

# Analysis of the domain structure of elongation factor-2 kinase by mutagenesis

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**Abstract** A number of elongation factor-2 kinase (eEF-2K) mutants were constructed to investigate features of this kinase that may be important in its activity. Typical protein kinases possess a highly conserved lysine residue in subdomain II which follows the GXGXXG motif of subdomain I. Mutation of two lysine residues, K340 and K346, which follow the GXGXXG motif in eEF-2K had no effect on activity, showing that such a lysine residue is not important in eEF-2K activity. Mutation of a conserved pair of cysteine residues C-terminal to the GXGXXG sequence, however, completely inactivated eEF-2K. The eEF-2K CaM binding domain was localised to residues 77–99 which reside N-terminal to the catalytic domain. Tryptophan 84 is an important residue within this domain as mutation of this residue completely abolishes CaM binding and eEF-2K activity. Removal of approximately 130 residues from the C-terminus of eEF-2K completely abolished autokinase activity; however, removal of only 19 residues inhibited eEF-2 kinase activity but not autokinase activity, suggesting that a short region at the C-terminal end may be important in interacting with eEF-2. Likewise, removal of between 75 and 100 residues from the N-terminal end completely abolished eEF-2K activity.

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**Key words:** Translation; Elongation factor-2; Eukaryotic elongation factor-2 kinase; Phosphorylation; Calcium/calmodulin-dependent kinase

## 1. Introduction

Calcium regulates a broad range of cellular processes including muscle contraction, glycogen metabolism, neurotransmitter release and cell proliferation [1]. Many of the effects of calcium are mediated by protein phosphorylation via activation of members of a family of  $\text{Ca}^{2+}$ /calmodulin-dependent serine/threonine protein kinases (CaMKs). These kinases include myosin light chain kinase, phosphorylase kinase, CaM kinases I, II and IV and a  $\text{Ca}^{2+}$ /CaM-dependent kinase which phosphorylates and activates CaM kinases I and IV [2–4]. The other CaM kinase is elongation factor-2 kinase (eEF-2K) which phosphorylates and inactivates eEF-2 [5–9]. eEF-2 mediates peptidyl-tRNA translocation from the A- to the P-site

in eukaryotic ribosomes and phosphorylation and inhibition of eEF-2 leads to the inhibition of mRNA translation [10].

A number of CaMKs share many structural and regulatory features, possessing a highly conserved catalytic domain followed by a calmodulin binding domain. They are also thought to be autoinhibited by binding of pseudosubstrate sequences to the active site in the absence of  $\text{Ca}^{2+}$ /CaM [2,11–13]. Recently the determination of the crystal structure of CaMK I [14] and mutation studies [15] have revealed the involvement of a number of specific residues in the autoinhibition of this kinase.

Although eEF-2K is  $\text{Ca}^{2+}$ /CaM-dependent, it is unrelated to the other CaMKs in terms of amino acid sequence and appears to represent a distinct family of protein kinases [16–18]. The only other characterised kinase that displays any homology to eEF-2K is *Dictyostelium* myosin heavy chain kinase (MHCK) which is not a  $\text{Ca}^{2+}$ /CaM-dependent kinase. The catalytic domain of MHCK possesses about 40% homology with a region in rat eEF-2K encompassing residues approximately 130–320, which presumably represents the eEF-2K catalytic domain [19,20].

In this paper we have used mutagenesis to investigate the structural organisation of eEF-2K and to define residues which may be important in the catalytic activity of eEF-2K.

## 2. Materials and methods

### 2.1. Materials

Biotinylated CaM was prepared in the presence of 1 mM calcium as described [21] using 1.2 mg of bovine brain CaM (Sigma). eEF-2 was purified from rabbit reticulocytes as previously described [22]. *pfu* polymerase was from Stratagene. Eugene 6 was purchased from Boehringer. Anti-myc (9E10) antibody was from Sigma. An antibody against the N-terminal region of rat eEF-2K was raised in rabbits by immunisation with GST-eEF-2K 1–99.

### 2.2. Binding of eEF-2K to biotinylated CaM

eEF-2K in Dulbecco's PBS (D-PBS) was dot-blotted onto nitrocellulose. The membrane was incubated with 12.5  $\mu\text{g}/\text{ml}$  of biotinylated CaM for 1 h. Binding of biotinylated CaM was detected by subsequent incubation with Extravidin-peroxidase (Sigma) and visualisation by ECL. All incubations and washes were done in D-PBS to ensure CaM remained bound. 1 mM calcium was also added to the ECL reagents. When required, 5 mM EGTA was added to D-PBS to chelate free calcium.

### 2.3. Expression of GST-eEF-2K

PCR was used to amplify cDNA encoding the first 99 residues of rat muscle eEF-2K [16] and to introduce a *Bam*HI site before the initiation codon using the sense primer 5'-TAGCTGGATCCC-CATGGCAGACGAAGACC-3' and an antisense primer with an *Eco*RI site, 3'-CCTCCAGATGGAATTCAGCCC-5'. The cDNA fragment was ligated into *Bam*HI/*Eco*RI-digested pGEX-5X-1 followed by ligation of an *Eco*RI fragment encoding the remainder of eEF-2K to generate pGEX-eEF-2K encoding full-length eEF-2K (1–724). GST-eEF-2K was expressed in BL21(DE3) cells by induction

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**Abbreviations:** CaM, calmodulin; CaMK,  $\text{Ca}^{2+}$ /CaM-dependent kinase; CHO.T, Chinese hamster ovary cells expressing human insulin receptor; eEF-2, eukaryotic elongation factor-2; eEF-2K, eEF-2 kinase; HEK 293, human embryonic kidney 293 cells; MHCK, myosin heavy chain kinase

with 0.2 mM IPTG for 4–5 h at ambient temperature. After purification using glutathione-agarose, about 1 mg of GST-eEF-2K was obtained per litre of bacterial culture. The full-length GST-eEF-2K had an apparent molecular weight of approximately 130 kDa as judged by SDS-PAGE.

Site-directed mutagenesis was carried out using a two-PCR method [23]. The second PCR was used to amplify the region of eEF-2K between the unique *Bsa*BI and *Kpn*I restriction sites within eEF-2K. The cDNAs were sequenced to verify mutagenesis using the T7 Sequenase kit (Amersham).

#### 2.4. Transient transfections and eEF-2K assay

eEF-2K with an N-terminal myc-epitope tag (EQKLISEEDL) inserted after the N-terminal methionine was constructed using PCR and was inserted into pcDNA.3.1 (Invitrogen). HEK 293 or CHO.T cells were transiently transfected with 3 µg of DNA using Fugene 6 in 6-cm plates. Transfected cells were left for 2 days before serum starvation overnight and then lysed in buffer as described [24]. Myc-eEF-2K from 5 µg of cell protein was immunoprecipitated with anti-myc antibody (2 µg coupled to protein G Sepharose) before measurement of eEF-2K activity as described [24]. Activity of GST-eEF-2K was assayed by incubation of 0.1 µg of the kinase with 1 µg of eEF-2 for 5 min before SDS-PAGE and autoradiography.

### 3. Results and discussion

Conventional protein kinases possess 11 catalytic subdomains that contain a number of highly conserved residues [25]. In subdomain I, which is at the N-terminal end of the catalytic domain, a GXGXXG motif is present which is followed by a conserved lysine residue in subdomain II. The original cloning of cDNA encoding rat eEF-2K [16] revealed that it had very little homology to conventional protein kinases. However, since eEF-2K also possessed a GXGXXG sequence, it was thought that the region containing this motif constituted the N-terminal end of the eEF-2K catalytic domain. It was therefore postulated that the first lysine residue C-terminal to the GXGXXG sequence (K340) was the invariant lysine in subdomain II. Mutation of this invariant lysine

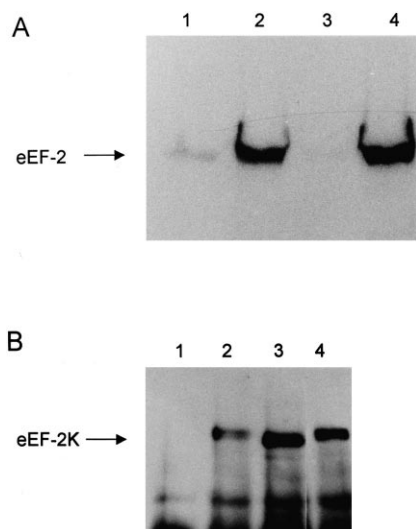


Fig. 1. Activity of eEF-2K mutants. A: HEK 293 cells were transfected with empty vector (lane 1) or myc-eEF-2K WT (lane 2), C313/317A (lane 3) or K340R (lane 4). After immunoprecipitation with anti-myc antibody, kinase activity was measured as described in Section 2. B: Myc-eEF-2K from 5 µg of cell extract was immunoprecipitated with anti-myc followed by SDS-PAGE, Western blotting and immunodetection with eEF-2K antibody. Samples applied to the lanes were as given in A.

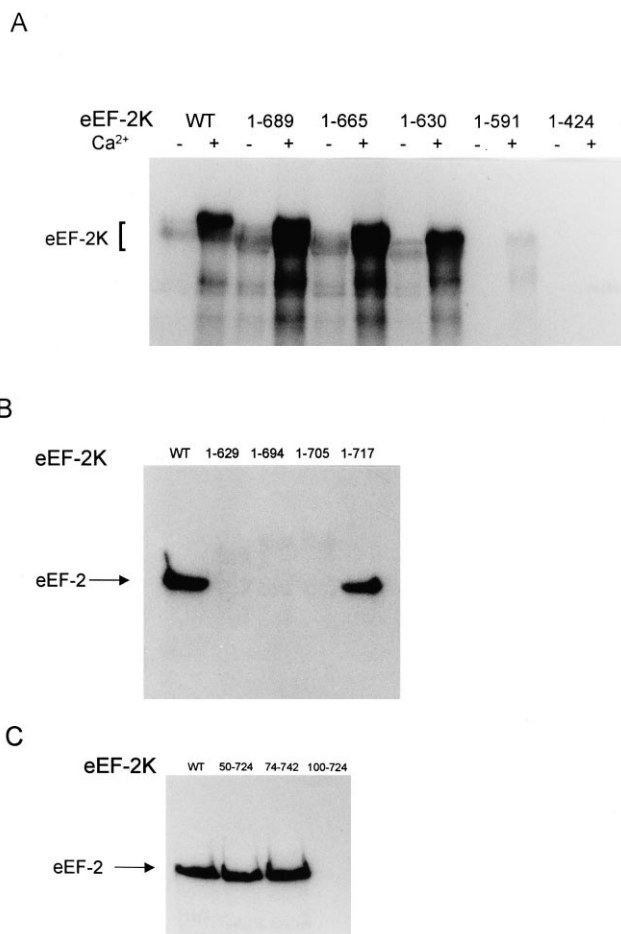


Fig. 2. Autophosphorylation and activity of GST-eEF-2K truncation mutants. A: Various C-terminal GST-eEF-2K truncation mutants were allowed to autophosphorylate in vitro for 40 min in the presence or absence of  $\text{Ca}^{2+}$  as indicated. The figure shows an autoradiograph of the gel after SDS-PAGE. B: The activity of various C-terminal eEF-2K truncation mutants against eEF-2 was assayed as described. C: Activities of various N-terminal eEF-2K truncation mutants were assayed as in B.

inactivates most protein kinases. We therefore mutated this residue to determine whether it was important in the catalytic activity of eEF-2K. Fig. 1 shows that myc-eEF-2K K340R expressed in HEK 293 cells was as active as wild-type myc-eEF-2K. We also mutated the next lysine residue in the sequence (K346) to rule out the possibility that this residue was important. However, again substitution of this residue was without effect (not shown).

Subsequent to the publication of the sequence of rat eEF-2K, the *Caenorhabditis elegans* eEF-2K sequence was published [17] as was the mapping of the *Dictyostelium* MHCK catalytic domain [20]. From these data several conclusions can be drawn: firstly the catalytic domain of rat eEF-2K resides approximately between residues 123 and 325; it has no homology with the catalytic domains of conventional protein kinases; the conserved residues between eEF-2K and MHCK appear to comprise eight subdomains [18]. Among the residues conserved between eEF-2K and MHCK are two cysteine residues (C313 and C317 in rat eEF-2K) which suggests a possible role in catalysis. A role for cysteine pairs in the catalytic activity of another atypical kinase (BCR) [26] has

been proposed which involves the formation of a phosphothiol intermediate with ATP. To test whether these two cysteine residues were important in catalytic activity they were both mutated to alanine residues. When expressed in bacteria as a GST-fusion protein (not shown) or myc-tagged in eukaryotic cells, eEF-2K C331/317 was completely inactive when tested against eEF-2 (Fig. 1), and in addition could not autophosphorylate (not shown). This suggests that those cysteine residues are involved in the catalytic activity of eEF-2K although these experiments alone cannot rule out the possibility that mutation of these residues interferes with the structural organisation of the catalytic domain. However, these results are similar to the BCR protein kinase in which deletion of either of two essential cysteine pairs results in complete inhibition of autokinase activity [26].

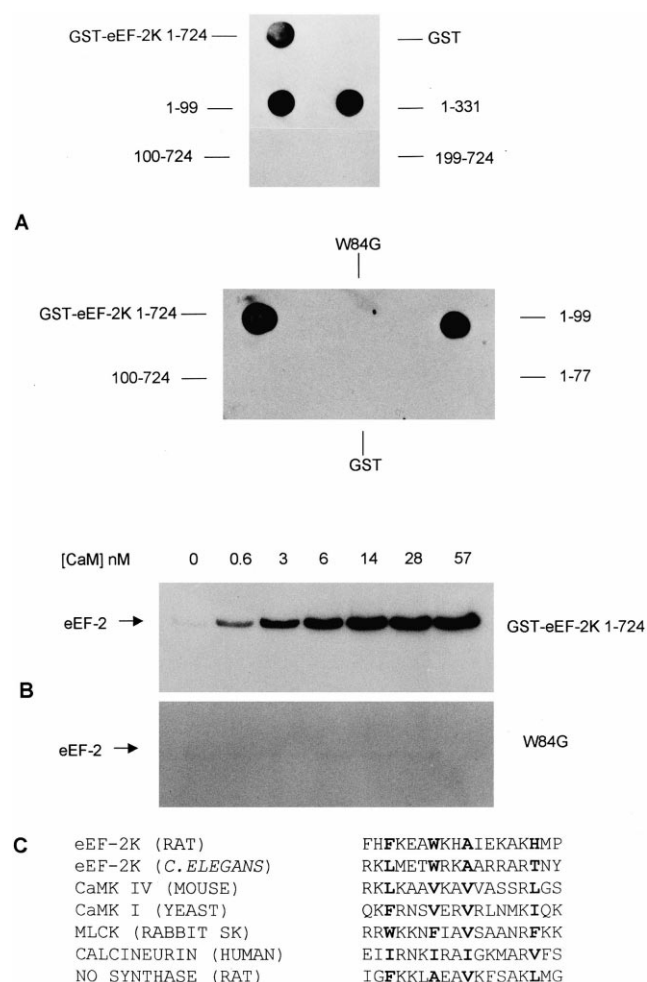


Fig. 3. Binding of calmodulin to eEF-2K truncation mutants. A: 1 µg of various GST-eEF-2K mutants was vacuum-blotted onto nitrocellulose membrane. The membrane was incubated with biotinylated CaM followed by Extravidin-peroxidase. CaM binding was visualised by ECL. GST alone was used as a negative control. The binding of CaM was strictly dependent upon the presence of free calcium in the incubation buffers (not shown). B: Activity of GST-eEF-2K W84G. Wild-type GST-eEF-2K and GST-eEF-2K W84G (0.1 µg) were incubated with eEF-2 (1 µg) in the presence of various concentrations of CaM as indicated for 5 min. After PAGE the dried gels were subjected to autoradiography. C: Alignment of eEF-2K CaM binding domain with that of other CaM binding proteins. The conserved hydrophobic residues in the 1-5-8-14 CaM binding domain are shown in bold. SK = skeletal muscle, MLCK = myosin light chain kinase; NO = nitric oxide.

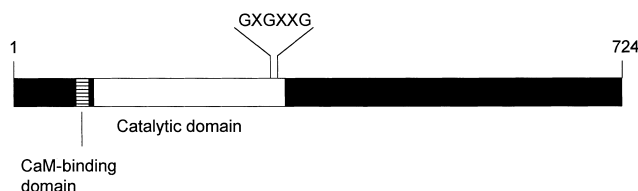


Fig. 4. Domain structure of eEF-2K. The relative positions of the CaM binding domain and the catalytic domain are shown as is the GXXGXXG motif within the catalytic domain.

In order to try to define the catalytic domain of eEF-2K we carried out N- and C-terminal truncation mutagenesis and determined the eEF-2 kinase and autokinase activity of the mutants. A 35 kDa fragment from the central region of MHCK which contained intrinsic kinase activity has been defined by truncation mutagenesis [20]. However, only limited truncation of eEF-2K from the C-terminal end of the molecule was found to inhibit eEF-2K activity. It was found that removal of as few as 19 C-terminal residues completely inhibited eEF-2K activity (Fig. 2). This was not simply due to disruption of ATP binding as truncation mutants with up to 100 residues removed could still autophosphorylate as well as the full-length protein (Fig. 2A). Further truncation of about 130 residues from the C-terminus did result in autokinase-defective eEF-2K (Fig. 2A). This is likely to be due to the disruption of ATP binding and was not due to the removal of autophosphorylation sites as all of these lie closer to the N-terminal end (Diggle et al., manuscript in preparation). Truncation of the N-terminal end of eEF-2K also resulted in its inactivation. However, deletion of a larger region of 75–100 residues is required to inactivate eEF-2K as GST-eEF-2K 74–724 was active while GST-eEF-2K 100–724 was inactive (Fig. 2C). It is possible that this is due to disruption of ATP binding or, as GST-eEF-2K 100–724 lacks the CaM binding domain, it is possible that this mutant is not activatable since it cannot bind CaM (see below).

We next investigated the regions of eEF-2K that are important in calmodulin binding. We had previously suggested that a possible CaM binding domain in eEF-2K was towards the C-terminal end (residues 593–609) based on the computer prediction that this region possessed amphipathic  $\alpha$ -helical structure. We determined to identify the CaM binding domain in GST-eEF-2K by using a filter binding assay to assess the ability of various truncation mutants of eEF-2K to bind biotinylated CaM [21]. Fig. 3 shows that the eEF-2K CaM binding domain is within the N-terminal 99 amino acids since, while GST-eEF-2K 1–99 could bind biotinylated CaM, any truncation mutant lacking the first 99 amino acids could not. Furthermore, the CaM binding domain was shown to be between residues 77 and 99 from the inability of GST-eEF-2K 1–77 to bind to biotinylated CaM (Fig. 3A).

To further prove that this region does constitute the CaM binding domain, one of the important hydrophobic residues (W84) was substituted by an alanine residue. Fig. 3 shows that GST-eEF-2K W84G was unable to bind biotinylated CaM and was completely inactive even at CaM concentrations that saturated wild-type eEF-2K.

Examination of the sequence between residues 77 and 99 suggests that since residue 95 is proline this may represent the extreme C-terminal end of the CaM binding domain as this residue would disrupt  $\alpha$ -helical structure. The sequence of the

domain is therefore likely to be FHFKEAWKHAIE-KAKHMP, which most closely conforms to the 1-5-8-14 type A CaM binding motif and also contains a number of basic residues which flank the hydrophobic residues which is a feature common to this type of CaM binding domain [27]. However, the sequence in eEF-2K does not entirely conform to this domain type as it lacks a hydrophobic residue at position 14 (the conserved hydrophobic residues are in bold). The corresponding region in *C. elegans* eEF-2K is homologous to the rat eEF-2K sequence (Fig. 3C) with position 14 also lacking a hydrophobic residue. This domain type is shared by a number of mammalian proteins including calcineurin, myosin light chain kinase and CaM kinase IV (see Fig. 3C) [27]. Fig. 4 shows a diagrammatic representation of eEF-2K domain structure.

#### 4. Conclusions

The fact that removal of relatively small regions from either the N- or C-terminus of GST-eEF-2K results in loss of kinase activity suggests that the maintenance of an active catalytic domain requires contacts with other regions of the molecule outside the catalytic domain. This is in contrast to a number of conventional protein kinases the catalytic domains of which can be isolated in an active form. Also the active catalytic domain of *Dictyostelium* MHCK, the only other characterised kinase with homology to eEF-2K, can also be isolated [20]. It is possible that this intractability is a consequence of improper folding of truncated forms of eEF-2K in bacteria and another expression system may be more suitable for the production of active truncated forms of eEF-2K.

While removal of approximately 130 residues from the C-terminal end of eEF-2K results in loss of autokinase activity, removal of only 19 residues results in loss of eEF-2K activity but not autokinase activity. This suggests that the extreme C-terminal end is essential for the interaction of eEF-2K with eEF-2. This region may directly interact with eEF-2 or possibly may be required for the oligomerisation of eEF-2K. However, as yet there is no evidence that oligomerisation of eEF-2K is required for its activity. Further work will be required to determine the requirement for the C-terminal region of eEF-2K for its activity. It is interesting to note that the interaction of p90 ribosomal S6 kinase (rsk) with extracellular signal-regulated kinase (ERK) is dependent upon a small region at the C-terminal end of p90 rsk and deletion of the C-terminal nine residues completely eliminated the interaction of the two kinases and prevented activation of p90 rsk by ERK in BHK cells [28].

The positioning of the CaM binding domain immediately N-terminal to the catalytic domain in eEF-2K highlights a further distinction between eEF-2K and the other CaMKs in which the CaM binding domain is C-terminal to the catalytic domain. In CaMK I, CaMK IV and CaMKK the CaM

binding domain is preceded and overlapped by an autoinhibitory domain. It is unclear as yet whether eEF-2K also possesses such an autoinhibitory domain and whether it is close to the CaM binding domain.

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