Mapping the Functional Domains of Elongation Factor-2 Kinase†

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ABSTRACT: A new class of eukaryotic protein kinases that are not homologous to members of the serine/threonine/tyrosine protein kinase superfamily was recently identified [Futey, L. M., et al. (1995) *J. Biol. Chem. 270*, 523−529; Ryazanov, A. G., et al. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4884−4889]. This class includes eukaryotic elongation factor-2 kinase, *Dictyostelium* myosin heavy chain kinases A, B, and C, and several mammalian putative protein kinases that are not yet fully characterized [Ryazanov, A. G., et al. (1999) *Curr. Biol. 9*, R43−R45]. eEF-2 kinase is a ubiquitous protein kinase that phosphorylates and inactivates eukaryotic translational elongation factor-2, and thus can modulate the rate of polypeptide chain elongation during translation. eEF-2 was the only known substrate for eEF-2 kinase. We demonstrate here that eEF-2 kinase can efficiently phosphorylate a 16-amino acid peptide, MH-1, corresponding to the myosin heavy chain kinase A phosphorylation site in *Dictyostelium* myosin heavy chains. This enabled us to develop a rapid assay for eEF-2 kinase activity. To localize the functional domains of eEF-2 kinase, we expressed human eEF-2 kinase in *Escherichia coli* as a GST-tagged fusion protein, and then performed systematic in vitro deletion mutagenesis. We analyzed eEF-2 kinase deletion mutants for the ability to autophosphorylate, and to phosphorylate eEF-2 as well as a peptide substrate, MH-1. Mutants with deletions between amino acids 51 and 335 were unable to autophosphorylate, and were also unable to phosphorylate eEF-2 and MH-1. Mutants with deletions between amino acids 521 and 725 were unable to phosphorylate eEF-2, but were still able to autophosphorylate and to phosphorylate MH-1. The kinases with deletions between amino acids 2 and 50 and 336 and 520 were able to catalyze all three reactions. In addition, the C-terminal domain expressed alone (amino acids 336−725) binds eEF-2 in a coprecipitation assay. These results suggest that eEF-2 kinase consists of two domains connected by a linker region. The amino-terminal domain contains the catalytic domain, while the carboxyl-terminal domain contains the eEF-2 targeting domain. The calmodulin-binding region is located between amino acids 51 and 96. The amino acid sequence of the carboxyl-terminal domain of eEF-2 kinase displays similarity to several proteins, all of which contain repeats of a 36-amino acid motif that we named "motif 36".

Despite the diversity of cellular processes regulated through protein phosphorylation, the vast majority of eukaryotic protein kinases share a similar catalytic domain structure. Both serine/threonine kinases and tyrosine kinases utilize a catalytic domain that consists of 12 conserved subdomains, and that folds into a characteristic two-lobed structure (*1*). There are, however, a number of reports demonstrating the existence of eukaryotic protein kinases that display little or no homology to members of the serine/threonine/tyrosine protein kinase superfamily, and therefore may have a different catalytic domain structure (*2*−*9*). One such unconventional protein kinase is eukaryotic elongation factor-2 kinase (eEF-2[1] kinase).

eEF-2 kinase, also known as Ca^{2+}/calmodulin-dependent protein kinase III, is a protein kinase present in virtually all tissues of vertebrates, as well as in various invertebrates (reviewed in refs *10* and *11*). The only known substrate of eEF-2 kinase is eEF-2 (*10*). eEF-2 is a 100 kDa protein that promotes ribosomal translocation, which is the reaction that results in movement of the ribosome along mRNA during translation (*12*). eEF-2 kinase phosphorylates eEF-2 predominantly on threonine 56, which results in inactivation of eEF-2 and an inhibition of protein synthesis (*13*). It was suggested that phosphorylation of eEF-2 by eEF-2 kinase represented the mechanism by which the protein synthesis rate can be regulated (*10*, *11*), although the exact physiological role of such a mechanism remains unclear.

The primary structure of eEF-2 kinase from several eukaryotic organisms was recently determined (*2*, *14*). Surprisingly, eEF-2 kinase does not display any homology to the other calmodulin-dependent protein kinases or to the other members of the eukaryotic protein kinase superfamily (*2*). On the other hand, the putative catalytic domain of eEF-2 kinase appears to be highly similar to the catalytic domain of the recently described myosin heavy chain kinases A and B (MHCK A and B) from *Dictyostelium* (*3*, *15*−*17*). Thus, eEF-2 kinase and *Dictyostelium* MHCK A and B represent a new class of protein kinases. This class also includes the

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[1] Abbreviations: eEF-2, eukaryotic elongation factor-2; MHCK A, myosin heavy chain kinase A; MHCK B, myosin heavy chain kinase B; MHCK C, myosin heavy chain kinase C; GST tag, glutathione *S*-transferase tag.

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Dictyostelium protein called myosin heavy chain kinase C (MHCK C), and several mammalian putative protein kinases that are not yet fully characterized (*18*).

It was reported that MHCK A and B could phosphorylate a 16mer peptide (MH-1) corresponding to their phosphorylation site in *Dictyostelium* myosin heavy chains (amino acids 2020−2035; *19*). Unexpectedly, as reported in this paper, we found that eEF-2 kinase can also phosphorylate MH-1, despite the fact that the amino acid sequence of MH-1 is very different from the sequence of the eEF-2 kinase phosphorylation site in eEF-2, and despite the fact that eEF-2 kinase cannot phosphorylate intact myosin heavy chains (*2*). Identification of a peptide substrate for eEF-2 kinase also enabled us to develop a rapid assay for eEF-2 kinase activity.

To gain insight into the structure and regulation of eEF-2 kinase, we performed the following experiments. We expressed human eEF-2 kinase in *Escherichia coli* as a GST fusion protein, and developed a protocol for purification of recombinant GST−eEF-2 kinase. Next, we produced a series of deletion mutants of eEF-2 kinase and analyzed the ability of these mutants to undergo autophosphorylation, to phosphorylate eEF-2 and a peptide substrate, and to bind calmodulin. This analysis revealed the location of the major functional domains of eEF-2 kinase. In addition, we found that the C-terminal domain of eEF-2 kinase contains a novel motif that is present in various prokaryotic and eukaryotic proteins.

MATERIALS AND METHODS

Expression and Purification of Recombinant Human eEF-2 Kinase in E. coli. Human eEF-2 kinase cDNA was cloned by RT-PCR from polyA+ mRNA prepared from the human glioma cell line T98G and cloned into pCR2.1 (Invitrogen) to form the construct pHGRH-1A (*2*). Then eEF-2 kinase cDNA was subcloned from pHGRH-1A into pGEX-2T (Pharmacia), which fused a glutathione *S*-transferase (GST) tag to the N-terminus of eEF-2 kinase. *E. coli* strain BL21-(DE3)pLysS cells were transformed with this construct. Cells were grown in LB with 50 μg/μL ampicillin and 30 μg/mL chloramphenicol at 37 °C until the optical density (measured at 600 nm) was 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM. Cells were induced for 3 h at 37 °C, and harvested by centrifugation. Cells were subjected to one freeze−thaw cycle, and lysed in phosphate-buffered saline [10 mM phosphate (pH 7.2), 2.7 mM KCl, and 138 mM NaCl] containing 1 mg/mL lysozyme and Complete protease inhibitor cocktail (Boehringer Mannheim). Lysis was carried out on ice for 30 min. The lysate was briefly sonicated to shear DNA, and then centrifuged at 4 °C and at 16000*g* for 30 min to separate the soluble and insoluble material. We found that only part of recombinant eEF-2 kinase remained soluble in the extracts, while a greater portion was insoluble and found in inclusion bodies. We purified both forms of eEF-2 kinase.

Soluble recombinant GST−eEF-2 kinase was purified from the lysate supernatant. All purification steps were carried out at 4 °C. A 50% slurry of glutathione-Sepharose was added to the supernatant (the bed volume being $^1/_{10}$ of the starting material volume), and binding was carried out overnight at 4 °C on a rocker. This material was put into a column, and was washed with 30 column volumes of PBS. The elution buffer consisted of 100 mM Tris-HCl (pH 8.0) and 20 mM reduced glutathione. The eluate was analyzed for protein content by 8% SDS−PAGE and staining with Coomassie Blue. The activity of the purified protein was analyzed by an eEF-2 kinase activity assay.

Insoluble eEF-2 kinase was purified from inclusion bodies, which were precipitated by centrifugation of the bacterial lysate. The pellet was resuspended in a buffer containing 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 7 mM β-mercaptoethanol, and 8 M urea and incubated on ice for 20 min. The denatured material was centrifuged for 30 min at 30000*g*. This suspension was dialyzed overnight at 4 °C against a buffer consisting of 20 mM Tris-HCl (pH 7.0). 100 mM NaCl, and 7 mM β-mercaptoethanol. The dialyzed material was centrifuged at 30000*g* for 30 min at 4 °C to remove any remaining insoluble material.

Electrophoretic analysis of purified recombinant GST−eEF-2 kinase obtained by both purification methods revealed essentially one band at approximately 125 kDa on a Coomassie-stained gel, which corresponds to the combined molecular masses of eEF-2 kinase and the GST tag (see Figure 3A, lane 1). The specific activity of eEF-2 kinase obtained by both of these methods was similar. All subsequent experiments were carried out independently using both forms of eEF-2 kinase.

Oligonucleotide-Directed Mutagenesis and Expression of Mutants of eEF-2 Kinase. Oligonucleotide-directed in vitro mutagenesis was performed using the Muta-Gene Phagemid *In Vitro* Mutagenesis kit from Bio-Rad according to the manufacturer's instructions. The kit is based on the in vitro mutagenesis method developed by Kunkel (*20*). pHGRH-1A was used for mutagenesis since the pCR2.1 vector has an f1 ori to generate the single-stranded DNA necessary for the mutagenesis protocol. 30mer oligonucleotides were synthesized which were complementary to a stretch of 15 nucleotides on each side of the region to be deleted. The mutated constructs were sequenced to verify that the proper deletion was made. An in vitro wheat germ extract-coupled transcription/translation system was used to verify that the protein was expressed from each construct. Mutants were subsequently subcloned into pGEX-2T to create GST fusions. Expression of mutant proteins in *E. coli* BL21(DE3)pLysS and subsequent purification were carried out as described above.

Peptide Synthesis. Peptides were synthesized commercially by Genosys. The EF-2 peptide has the following sequence: SARAGETRFTDTRKDE. The MH-1 peptide has the following sequence: RKKFGESEKTKTKEFL.

Activity Assay of eEF-2 Kinase Mutants with eEF-2 as a Substrate. Recombinant eEF-2 kinase was incubated with purified rabbit reticulocyte eEF-2 (1.0 μg) in a buffer consisting of 50 mM Hepes-KOH (pH 6.6), 10 mM magnesium acetate, 5 mM DTT, 100 μM $CaCl_2$, 0.5 μg of calmodulin, 60 μM ATP, and 1.0 μCi of [γ-^{32}P]ATP (specific activity of 6000 Ci/mmol). The total volume of the reaction was 50 μL. The reaction was carried out at 30 °C for 10 min, and was terminated by incubation in an ice/water bath and addition of Laemmli sample buffer. The reaction mixture was heated for 3 min. Samples were analyzed by 8% SDS−PAGE and autoradiography. To assay for autophosphoryla-

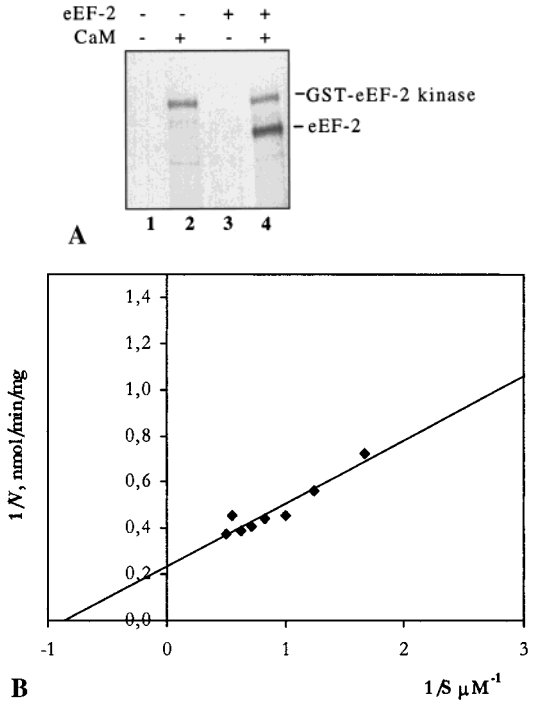

FIGURE 1: Enzymatic activity of human GST-tagged eEF-2 kinase expressed in *E. coli*. (A) Autoradiograph of eEF-2 kinase assays with recombinant GST-tagged eEF-2 kinase in crude bacterial lysates (10 μg of total protein). Kinase assays were carried out as described in Materials and Methods. Reaction mixtures were incubated for 10 min and analyzed by 8% SDS−PAGE. Reactions were carried out with and without eEF-2 (0.5 μg) and with and without calmodulin (0.5 μg). (B) Determination of the kinetic parameters of eEF-2 phosphorylation by purified GST−eEF-2 kinase. Experiments were performed as described in Materials and Methods.

tion activity, kinase assays were carried out as described above except that eEF-2 was omitted from the reaction mixture.

Activity Assay of eEF-2 Kinase Mutants with a Peptide Substrate. Recombinant eEF-2 kinase was incubated with 100 μM peptide substrate in a buffer consisting of 50 mM Hepes-KOH (pH 6.6), 10 mM magnesium acetate, 5 mM DTT, 100 μM $CaCl_2$, 0.5 μg of calmodulin, 100 μM ATP, and 1.0 μCi of [γ-^{32}P]ATP. The total volume of the reaction was 50 μL. The reaction was carried out at 30 °C for 10 min, and terminated by incubation in an ice/water bath. An aliquot of each reaction mixture was spotted onto a 2 cm × 2 cm square of phosphocellulose paper, and washed 5 × 4 min in 75 mM phosphoric acid. After a 30 s rinse in 95% ethanol, the filter papers were dried and counted by Cerenkov counting.

Determination of Kinetic Parameters of eEF-2 Kinase. Recombinant eEF-2 kinase was incubated with various amounts of peptide or purified eEF-2. The reaction mixture containing eEF-2 is the same as described above for the activity assay with eEF-2 as a substrate, except for addition of 500 μM ATP, 25 μCi of [γ-^{32}P]ATP, and 0.3 μg of eEF-2 kinase. The reaction volume was 20 μL. Reactions were carried out for 10 min at 30 °C, and terminated by incubation in an ice/water bath and addition of Laemmli sample buffer. Samples were analyzed by 8% SDS−PAGE and autoradiography. Bands corresponding to radiolabeled eEF-2 were excised from the gel, and the amount of labeling was quantitated by Cerenkov counting.

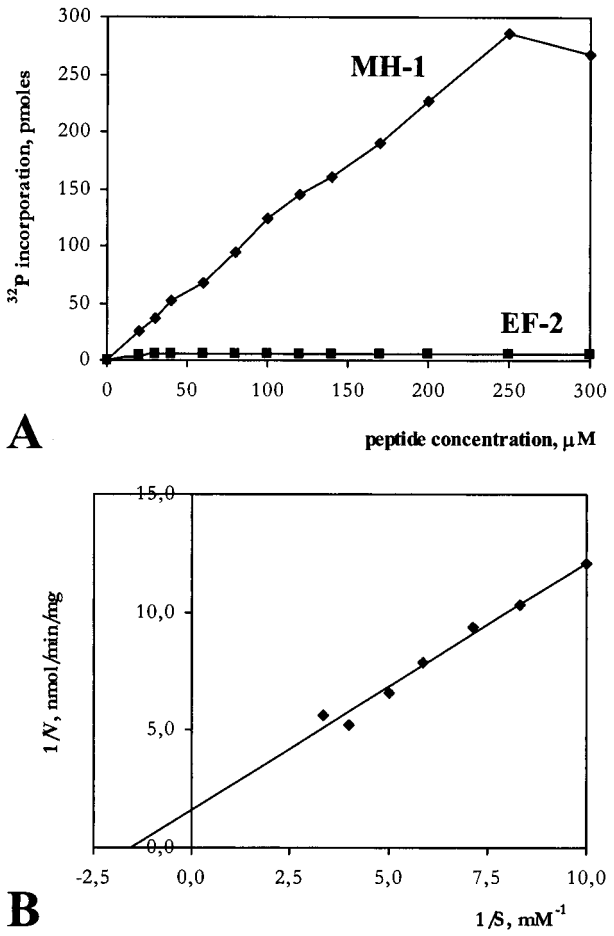

FIGURE 2: Phosphorylation of synthetic peptides by eEF-2 kinase. (A) Phosphorylation of peptides MH-1 and EF-2 by purified GST−eEF-2 kinase. Reactions were performed as described in Materials and Methods. Diamonds represent phosphorylation of MH-1, and squares represent phosphorylation of EF-2. (B) Determination of the kinetic parameters for MH-1 phosphorylation by GST−eEF-2 kinase.

The reaction mixture containing MH-1 as a substrate is also the same as described above for the activity assay with a peptide substrate except for addition of 500 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 0.3 μg of eEF-2 kinase. The assay was also carried out using the same method described above.

Cloning of the C-Terminus of eEF-2 Kinase. The C-terminus of eEF-2 kinase was cloned by PCR using wild-type human eEF-2 kinase as the template. The sequences of the primers that were used were as follows: forward, 5′-GTGAATCAGAACACCAAGCTGCTGCAATCA-3′; and reverse, 5′-TTATTCCTCCATCTGGGCCCAGGCCTCTTC-3′. The PCR conditions were as follows: 30 s at 94 °C, 30 s at 58 °C, and 3 min at 72 °C for 30 cycles, followed by a 10 min final extension at 72 °C. The PCR product was cloned into the PCRII-TOPO vector (Invitrogen) per the manufacturer's instructions. The insert was then subcloned into the *Eco*RI site in pGEX-2T (Pharmacia). Expression of the recombinant protein was carried out as described above.

eEF-2/GST−eEF-2 Kinase Coprecipitation Assay. A reaction mixture containing 5 μg of GST-tagged eEF-2 kinase, 2 μg of eEF-2, 20 μL of glutathione-Sepharose, 50 mM Hepes-KOH (pH 6.6), 10 mM magnesium acetate, and 5 mM DTT was incubated for 2 h at 4 °C. The Sepharose beads were collected by centrifugation and washed five times with 1 mL of wash buffer containing 50 mM Hepes-KOH

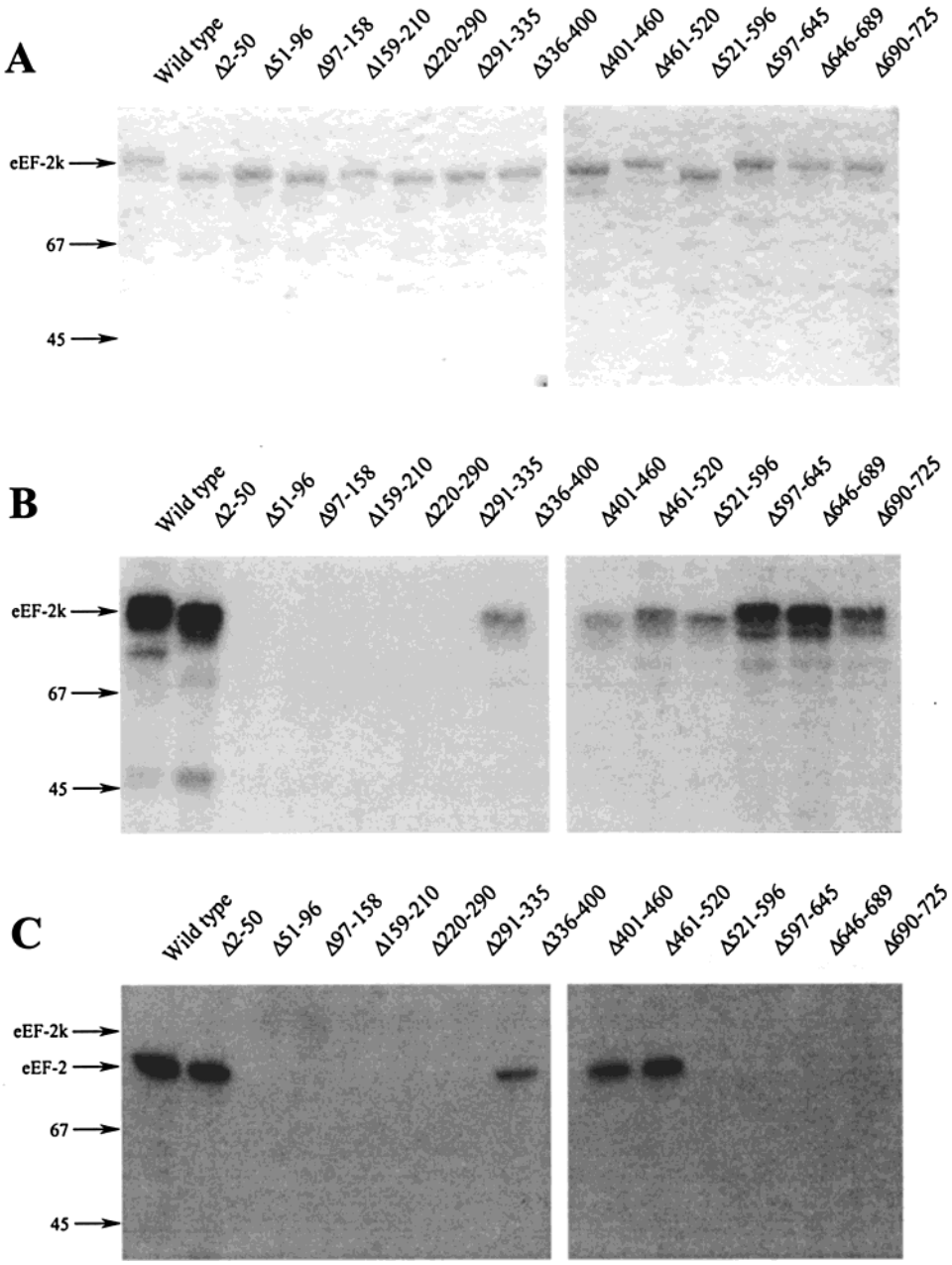

FIGURE 3: Enzymatic activity of eEF-2 kinase deletion mutants. (A) Coomassie Blue-stained gel of purified GST−eEF-2 kinase mutants after 8% SDS−PAGE. Labels above the lanes designate the amino acids deleted in the mutant being assayed. (B) Autoradiograph of autophosphorylated GST−eEF-2 kinase. The assay was performed as described in Materials and Methods. The incubation time of all reactions was 10 min. The Coomassie Blue-stained gel corresponding to this autoradiograph is shown in panel A. (C) Autoradiograph of the eEF-2 kinase assay using purified GST−eEF-2 kinase deletion mutants. The eEF-2 kinase assay was performed as described for panel B, except 12.5-fold less eEF-2 kinase and 1.0 μg of purified eEF-2 were used.

(pH 6.6), 10 mM magnesium acetate, 5 mM DTT, and 0.005% Triton X-100. Then 1 μCi of [*adenylate*-^{32}P]NAD (NEN) and diphtheria toxin (Calbiochem) in 10 μL of wash buffer were added to the beads, and the mixture was incubated for 15 min at 30 °C. The reaction was terminated by addition of Laemmli buffer. Samples were analyzed by 10% SDS−PAGE and autoradiography.

Calmodulin Binding Assay. Calmodulin binding activity was determined by the ability of the recombinant eEF-2 kinase deletion mutants to bind to calmodulin−agarose, which was kindly provided by D. Wolff (University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School). Calmodulin−agarose was prepared by coupling bovine brain calmodulin to Affi-gel 15 per the manufacturer's protocol. One milliliter of calmodulin−agarose was put into a HR5/5 FPLC column, and then the column was equilibrated with 50 mM MOPS (pH 7.4), 150 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM EGTA. Approximately 100 μg of mutant recombinant eEF-2 kinase was applied to the column in the same buffer. eEF-2 kinase was eluted with 50 mM MOPS (pH 7.4), 150 mM NaCl, and 5 mM EGTA. The eluate was analyzed by SDS−PAGE and Coomassie Blue staining.

RESULTS

Expression of Recombinant Human eEF-2 Kinase in E. coli. Human eEF-2 kinase was expressed with an N-terminal GST tag. We observed that recombinant eEF-2 kinase

represented the major protein band after SDS−PAGE analysis of crude bacterial lysates, so initially we investigated the enzymatic activity of eEF-2 kinase using crude lysates (Figure 1A). As can be seen in Figure 1A, GST−eEF-2 kinase undergoes autophosphorylation upon incubation with [γ-^{33}P]ATP and can phosphorylate eEF-2. Both the ability of eEF-2 kinase to autophosphorylate and phosphorylate eEF-2 were strictly calmodulin-dependent (lanes 1 and 3 vs lanes 2 and 4). All subsequent experiments were performed with purified recombinant GST−eEF-2 kinase.

GST−eEF-2 kinase was purified as described in Materials and Methods, and kinetic parameters of eEF-2 phosphorylation by GST−eEF-2 kinase were measured. As can be seen in Figure 1B, GST−eEF-2 kinase phosphorylates eEF-2 with a K_m of 1.2 μM and a V_{max} of 4 nmol min^{-1} mg^{-1}.

Identification of a Peptide Substrate for eEF-2 Kinase. Attempts to use synthetic peptide substrates corresponding to the eEF-2 kinase phosphorylation site in eEF-2 have been unsuccessful. As shown in Figure 2A, eEF-2 kinase was unable to phosphorylate a 16mer peptide (EF-2) corresponding to the phosphorylation site in mammalian eEF-2 (amino acids SARAGETRFTDTRKDE). However, two protein kinases that are structurally related to eEF-2 kinase, MHCK A and B, were shown to be able to phosphorylate a peptide [MH-1 (RKKFGESEKTKTKEFL)] corresponding to their phosphorylation site in *Dictyostelium* myosin heavy chains (*16*, *19*). Unexpectedly, we found that eEF-2 kinase could also phosphorylate MH-1 (Figure 2A) with a K_m of 660 μM and a V_{max} of 1.5 nmol min^{-1} mg^{-1} (Figure 2B).

Expression of eEF-2 Kinase Deletion Mutants and Analysis of Their Activity. Initial attempts to obtain truncation mutants revealed that deletion of even 20 amino acids from the C-terminus of eEF-2 kinase prevented it from phosphorylating eEF-2. Thus, we decided to use in vitro mutagenesis to create deletion mutants. We generated 13 mutants with deletions ranging from 36 to 76 amino acids that systematically span the entire eEF-2 kinase molecule. All 13 mutants were expressed as GST-tagged proteins and either purified from inclusion bodies or affinity-purified using glutathione-Sepharose.

The 13 deletion mutants were assayed for the ability to undergo autophosphorylation and to phosphorylate eEF-2 (Figure 3B,C). Mutants with deletions between amino acids 51 and 335 were not able to phosphorylate eEF-2 or to undergo autophosphorylation (Figure 3B,C). On the other hand, deletions between amino acids 521 and 725 caused a loss of eEF-2 kinase activity, but not a loss of autophosphorylation activity. This suggests that the catalytic domain is located between amino acids 51 and 335 while the region between amino acids 521 and 725 is important for eEF-2 recognition. The region between amino acids 336 and 520 probably serves as a linker between the two domains. It is unnecessary for catalytic activity since any deletions made in this region did not inactivate eEF-2 kinase. Although we used similar amounts of the various eEF-2 kinase mutants in our assays (Figure 3A), there was a considerable quantitative difference in autophosphorylation activity between the mutants, which cannot be ascribed to the difference in the amount of protein. The lower-than-expected activity of some mutants may be due to the fact that some of the autophosphorylation sites are deleted in these mutants. It is also possible that the fraction of correctly folded protein varies in the different mutant samples.

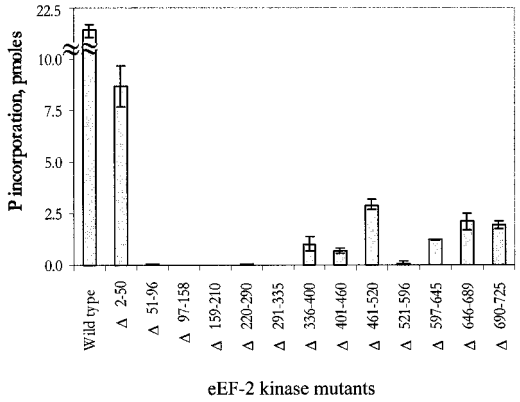

FIGURE 4: Phosphorylation of MH-1 by eEF-2 kinase deletion mutants. The histogram depicts the ability of wild-type eEF-2 kinase and the eEF-2 kinase deletion mutants to phosphorylate a peptide substrate. The assays were carried out as described in Materials and Methods using the MH-1 peptide as a substrate. The incubation time of all reactions was 10 min. The histogram depicts the picomoles of phosphate incorporated into the peptide by the various mutants. The amount of eEF-2 kinase used in the reactions was the same as in Figure 3B.

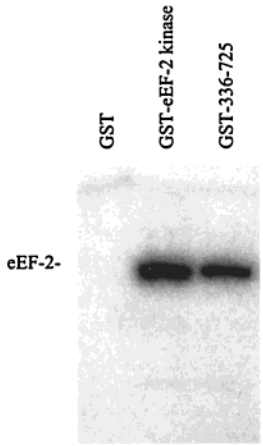

FIGURE 5: Interaction between eEF-2 and the C-terminus of eEF-2 kinase. The GST coprecipitation experiment was performed as described in Materials and Methods. [^{32}P]ADP-ribosylated eEF-2 was detected by autoradiography. GST alone was used as a negative control.

We next analyzed the ability of the deletion mutants to phosphorylate a synthetic peptide substrate, MH-1. As is shown in Figure 4, only five mutants were unable to phosphorylate MH-1, and the deletions in these mutants were within amino acids 51−335, all of which are within the putative catalytic domain. Mutants with deletions in the C-terminal domain were able to phosphorylate MH-1, thus confirming that this domain is not involved in the catalysis of phosphotransfer, but probably serves as an eEF-2 targeting domain. The mutant with a deletion between amino acids 521 and 596 had low peptide phosphorylating activity. The reason for this low activity is unclear.

We also analyzed the ability of the C-terminal region of eEF-2 kinase to bind eEF-2 using a coprecipitation assay. We expressed a GST-tagged construct containing amino acids 336−725 of eEF-2 kinase. As can be see in Figure 5, eEF-2 coprecipitates with the recombinantly expressed C-terminus of eEF-2 kinase. This result demonstrates that the C-terminal region of eEF-2 kinase can directly interact with eEF-2.

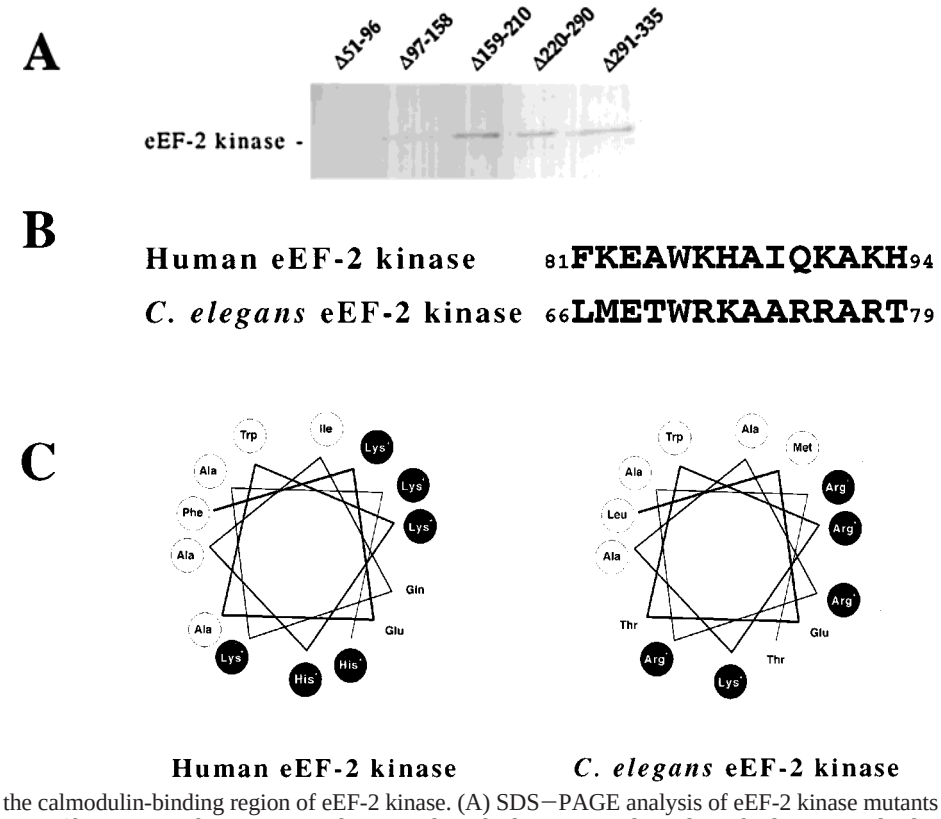

FIGURE 6: Mapping the calmodulin-binding region of eEF-2 kinase. (A) SDS−PAGE analysis of eEF-2 kinase mutants after chromatography on calmodulin−agarose. Chromatography was carried out as described in Materials and Methods. Material adsorbed to calmodulin−agarose and eluted with buffer containing EGTA was analyzed by 8% SDS−PAGE. Bands were visualized by Coomassie Blue staining. (B) Sequence of the putative calmodulin-binding region from human and *C. elegans* eEF-2 kinase. (C) Helical wheel representation of the putative calmodulin-binding region sequence of human and *C. elegans* eEF-2 kinase. Filled circles represent positively charged amino acids, and open circles represent hydrophobic amino acids.

Location of the Calmodulin-Binding Region. In all mutants that are able to undergo autophosphorylation, this phosphorylation was strictly calcium/calmodulin-dependent (data not shown), suggesting that these mutants can interact with calmodulin, and therefore, the calmodulin-binding region is located somewhere within amino acids 51−335. To locate the calmodulin-binding region more precisely, we analyzed the five mutants with deletions between amino acids 51 and 335. As shown in Figure 6A, the only eEF-2 kinase mutant that is unable to bind to calmodulin−agarose contained a deletion between amino acids 51 and 96. The actual calmodulin-binding site is probably located within amino acids 81−94 (Figure 6B). According to secondary structure predictions carried out using the ALB-Globule program (*21*), this stretch of amino acids is predicted to form an amphipathic α-helix (Figure 6C). The homologous sequence from *Caenorhabditis elegans* eEF-2 kinase is also predicted to form an amphipathic α-helix (see Figure 6B,C).

DISCUSSION

eEF-2 kinase belongs to an emerging new class of protein kinases that are structurally and evolutionarily unrelated to the eukaryotic serine/threonine/tyrosine protein kinases. In addition to eEF-2 kinase, this new class includes *Dictyostelium* MHCK A, B, and C and several mammalian putative protein kinases (*18*). All conventional eukaryotic protein kinases share a similar catalytic domain structure, which can be divided into 12 conserved subdomains. They also possess highly conserved motifs, such as the DXXXN and DFG motifs, which are essential for the catalysis of phosphotransfer. In contrast, eEF-2 kinase has none of these motifs.

Mutational analysis revealed that eEF-2 kinase is likely to be composed of two domains connected by a linker region (Figure 7). We previously predicted that the catalytic domain is located between amino acids 75 and 335 because this region is homologous among eEF-2 kinases cloned from different species (*2*), and it was experimentally demonstrated in the case of MHCK A that the homologous region corresponds to the catalytic domain (*15*). Consistent with this idea, we found that any deletion in this region results in the complete inactivation of eEF-2 kinase.

Redpath et al. (*14*) cloned rat skeletal muscle eEF-2 kinase, which is approximately 90% identical to human eEF-2 kinase, and they proposed that the catalytic domain is located between amino acids 288 and 554 because this region is slightly similar to the catalytic domain of cAMP-dependent protein kinase. In a previous paper (*2*), we showed that this region is the least homologous among eEF-2 kinases from various species, and thus is unlikely to represent the catalytic domain. In this work, we show that any deletion within this region does not affect eEF-2 kinase activity, demonstrating that this region does not contain the catalytic domain.

What is the function of the C-terminal domain of eEF-2 kinase? Since eEF-2 kinase with deletions in this region retains the ability to undergo autophosphorylation and to phosphorylate the MH-1 peptide, this portion of the molecule is not part of the catalytic domain. However, any deletion in this region results in the complete inhibition of eEF-2 phosphorylation. In addition, the C-terminal region expressed alone binds eEF-2 in a coprecipitation assay. These results suggest this domain may be involved in eEF-2 recognition.

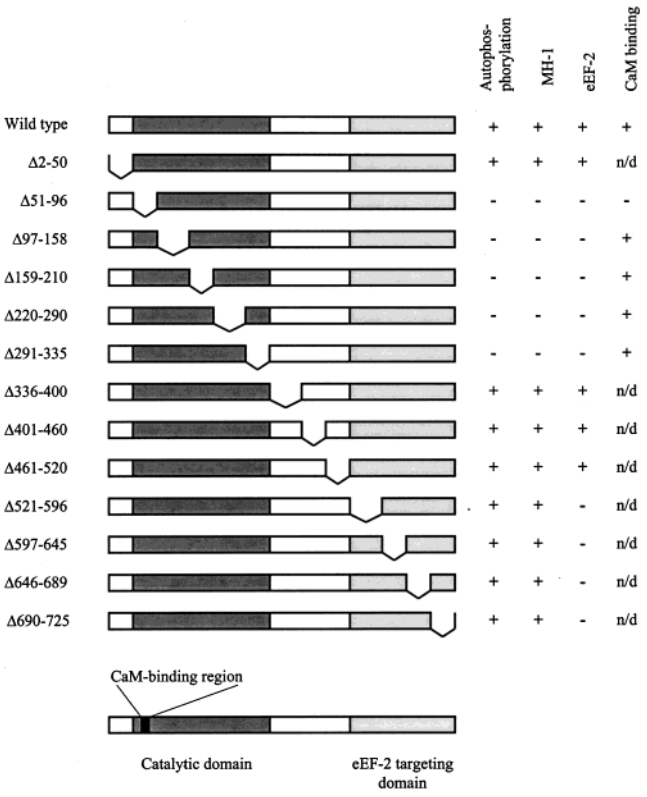

FIGURE 7: Schematic representation of wild-type and mutant eEF-2 kinases. Dark gray areas represent the catalytic domain. Light gray areas represent the eEF-2 targeting domain. Numbers at the top of the schematic represent the amino acids at the boundaries of each of these regions. eEF-2 kinase activity and autophosphorylation activity of each mutant are summarized on the right with + representing the presence of activity and − representing the absence of activity. A schematic representation of the major functional domains of eEF-2 kinase as determined in this work is shown at the bottom.

This situation would be analogous to that of MHCK A and B, in which it was shown that upon deletion of the C-terminal WD repeat domain, these kinases almost completely lost the ability to phosphorylate myosin heavy chains, but were still able to phosphorylate a synthetic peptide (*16*, *17*). This suggests that the C-terminal WD repeat-containing region of MHCK A and B is important for targeting these kinases to myosin.

We noticed the presence of a novel motif in the C-terminal domain of eEF-2 kinase. Database searches for proteins homologous to the C-terminal domain of human eEF-2 kinase revealed a similarity to several eukaryotic and prokaryotic proteins. These include the hypothetical *E. coli* protein YBEQ (GenBank entry P77234; see Figure 8A), the murine protein SEL 1L (*22*; GenBank entry AAD05210), and the hypothetical *Helicobacter pylori* 29 kDa protein (GenBank entry AAB47276). All these proteins contain several repeats of a novel 36-amino acid sequence ($^{D}/_{N}$YXXAFFW$^{Y}/_{L}$KKAAEQGHXXAQNNL$^{G}/_{A}$W-MYXXGEGVXK), which we named "motif 36" (Figure 8B). Elements of motif 36 are clearly present in the C-terminal portion of eEF-2 kinase (Figure 8B). Database analysis revealed that there are a number of other prokaryotic and eukaryotic proteins that contain 2−10 repeats of motif 36, and to date, none of these proteins have a well-defined function. In only two instances, proteins containing this motif have been studied; one of these is the *sel-1* gene product from *C. elegans*, which is a negative regulator of LIN-12 and GLP-1 (*23*), and which is the nematode homologue of murine SEL1L (*22*). LIN-12 and GLP-1 are transmembrane receptors that are involved in specifying cell fate decisions during development. Although the exact mechanism of SEL-1 action is still unclear, it was suggested that it might bind LIN-12 and GLP-1, and thereby target them for degradation (*23*, *24*). The other protein that has been studied is the *Saccharomyces cerevisiae HRD3* gene product that is involved in HMG-CoA reductase turnover (*25*). It has been suggested that the function of Hrd3 is to bind HMG-CoA reductase and target it for degradation (*25*). Thus, the C-terminal portion of eEF-2 kinase contains a novel and widely occurring motif, which may be involved in mediating protein−protein interactions.

We have also tentatively mapped the calmodulin-binding domain to the region between amino acids 51 and 96. The actual binding site for calmodulin is probably within amino acids 81−94, which are homologous to the amino acids in the corresponding region of *C. elegans* eEF-2 kinase (Figure 6). Amino acids 81−94 in human eEF-2 kinase and the corresponding region in *C. elegans* eEF-2 kinase are strongly predicted to form a basic amphipathic α-helix, which is a characteristic feature of calmodulin-binding domains (reviewed in refs *26* and *27*). Rhoads and Friedberg analyzed the sequences of various calmodulin-binding proteins, and identified consensus calmodulin-binding motifs (*28*). The sequence of human eEF-2 kinase from amino acids 81−94 (FKEAWKHAIEKAKH) resembles a type A, 1−8−14 motif, which is present in many calmodulin-dependent enzymes (*28*). Usually, deletion of the calmodulin-binding regions in calmodulin-dependent enzymes does not result in a complete inhibition of enzymatic activity. However, removal of amino acids 51−96 from eEF-2 kinase resulted in inactivation of eEF-2 kinase. This may be due to the fact that certain residues that are important for catalysis are also located within this region, or perhaps removal of this relatively large portion of the molecule precludes the correct folding of the catalytic domain. While we were writing this paper, a paper by Diggle et al. (*29*) was published, which provided an analysis of the location of the calmodulin-binding site in rat eEF-2 kinase. Their results are consistent with ours. They suggest that the calmodulin-binding site is located between amino acids 77 and 99 of rat eEF-2 kinase, which correspond to amino acids 78−100 of human eEF-2 kinase. In addition, they found that substitution of tryptophan 84 (corresponding to tryptophan 85 in human eEF-2 kinase) with an alanine residue completely abolished calmodulin binding. Overall, their mutational analysis of rat eEF-2 kinase is consistent with our results.

We have found that eEF-2 kinase can phosphorylate a peptide corresponding to the MHCK A phosphorylation site in *Dictyostelium* myosin II heavy chains. This result was unexpected for several reasons. As we found earlier, eEF-2 kinase cannot phosphorylate intact *Dictyostelium* myosin II heavy chains (*2*). In addition, eEF-2 kinase cannot phosphorylate a peptide corresponding to its phosphorylation site in eEF-2. However, this surprising finding can be explained if eEF-2 kinase recognizes the secondary structure rather than the primary structure of its substrate. We previously suggested that eEF-2 kinase and the related protein kinases possess an unusual catalytic domain because they are adapted

A

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Human eEF-2 kinase:  588 KETEENKTKGFDYLLKAAEAGDRQSMILVARAFDSGQNLSPDRCQDWLEALHWY 641
        consensus        K   +NKT    + LK+A+ G+R +   +A  +++G+ +     QD+ +A++WY
      E. coli YBEQ:  184 KGVAQNKTLAAFWYLKSAQQGNRHAQFQIAWDYNAGEGVD----QDYKQAMYWY 233


Human eEF-2 kinase:  679 GYGLEKDPQRSGDLYTQAAEAAMEAMKGRLANQYY 713
        consensus        G G+EKD Q + + +T+AAE          LA  Y+
      E. coli YBEQ:  255 GQGVEKDYQAAFEWFTKAAECNDATAWYNLAIMYH 289
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B

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E. coli YBEQ:    1 MIMIFTSSCCDNLSIDEIIER-AEKGDCEAQYIVGFYYNRDSAIDSP
                   DDEKAFYWLKLAAEQGHCEAQYSLGQKYTEDKSRHK
                   DNEQAIFWLKKAALQGHTFASNALGWTLDRGEAP--
                   NYKEAVVWYQIAAESGMSYAQNNLGWMYRNGNGVAK
                   DYALAFFWYKQAALQGHSDAQNNLADLYEDGKGVAQ
                   NKTLAAFWYLKSAQQGNRHAQFQIAWDYNAGEGVDQ
                   DYKQAMYWYLKAAAQGSVGAYVNIGYMYKHGQGVEK
                   DYQAAFEWFTKAAECNDATAWYNLAIMYHYGEGRPV
                   DLRQALDLYRKVQSSGTRDVSQEIRETEDLL 327

Consensus:         DYXQAFFWYKKAAEQGHXXAQNNLGWMYXXGEGVXK
                   N       L            Y IA

Human eEF-2 kinase:  547 DQESAVFHLEHAANLGELEAIVGLGLMYSQLPHHILADVSLKETEE
                   NKTKGFDYLLKAAEAGDRQSMILVARAFDSGQNLSPDRCQ
                   DWLEALHWYNTALEMTD----CDEG-----GEYDGMQ
                   DEPR-YMMLAREAEM------LFTG-----GYGLEK
                   DPQRSGDLYTQAAEAAMEAMKGRLANQYYQKAEEAWAQMEE 725
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FIGURE 8: Motif 36. (A) Sequence alignment of portions of the C-terminal domain of eEF-2 kinase and portions of the *E. coli* hypothetical protein YBEQ. Identical amino acids are shown, and conserved amino acids are indicated with a +. (B) Amino acid sequence of the *E. coli* protein YBEQ demonstrating that this protein contains multiple repeats of a 36-amino acid sequence motif. Elements of motif 36 are also present in the C-terminal domain of eEF-2 kinase. Invariant amino acids of motif 36 are shown in bold.

to recognize an α-helical conformation in their substrates (*18*). Therefore, we named this class of protein kinases the α-kinases (*18*). It is possible that eEF-2 kinase can phosphorylate MH-1 because it is derived from an α-helical coiled-coil region of myosin heavy chains (*30*), and thus is likely to retain some α-helicity. On the other hand, the peptide corresponding to the eEF-2 kinase phosphorylation site in eEF-2 probably does not have any structure, and can form an α-helix only within the three-dimensional context of eEF-2. It is also possible, however, that for eEF-2 phosphorylation to occur, eEF-2 kinase must interact with a region of eEF-2 distinct from the phosphorylation site. This may explain why the eEF-2 peptide cannot serve as a substrate for eEF-2 kinase. The MH-1 peptide may be sufficiently different from the eEF-2 peptide, and may only need to bind at the active site to undergo phosphorylation.

In conclusion, the results presented here suggest that eEF-2 kinase consists of two domains. The catalytic domain is located in the N-terminal portion of the molecule, while the eEF-2 targeting domain is located in the C-terminal region. The two domains are connected by a linker region. The structure of the catalytic domain of eEF-2 kinase is clearly different from that of the conventional eukaryotic protein kinases. There is, however, another superfamily of protein kinases represented by the histidine protein kinases of bacterial two-component systems (*31*). Members of this superfamily have also been identified in eukaryotes (*32*). Interestingly, protein kinases have been found that contain a histidine kinase-like catalytic domain, but phosphorylate their substrates on serine residues (*5*, *6*). Recently, the three-dimensional structure of the catalytic domain of two different bacterial histidine kinases, EnvZ and CheA, was determined (*33*, *34*). The histidine kinase catalytic domain appears to be totally different in structure when compared to the catalytic domain of the conventional eukaryotic protein kinases, and utilizes a fold similar to that of DNA gyrase B and Hsp90. Is it possible that eEF-2 kinase also utilizes this fold? We mentioned, in our previous publication, that there is a certain similarity between the structure of the α-kinases and the structure of the histidine kinase catalytic domain (*18*). In the catalytic domain of both types of protein kinase, the glycine rich region, which presumably binds the phosphates of ATP, is located near the C-terminus of the catalytic domain. We also recently analyzed the predicted secondary structure of the eEF-2 kinase catalytic domain using the ALB-Globule program (*21*), and noticed that the distribution of predicted secondary structure elements in eEF-2 kinase is strikingly similar to the distribution of secondary structure elements in EnvZ as determined by NMR (*33*). Moreover, it was recently demonstrated that the histidine residue phosphorylated by histidine kinases is located within an α-helix, indicating that the catalytic domain of histidine kinases is adapted to recognize α-helices (*35*, *36*). These results suggest the interesting possibility that eEF-2 kinase, as well as the α-kinases in general, is structurally related to the bacterial histidine kinases and that they have a common

evolutionary origin. Further structural analysis of eEF-2 kinase will be necessary to test this hypothesis.

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