of 50 nM to ~10−100 μM, can lead to a 10-fold enhancement of eEF-2K activity in the presence of 2 μM CaM. Thus, these data suggest that significant basal activity of eEF-2K can be supported at low physiological $[Ca^{2+}]_i$ values if the concentration of cellular CaM is sufficiently high. Interestingly, the characterization of eEF-2K from rabbit reticulocytes and rat pancreas by Mitsui et al. indicated that the native enzyme exhibits half-maximal activation at <1 nM CaM.[16] One explanation for this low K_c^{app} value could be that certain post-translational modifications occurring in vivo may increase the affinity of CaM for the kinase.[29]

Our studies have identified five Ca^{2+}/CaM-stimulated autophosphorylation sites in eEF-2K: Thr-348, Thr-353, Ser-445, Ser-474, and Ser-500. Three of these sites, Thr-348, Ser-445, and Ser-474, have been identified as possible phosphorylation sites during the large-scale analysis of proteins phosphorylated in vivo.[40−43] Mutagenesis studies support the notion that the phosphorylations of Thr-353, Ser-445, and Ser-474 are not essential for eEF-2K activity (Figure 4). Phosphorylations of Thr-348 and Ser-500, which are both early events following stimulation by Ca^{2+}/CaM, are discussed further below.

Calcium/Calmodulin-Stimulated Autophosphorylation of Thr-348 Triggers Substrate Phosphorylation. Autophosphorylation of eEF-2K exhibits a complex progression, with an initial rapid autophosphorylation phase followed by a slower phase. We have shown that the activity of eEF-2K against a peptide substrate is independent of autophosphorylation events that occur after the first minute (Figure 2E). Mutation of Thr-348 to Ala or Asp renders eEF-2K inactive toward the peptide substrate (Figure 4), suggesting that the initial rapid phosphate incorporation observed in Figure 2C involves the phosphorylation of Thr-348. To analyze this further, we monitored phosphorylation of Thr-348 both by a mass spectrometry approach and by immunoblotting using an antibody that specifically detects phosphorylated Thr-348. These studies confirmed that rapid incorporation of phosphate occurs at Thr-348 within the first minute following stimulation, affirming the importance of phosphorylation at this site in the activation of the kinase (Figure 5). Indeed, during the large-scale mass spectrometry analysis of proteins phosphorylated in

Scheme 1. Regulation of eEF-2K Activity by Multisite Phosphorylation[a]



[a]Summary of the various phosphorylated residues on eEF-2K. Components are color-coded as follows. Red indicates portions suggested to be involved in the negative regulation of eEF-2K activity through an inhibitory phosphorylation (these sites include Ser-78, Ser-359, Ser-366, and Ser-396). Regulation through the mTOR pathway involves the phosphorylation of Ser-366 by p70 S6 kinase and the phosphorylation of Ser-359 and Ser-78 by at least two additional unknown kinases.[22−24] It has been postulated that the phosphorylation of Ser-78 acts to hinder the binding of CaM to eEF-2K.[24] The cdc2−cyclin B complex has been shown to modulate eEF-2K activity via Ser-359 in a manner that is dependent on the cell cycle as well as amino acid availability and is perhaps controlled by mTOR.[25] Regulation through the MAPK cascade occurs via the phosphorylation of Ser-366 by $p90^{RSK1}$ in an ERK-dependent fashion.[22] In addition, the stress-activated protein kinases p38α and p38δ inhibit eEF-2K via phosphorylation of Ser-396.[23] p38δ is also known to phosphorylate eEF-2K on Ser-359.[21] Green indicates portions suggested to be involved in the positive regulation of eEF-2K activity through an activating phosphorylation (these sites include Ser-398 and Ser-500). Phosphorylation of Ser-398 by the energy supply regulator AMPK is known to activate eEF-2K.[29] The cAMP-dependent PKA has also been shown to activate eEF-2K via phosphorylation of Ser-500 and in the process imparts Ca^{2+}-independent activity to the kinase.[26−28] Blue indicates portions involved in autophosphorylation of eEF-2K (these sites include Thr-348, Thr-353, Ser-445, Ser-474, and Ser-500). Of the five autophosphorylation sites, only Thr-348 appears to be essential for activity against its substrate. Ser-500 is an autophosphorylation site that could be the key residue responsible for autophosphorylation-induced Ca^{2+}-independent [CaM-dependent (this work)] activity.[16,17] The role of the phosphorylation of Ser-377 by MAPKAP-K2 has not yet been determined.[23]

vivo, Thr-348 has previously been identified as a possible phosphorylation site in a variety of mouse tissues, including pancreas, spleen, and testis.[44]

Sequence alignment studies by Crawley et al. have indicated that the corresponding residue in MHCK A is Thr-825, the phosphorylation of which has also been shown to be a necessity for activity.[20] In an intriguing mechanism, Crawley et al. proposed a model in which phosphorylated Thr-825 acts as a ligand for a P_i-binding pocket in the catalytic domain of MHCK A. This interaction is predicted to have an allosteric effect that induces a conformational change in the active site of MHCK A, thus promoting its activity. Several residues of MHCK A have been demonstrated to be involved in this process, and the corresponding residues are conserved in eEF-2K.[20] Therefore, as suggested by Crawley et al.,[20] it is reasonable to propose that eEF-2K is regulated in a similar manner, but because activation also depends on its interaction with Ca^{2+}/CaM, a more complex activation process is likely. Furthermore, both T348A and T348D undergo robust Ca^{2+}/CaM-stimulated autophosphorylation, suggesting that the role of the Thr-348 phosphorylation is to somehow trigger the additional function of phosphorylating an exogenous substrate.

Evidence That Calcium/Calmodulin-Stimulated Autophosphorylation of Ser-500 Induces Paradoxical Calcium-Independent Activity That Requires Calmodulin. eEF-2K has previously been shown to be phosphorylated by cAMP-dependent protein kinase (PKA) both in vitro and indirectly in vivo.[26−28] This phosphorylated residue has been identified as Ser-500 (Ser-499 in rat) and has been suggested to play an important role in inducing Ca^{2+}-independent activity in eEF-2K.[28] Autophosphorylation of eEF-2K has also been reported to elicit Ca^{2+}-independent activity.[16,17] Our mass spectrometry analysis identified Ser-500 as a site that becomes phosphorylated upon Ca^{2+}/CaM stimulation of eEF-2K. Analysis of the phosphorylation using an antibody that is specific for eEF-2K when phosphorylated at Ser-500 revealed the time course for Ser-500 phosphorylation following Ca^{2+}/CaM stimulation. Interestingly, under the experimental conditions, the phosphorylation of Ser-500 appears to exhibit a lag phase but can be detected within 5 min of addition of Ca^{2+}/CaM to eEF-2K, with ~50% (compared to the maximal level detected after 3 h) being phosphorylated after 15 min (Figure 6C). As noted earlier, our studies confirm that Ca^{2+}/CaM-stimulated autophosphorylation induces Ca^{2+}-independent activity of eEF-2K, and that the S500A mutant autophosphorylates, but does not become Ca^{2+}-independent (unpublished data).

We report here that the substitution of an Asp for Ser-500 (to generate eEF-2K S500D) renders the kinase Ca^{2+}-independent (Figure 6D). Paradoxically, this activity requires

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the presence of CaM in the buffer (Figure 6D). In contrast, both the wild-type enzyme and the S500A mutant exhibit significantly lower activity in the presence of 1 mM EGTA (without Ca^{2+} and with CaM). It should be noted that under the conditions of the experiment shown in Figure 6D, autophosphorylation of eEF-2K at Ser-500 is predicted to be minor when both Ca^{2+} and CaM are absent from the buffer.

CaMK-II and CaMK-IV are hypothesized to act as molecular switches, where a momentary increase in $[Ca^{2+}]_i$ permits CaM to modulate the activity of the kinase through autophosphorylation.[17,45] In the process, the autophosphorylation equips the enzyme with Ca^{2+}-independent activity; thus, when $[Ca^{2+}]_i$ declines to the basal level, the kinase continues to possess significant activity until it is dephosphorylated by cellular phosphatases.[17,45] A similar mechanism of eEF-2K regulation, which would depend on significant phosphorylation of Ser-500, remains to be demonstrated in vivo.

CONCLUSION

Elevation of $[Ca^{2+}]_i$ is presumed to activate eEF-2K following Ca^{2+}/CaM binding.[7−10] This study identified five major Ca^{2+}/CaM-stimulated autophosphorylation sites in eEF-2K: Thr-348, Thr-353, Ser-445, Ser-474, and Ser-500. Phosphorylation of Thr-348 occurs within seconds and appears to be necessary for substrate phosphorylation, but not autophosphorylation. Phosphorylation of Ser-500, which occurs within a few minutes but lags behind the phosphorylation of Thr-348, is associated with Ca^{2+}-independent activity of eEF-2K. No function for the phosphorylation of Thr-353, Ser-445, and Ser-474 has been delineated. Many questions about the molecular mechanisms that underlie the control of eEF-2K through multisite phosphorylation remain to be addressed. In addition to autophosphorylation, several protein kinases are known to regulate eEF-2K. The various sites of phosphorylation in eEF-2K have been summarized in Scheme 1. AMPK and protein kinase A are reported to activate eEF-2K through mechanisms associated with the phosphorylation of Ser-398 and Ser-500, respectively.[26−29] Inactivation of eEF-2K is associated with the phosphorylation of Ser-78, Ser-359, Ser-366, or Ser-396, caused by a number of upstream kinases, including p38α, p38δ, p70 S6 kinase, $p90^{RSK1}$, MAPKAP-K2, cdc2, and at least two other unidentified kinases regulated by mTOR.[21−25] The availability of highly purified eEF-2K is expected to facilitate a better understanding of the mechanisms governing the post-translational control of this important protein kinase.

ASSOCIATED CONTENT

Supporting Information

Protocols for the purification of eEF-2K and mass spectrometry protocols for the identification of phosphorylation sites, results of the characterization of eEF-2K, and CID mass spectra of ^{348}TILR^{351} and ^{348}pTILR^{351} peptides used to measure incorporation of phosphate at Thr-348. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

[a]During the review of the manuscript, a research article identifying some of the autophosphorylation sites in eEF-2K was accepted for publication.[1] Some similarities between the two manuscripts include the detection of Thr-348, Thr-353, and Ser-445 as major autophosphorylation sites. Additionally, it was suggested that eEF-2K autophosphorylation at Thr-348 is required for activity against its substrate. However, in addition to the different approaches used, several significant differences exist between the two studies. The first is the stoichiometry of incorporation of phosphate into eEF-2K. We have found that 4 mol of phosphate is incorporated per mole of eEF-2K in the presence of Ca^{2+}/CaM, while Pyr Dit Ruys et al. report 8 mol of phosphate/mol of eEF-2K. The second difference is an increase in the level of incorporation of phosphate at Thr-348. Pyr Dit Ruys et al. report no significant increase in the level of phosphorylation at Thr-348 in vitro, as it is constitutively phosphorylated. However, coexpression of eEF-2K with λ-phosphatase afforded us an enzyme that, upon incubation with Ca^{2+}/CaM, allowed detection of rapid incorporation of phosphate at Thr-348 by mass spectrometry and immunoblotting analysis, which supports the claim that phosphorylation at Thr-348 is stimulated by Ca^{2+}/CaM and is required for activity against eEF-2. The third difference is the detection of Ser-500 as a significant autophosphorylation site. Pyr Dit Ruys et al. indicated that Ser-500 is not a significant site of autophosphorylation; however, our studies indicate that phosphate is incorporated at this site within the first 5 min of autophosphorylation, and moreover, mutational analysis suggests that phosphorylation of Ser-500 induces significant Ca^{2+}-independent activity that is dependent on CaM. Some of these differences could be due to the difference in enzyme preparations; our study employs a tagless recombinant eEF-2K verified as highly purified and monomeric by light scattering.[2] Pyr Dit Ruys et al. use a GST-tagged form of the enzyme.

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ABBREVIATIONS

ACN, acetonitrile; AMPK, AMP-activated protein kinase; BSA, bovine serum albumin fraction V; CaM, calmodulin; CaMK, calcium/calmodulin-dependent protein kinase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; eEF-2, eukaryotic elongation factor 2; eEF-2K, eukaryotic elongation factor 2 kinase; EGTA, ethylene glycol tetraacetic acid; ERK, extracellular signal-regulated kinases; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAPK, mitogen-activated

protein kinase; MAPKAP-K, mitogen-activated protein kinase-activated protein kinase; MEK, MAPK/ERK kinase; MHCK A, myosin II heavy chain kinase A; MPA, mobile phase A; MPB, mobile phase B; MS, mass spectrometry; mTOR, mammalian target of rapamycin; Ni-NTA, nickel-nitrilotriacetic acid; PKA, cAMP-dependent protein kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEV, tobacco etch virus; Trx-His_6-tag, thioredoxin-six-histidine tag.

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