Phosphorylation of elongation factor-2 kinase on serine 499 by cAMP-dependent protein kinase induces Ca^{2+}/calmodulin-independent activity

Tricia A. DIGGLE*[1], Tatiana SUBKHANKULOVA*, Kathryn S. LILLEY†, Nita SHIKOTRA*, Anne E. WILLIS* and Nicholas T. REDPATH‡[2]

*Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K., †Protein and Nucleic Acid Laboratory, University of Leicester, University Road, Leicester LE1 7RH, U.K., and ‡MRC Laboratory for Molecular Cell Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Elongation factor-2 kinase (eEF-2K) negatively regulates mRNA translation via the phosphorylation and inactivation of elongation factor-2 (eEF-2). We have shown previously that purified eEF-2K can be phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA) and that this induces significant Ca^{2+}/calmodulin (CaM)-independent eEF-2K activity [Redpath and Proud (1993) Biochem. J. **293**, 31–34]. Furthermore, elevation of cAMP levels in adipocytes also increases the level of Ca^{2+}/CaM-independent eEF-2K activity to a similar extent, providing a mechanistic link between elevated cAMP and the inhibition of protein synthesis [Diggle, Redpath, Heesom and Denton (1998) Biochem. J. **336**, 525–529]. Here we describe the expression of glutathione S-transferase (GST)-eEF-2K fusion protein and the identification of two serine residues that are phosphorylated by PKA *in vitro*. Endoproteinase Arg-C digestion of GST-eEF-2K produced two phosphopeptides that were separated by HPLC and sequenced. ^{32}P Radioactivity release from these peptides indicated that the sites of phosphorylation were Ser-365 and Ser-499, both of which lie C-terminal to the catalytic domain. Mutation of these sites to non-phosphorylatable residues indicated that both sites need to be phosphorylated to induce Ca^{2+}/CaM-independent eEF-2K activity *in vitro*. However, expression of Myc-tagged eEF-2K in HEK 293 cells, followed by treatment with chlorophenylthio-cAMP (CPT-cAMP), showed that Ser-499 phosphorylation alone induced Ca^{2+}/CaM-independent eEF-2K activity in cells. Co-expression of wild-type eEF-2K with luciferase resulted in a 2–3-fold reduction in luciferase expression. Expression of eEF-2K S499D resulted in a 10-fold reduction in luciferase expression despite the fact that this mutant was expressed at very low levels. This indicates that eEF-2K S499D is constitutively active when expressed in cells, thus leading to the suppression of its own expression. Our data demonstrate an important role for the phosphorylation of Ser-499 in the activation of eEF-2K by PKA and the inhibition of protein synthesis.

Key words: mRNA, protein synthesis, signal transduction, translation.

INTRODUCTION

Elongation factor-2 kinase (eEF-2K) is a negative regulator of eukaryotic mRNA translation. It phosphorylates and inactivates elongation factor-2 (eEF-2), thereby reducing the rate of peptide chain elongation [1–3]. eEF-2K is a Ca^{2+}/calmodulin (CaM)-dependent protein kinase that has recently been cloned from rat [4], human, mouse and *Caenorhabditis elegans* [5]. It is a novel type of kinase that is unrelated to other serine/threonine kinases. The catalytic domain was reported originally to lie within a central region of eEF-2K encompassing residues 288–554 in rat eEF-2K [4]. However subsequent cloning of the *C. elegans* homologue of eEF-2K [5] has revealed that the only region of extensive homology, which by implication represents the catalytic domain, resides in the N-terminal half of eEF-2K. This region shares over 40 % identity with the catalytic domains of *Dictyostelium* myosin heavy-chain kinases (MHCKs) A and B, the only other characterized kinases with homology to eEF-2K [6,7]. The catalytic domains of eEF-2K and MHCK possess none of the conserved catalytic subdomains present in conventional serine/threonine kinases apart from a GXGXXG motif that is involved in binding Mg^{2+}/ATP in conventional protein kinases. However, this motif is present at the extreme C-terminal end of eEF-2K and MHCK catalytic domains, rather than at the N-terminal end as in conventional protein kinases. It is unclear as yet whether this GXGXXG motif has the same role in eEF-2K and MHCK as it has in other protein kinases.

Native eEF-2K (purified from rabbit reticulocytes) can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (PKA) [8]. This phosphorylation converts eEF-2K into a partially autonomous (Ca^{2+}/CaM-independent) kinase. The maximal level of autonomous activity that can be observed with PKA-phosphorylated eEF-2K is about 40 % of the activity measured in the presence of Ca^{2+}/CaM [8]. We have shown recently that elevation of cAMP by β-adrenergic agonists in adipocytes also activates eEF-2K by increasing its activity at low calcium concentrations resulting in an increase in eEF-2 phosphorylation and a decrease in the rate of peptide chain elongation [9]. This is therefore likely to be the mechanism involved in the inhibition of protein synthesis by hormones such as glucagon and other agents that increase the level of cytoplasmic cAMP [10,11].

The activity of eEF-2K can be regulated acutely by phosphorylation in two other ways: Ca^{2+}/CaM-dependent autophosphorylation of eEF-2K also induces autonomy [12] and eEF-2K can be inhibited in response to insulin in Chinese hamster ovary cells expressing human insulin receptor and in adipocytes [9,13], by a mechanism that is not clear but which is

Abbreviations used: CaM, calmodulin; eEF-2, eukaryotic elongation factor-2; eEF-2K, eEF-2 kinase; GST, glutathione S-transferase; IMAC, immobilized metal-ion affinity chromatography; MHCK, myosin heavy-chain kinase; PKA, cAMP-dependent protein kinase; CPT-cAMP, chlorophenylthio-cAMP.

[1] Present address: GlaxoWellcome R&D, Gunnells Woods Road, Stevenage, Herts. SG1 2NY, U.K.
[2] To whom correspondence should be addressed (e-mail n.redpath@ucl.ac.uk).

likely to involve changes in eEF-2K phosphorylation. It is therefore important that sites of phosphorylation in eEF-2K are identified and the effects of phosphorylation of each site on eEF-2K activity determined. To this end we have identified two sites in eEF-2K that are phosphorylated by PKA *in vitro*. Mutation of these sites has been used to determine which sites of phosphorylation are responsible for the induction of autonomy and the effects of eEF-2K phosphorylation on translation *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

eEF-2 was purified from rabbit reticulocytes as described previously [14]. The catalytic subunit of PKA was purified from pig heart as described in [8], except that the hydroxyapatite step was omitted. Centrifree concentrators were from Millipore. PCR was carried out using *Pfu* polymerase (Stratagene). Arylamine discs were purchased from Perkin Elmer and sequencing-grade endoproteinase Arg-C was from Promega. Fugene 6 was purchased from Bio-Rad. Anti-Myc (9E10) antibody was from Sigma. An antibody against the N-terminal region of rat eEF-2K was raised in rabbits by immunization with glutathione S-transferase (GST)-eEF-2K 1-99.

Expression of recombinant GST-eEF-2K

PCR was used to amplify cDNA encoding the first 99 residues of rat muscle eEF-2K [4] 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 *Escherichia coli* BL21(DE3) cells by induction with 0.2 mM isopropyl β-D-thiogalactoside 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 mass of approx. 130 kDa as judged by SDS/PAGE.

Site-directed mutagenesis was carried out using a two-PCR method [15]. 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).

Phosphorylation of GST-eEF-2K by PKA and purification of [^{32}P]phosphopeptides

GST-eEF-2K (450 μg) was incubated with PKA (35 m-units/ml; 1 unit incorporates 1 μmol of phosphate/min into histone 2A) for 60 min at 30 °C in 50 mM Mops, pH 7.2, containing 11 mM $MgCl_2$, 0.4 mM EGTA, 2 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA), 200 μM ATP (800 c.p.m./pmol), 5 mM dithiothreitol, 10 % glycerol, 0.5 mM benzamidine and 1 μg/ml each of pepstatin, antipain and leupeptin. The reaction was stopped by addition of EDTA to 20 mM. ATP, $MgCl_2$ and EDTA were removed by gel filtration using a PD-10 column and the sample concentrated in an Ultrafree-MC filter (Millipore). The buffer was exchanged to 100 mM *N*-ethylmorpholine, pH 7.6, containing 1 mM $CaCl_2$ and 2 mM dithiothreitol and the sample digested overnight with 20 μg of endoproteinase Arg-C at 37 °C. The pH was adjusted to below 5.5 and the phosphopeptides purified further using immobilized metal-ion affinity chromatography (IMAC) [16]. The digest was applied to Fe^{3+}-Sepharose (50 μl) equilibrated in 50 mM Mes, pH 5.5, containing 1 M NaCl. Unbound peptides were removed by washing in 2 mM Mes, pH 6.0. Phosphopeptides were eluted in 500 mM $NaHCO_3$, pH 8.0, and dried in a Univap (Uniscience). ^{32}P-Labelled peptides were separated by reversed-phase HPLC (Luna C_{18}, 15 cm × 2 mm column; 0.1 % trifluoroacetic acid in water/0.33 % acetonitrile per min). Peptides were purified further as necessary using the same column but in 10 mM ammonium acetate, pH 6.5 (0.5 % acetonitrile/min). Sequencing of peptides coupled to Sequelon arylamine membrane was performed by Edman degradation using an Applied Biosystems 476A sequencer. In order to determine the site(s) of phosphorylation, a second identical sample was analysed and ^{32}P radioactivity measured after each cycle of Edman degradation.

Two-dimensional phosphopeptide mapping of phosphorylated GST-eEF-2K

GST-eEF-2K (20 pmol) was phosphorylated for 20 min as described above, separated by SDS/PAGE and transferred to nitrocellulose membrane. GST-eEF-2K was visualized by brief incubation in Ponceau S and the appropriate bands were sliced from the membrane. Membrane pieces were washed twice in 1 ml of water and incubated in 0.5 % polyvinylpyrrolidone as described in [17]. These were then digested with 3 μg of endoproteinase Arg-C in buffer, as above, in a total volume of 40 μl at 37 °C for 16 h. The supernatants were removed to fresh tubes, dried in a Univap as above and washed twice, once in 25 μl of water and then in chromatography buffer. Two-dimensional phosphopeptide mapping was performed as described in [12] except that electrophoresis was carried out at 1000 V for 30 min.

Transient transfections

eEF-2K with an N-terminal Myc-epitope tag (EQKLISEEDL) inserted after the N-terminal methionine was constructed using PCR and was inserted into pcDNA3.1 (Invitrogen). HEK 293 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. When required, cells were then treated with or without 1 mM chlorophenylthio-cAMP (CPT-cAMP) for 10 min and extracted [13]. 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 in [9]. For Western analysis, Myc-eEF-2K from 300 μg of cell extract was immunoprecipitated with 9E10 before SDS/PAGE and transfer on to PVDF membrane (Millipore). Myc-eEF-2K was then detected using anti-GST-eEF-2K 1-99 antibody.

Co-expression of eEF-2K with luciferase was carried out using 2 μg of pGL3 encoding firefly luciferase. Co-transfection conditions were as for eEF-2K alone. Cells were extracted in 100 μl of extraction buffer and 5 μl of this was used to measure luciferase activity using the Stop & Glo assay kit (Promega). To assess the level of luciferase, total cellular RNA was prepared and analysed as described in [18]. RNA (4 μg) was blotted and probed for luciferase (2.37 kb), followed by stripping and re-probing for GAPDH (1.6 kb).

RESULTS AND DISCUSSION

In order to map the sites phosphorylated in eEF-2K by PKA, GST-eEF-2K was constructed and expressed in *E. coli*. The expression was carried out at room temperature (approx. 22 °C), since it was found that GST-eEF-2K expressed at 37 °C was

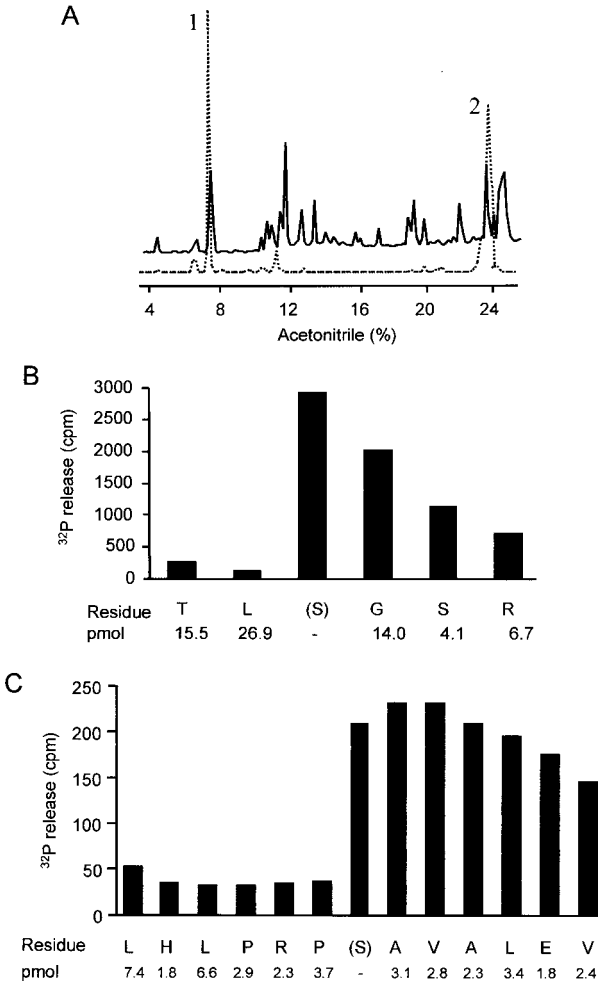

Figure 1 Sequence and ^{32}P release of phosphopeptides from GST-eEF-2K phosphorylated by PKA

^{32}P-Labelled GST-eEF-2K phosphorylated by PKA was digested with 20 μg endoproteinase Arg-C. (**A**) The phosphopeptides were partially purified by IMAC and chromatographed on a C_{18} column as described in the Experimental procedures section. The solid line shows absorbance at 212 nm and the dashed line ^{32}P radioactivity in arbitrary units. (**B**) Phosphopeptide 1 was coupled to an arylamine membrane without further purification and subjected to Edman degradation. A second sample (20000 c.p.m.) was sequenced to follow ^{32}P release. The panel shows the sequence using the single letter code for amino acids and ^{32}P radioactivity released after each cycle of degradation. The recovery from each round, in pmol, is indicated. (**C**) Peptide 2 was purified further by HPLC in ammonium acetate as described in the Experimental procedures section and subjected to Edman degradation and ^{32}P radioactivity release (13839 c.p.m. coupled) as for phosphopeptide 1. The parentheses around the serines indicate that no residue was detected in these rounds of Edman degradation, which is consistent with the residue being phosphorylated. Serine residues were assigned to these rounds from the known sequence of rat eEF-2K.

completely insoluble. At the lower temperature approx. 50% of the expressed GST-eEF-2K was obtained in a soluble form (results not shown). GST-eEF-2K was however sensitive to degradation and the preparations purified by glutathione-agarose displayed a number of lower-molecular-mass species which amounted to up to 25% of the total protein.

In order to obtain phosphopeptides for sequencing, GST-eEF-2K was phosphorylated with PKA, digested with endoproteinase Arg-C and the peptides were separated by HPLC as described in the Experimental procedures section. IMAC [16] was used to partially purify phosphopeptides before HPLC.

Endoproteinase Arg-C cleavage resulted in two major, well-separated peaks of ^{32}P radioactivity eluted at 7% and 23% acetonitrile, and termed 1 and 2 respectively (Figure 1A). Endoproteinase Arg-C digestion was chosen since, when tryptic digestion was performed, the two peaks were difficult to separate and eluted very close together at 23% acetonitrile. Both peptides were phosphorylated on serine residues (results not shown). Phosphopeptide 1 was sequenced by Edman degradation and shown to have the sequence TLSGSR, corresponding to residues 363–368 of the rat eEF-2K sequence [4] (Figure 1B). This peptide was then subjected to Edman degradation again, with the release of ^{32}P being determined after every cycle. Initial release of radioactivity was found in round three (Figure 1B), indicating that Ser-365 was phosphorylated by PKA. Peak 2 was purified further by chromatography at pH 6.5 and sequenced as before. This peptide corresponded to residues 493–507 of rat eEF-2K. Release of ^{32}P was found to occur in round seven, indicating that Ser-499 was also phosphorylated by PKA. It was found that there was considerable carry over of ^{32}P for a number of cycles subsequent to round seven (Figure 1C). This was due to the presence of two proline residues N-terminal to Ser-499. Proline residues are relatively inefficiently cleaved during Edman degradation leading to carry over into the next round. The sequencing programme used was not optimized for proline-containing peptides. The release of ^{32}P in rounds subsequent to Ser-499 was not due to the presence of other phosphorylated residues, since Ser-499 was the only phosphorylatable residue in the peptide. Additionally, matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS verified the purity and molecular mass of this peptide containing one phosphate group (results not shown). Further experiments, described below, established that Ser-499 was indeed phosphorylated by PKA.

The sites phosphorylated by PKA have the minimum consensus-sequence requirement of an arginine residue in the -1 to -3 positions relative to the target site, within a relatively hydrophobic context [19,20]. Indeed, *in vivo*, up to one-third of PKA phosphorylation sites do not possess the optimum consensus sequence of RRXS, instead having only one arginine residue in the -1, -2 or -3 position [20]. This suggests that both of these residues (Ser-365 and Ser-499) are equally good potential candidates for phosphorylation *in vivo*.

To establish conclusively that both Ser-365 and Ser-499 in GST-eEF-2K were phosphorylated by PKA, these sites were mutated to non-phosphorylatable residues. GST-eEF-2K S365A and S499A mutants were expressed and phosphorylated by PKA *in vitro*, followed by endoproteinase Arg-C digestion and two-dimensional phosphopeptide mapping. Figure 2 shows that phosphorylation of GST-eEF-2K S365A by PKA results in the appearance of only peptide 2, whereas phosphorylation of the S499A mutant produced only peptide 1, thus demonstrating that assignment of the two sites phosphorylated by PKA was correct. This result also demonstrated that the phosphorylation of both sites occurred independently, showing that phosphorylation of one site was not dependent on phosphorylation of the other. A minor phosphopeptide (peptide 3) that ran near the origin was observed that accounted for less than 10% of the total radioactivity incorporated into sites 1 and 2. Our studies indicate that this site is probably contained within the peptide corresponding to residues 433–447 of rat eEF-2K and may represent low-level phosphorylation of Ser-434 (results not shown).

To investigate which site(s) of phosphorylation was responsible for the induction of autonomous activity *in vitro*, wild-type GST-eEF-2K and the two serine mutants were phosphorylated by PKA. eEF-2K activity was then measured with and without Ca^{2+}/CaM (Figure 2E). Phosphorylation of wild-type GST-eEF-

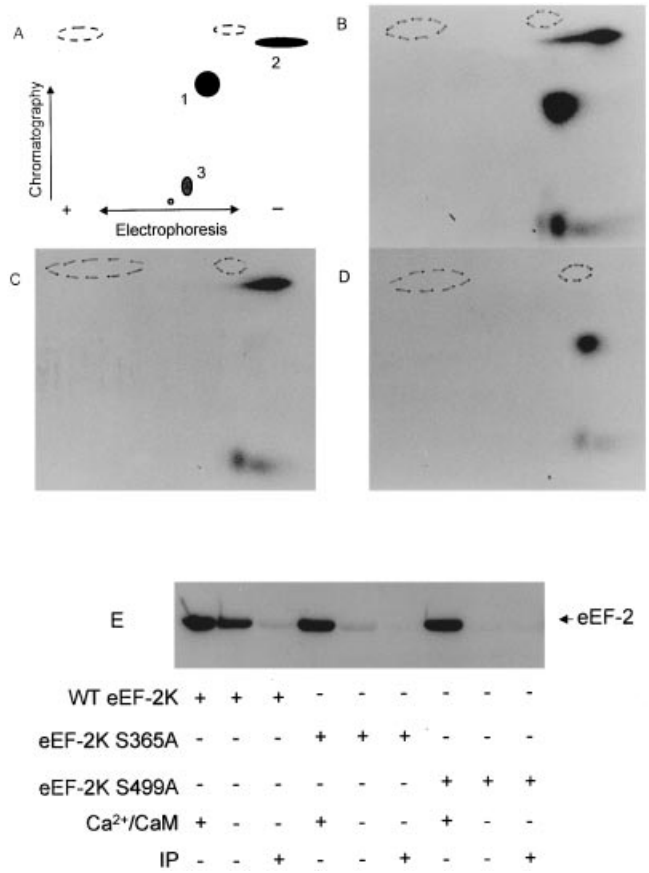

Figure 2 Two-dimensional phosphopeptide maps of GST-eEF-2K phosphorylated by PKA and effect of phosphorylation by PKA on eEF-2K activity *in vitro*

GST-eEF-2K was phosphorylated with PKA and cleaved with endoproteinase Arg-C for mapping as described. (**A**) A schematic representation of the phosphorylated peptides. Peptide 1 is TLpSGSR and peptide 2 is LHLPRPpSAVALEV. Peptide 3 is likely to be due to autophosphorylation of eEF-2K, which occurs at a low level in the absence of calcium. The other panels are autoradiographs of two-dimensional phosphopeptide maps of wild type GST-eEF-2K (**B**), GST-eEF-2K S365A (**C**) and GST-eEF-2K S499A (**D**). (**E**) Wild-type (WT), S365A or S499A GST-eEF-2K were preincubated with PKA for 15 min as in Figure 1. eEF-2K activity was then measured using eEF-2 and [γ-^{32}P]ATP in a final volume of 30 μl, in the presence or absence of Ca^{2+}/CaM as indicated, for 10 min. Samples were subjected to SDS/PAGE and ^{32}P-labelled eEF-2 was revealed by autoradiography. In some preincubation reactions 2 μM PKA inhibitor peptide (IP) was included to prevent phosphorylation of eEF-2K by PKA. The activities of the S365A and S499A mutants in the presence of Ca^{2+}/CaM were not significantly different from that of wild-type eEF-2K.

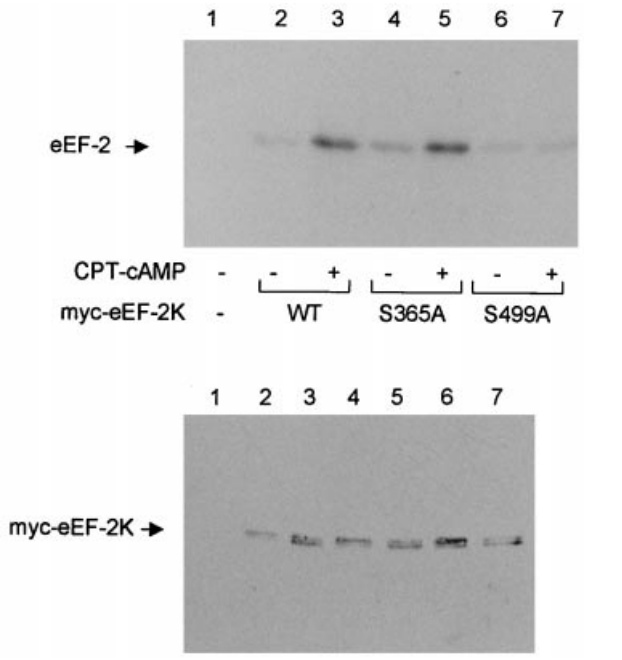

Figure 3 Induction of autonomous eEF-2K activity in HEK 293 cells treated with CPT-cAMP

HEK 293 cells were transfected with empty pcDNA3.1 (lane 1) or containing wild-type eEF-2K (lanes 2 and 3), eEF-2K S365A (lanes 4 and 5) or eEF-2K S499A (lanes 6 and 7). After immunoprecipitation of Myc-eEF-2K from 5 μg of cell extract, activity was measured in the absence of Ca^{2+}/CaM as described in the Experimental procedures section. To assess the level of expression of Myc-tagged eEF-2K in the cells used, Myc-eEF-2K from 300 μg of cell extract was immunoprecipitated with anti-Myc antibody and probed after Western blotting with an antibody raised against the N-terminal 99 amino acids of eEF-2K.

2K with PKA resulted in autonomous activity, amounting to $32.3 \pm 4.5\%$ ($n = 7$) of the maximal activity. In contrast, phosphorylation of GST-eEF-2K S365A or S499A resulted in much reduced autonomous activity ($12.3 \pm 3.7\%$, $n = 11$, and $2.4 \pm 0.9\%$, $n = 9$, respectively), indicating that, *in vitro*, phosphorylation of both residues is required for the activation of eEF-2K. The mutations themselves were not inhibitory as both serine mutants displayed activity similar to the wild type in the presence of Ca^{2+}/CaM.

We next investigated whether both sites of phosphorylation were required to induce eEF-2K autonomy *in vivo*. To do this we transfected HEK 293 cells with plasmids containing Myc-tagged wild-type eEF-2K or eEF-2K harbouring S365A and S499A mutations. After treatment of the cells with CPT-cAMP, Myc-tagged eEF-2K was immunoprecipitated and assayed in the absence of Ca^{2+}/CaM. Figure 3 shows that both wild-type eEF-2K and eEF-2K S365A became autonomous after treatment of the cells with CPT-cAMP. However, CPT-cAMP was without effect on eEF-2K S499A. This indicates that, in cells, only S499 phosphorylation by PKA is required for autonomy. The reason for the discrepancy between the results *in vitro* and *in vivo* is unclear. It is also unclear whether, *in vivo*, PKA phosphorylates both sites or only S499. If it does phosphorylate S365 the above results suggest that phosphorylation of this site *in vivo* does not affect the activity of eEF-2K.

We next mutated the sites of phosphorylation in eEF-2K to acidic residues to mimic phosphorylation to determine what effects these mutations had on protein synthesis in cells. The replacement of an activatory site of phosphorylation with an acidic residue should result in Ca^{2+}/CaM-independent and hence constituitively active eEF-2K. This may therefore be expected to lead to the inhibition of protein synthesis.

We attempted to express these mutants in HEK 293 cells; however, whereas Myc-eEF-2K S365D could be expressed successfully, as determined by immunoprecipitation and detection by Western blotting, Myc-eEF-2K S499D was expressed only at less than 1% of the level of expression of wild-type eEF-2K. The poor expression was not due to errors in the cDNA sequence, as this was verified by direct sequencing (results not shown). A second, independent Myc-eEF-2K S499D cDNA clone was then tested for expression, but the results were the same; less than 1% of the expression level of wild-type and other mutants, including S365D. Mutation of Ser-499 was not inhibitory for expression,

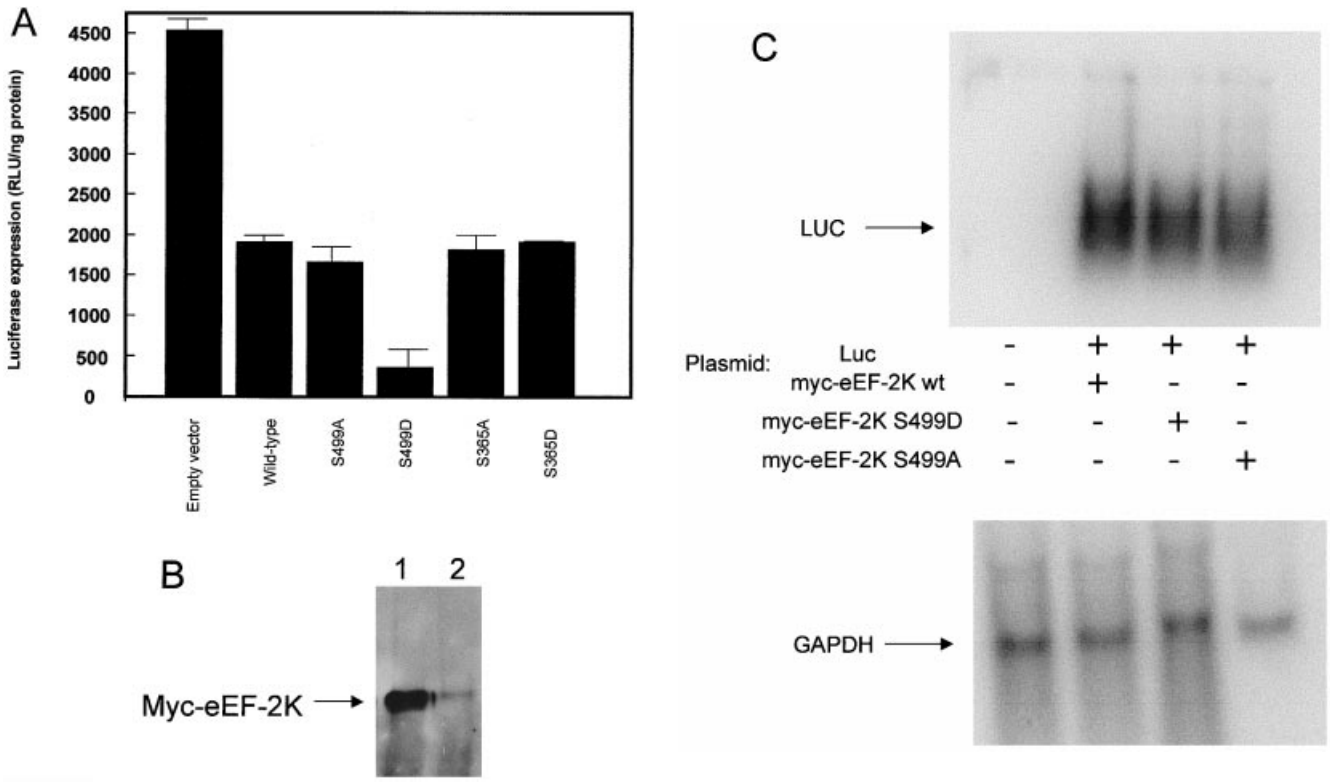

Figure 4 Inhibition of luciferase expression by expression of eEF-2K

(**A**) pGL3 encoding firefly luciferase (2 μg) was co-expressed with 3 μg of pcDNA3.1 (empty vector) or vector encoding various forms of eEF-2K in HEK 293 cells. Cells were then extracted and luciferase expression measured as described in the Experimental procedures section. The measurements were done in triplicate and the results shown are representative of at least 10 separate experiments. RLU, relative light units. (**B**) Myc-eEF-2K was immunoprecipitated from transfected HEK 293 cells followed by SDS/PAGE and immunodetection as in the Experimental procedures section. The panel shows typical relative expression levels of Myc-eEF-2K wild-type (lane 1) and Myc-eEF-2K S499D (lane 2). Myc-eEF-2K S499A, S365A and S365D were typically expressed at levels similar to the wild type. (**C**) Luciferase was co-expressed with wild-type (wt), S499D and S499A eEF-2K in HEK 293 cells followed by Northern blotting for luciferase (LUC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNAs, as described in the Experimental procedures section.

as Myc-eEF-2K S499A was expressed at the level of wild-type eEF-2K (see Figure 4 and the discussion below).

Another explanation for the lack of Myc-eEF-2K S499D expression could be that this form of eEF-2K was indeed constitutively active and that a low level of expression led to eEF-2 phosphorylation and the inhibition of further protein synthesis and hence Myc-eEF-2K S499D expression.

To test whether Myc-eEF-2K expression in cells led to the reduction in protein synthesis, various Myc-eEF-2K mutants were co-expressed with a luciferase reporter gene in HEK 293 cells and the level of luciferase protein expressed was assayed. Figure 4(A) shows that expression of wild-type, S499A, S365A and S365D eEF-2K resulted in a 2–3-fold reduction in luciferase expression compared with empty vector control. The inhibition of luciferase by expression of the wild type is due to the fact that eEF-2K is active under basal cellular conditions, as illustrated by the basal level of eEF-2 phosphorylation (see for example [13]). Expression of eEF-2K S499D reduced luciferase expression by a further 4-fold. However, the level of expression of eEF-2K S499D was less than 1% of the expression level of wild-type eEF-2K (Figure 4B) and of the various other mutants (including eEF-2K S365D; results not shown). This suggests that, when expressed in HEK 293 cells, eEF-2K S499D is at least 400 times more active than wild-type eEF-2K under cellular conditions. It is therefore likely that the low expression of eEF-2K S499D is due to this mutant being highly constitutively active, thus leading to the inhibition of mRNA translation and thereby inhibiting further expression of itself. Very similar results were obtained when β-galactosidase was used as a reporter (results not shown). The low level of luciferase activity observed when co-expressed with eEF-2K S499D was not due to a reduced level of luciferase DNA transcription, as this was found to be unaffected by co-expression with eEF-2K S499 (Figure 4C).

Whereas overexpression of eEF-2K in cells would be expected to lead to the inhibition of mRNA translation, this is the first direct demonstration that this is indeed the case and indicates that the eEF-2/eEF-2K system can act as a controller of mRNA translation *in vivo*.

Both sites of phosphorylation lie within a central region of eEF-2K that is C-terminal to the catalytic domain. This region of mammalian eEF-2K has little homology with the *C. elegans* homologue of eEF-2K [5], which suggests that this region has no role in the catalytic mechanism of eEF-2K. It is also interesting to note that neither serine residue phosphorylated by PKA in mammalian eEF-2K is conserved at the equivalent position in *C. elegans*. This suggests that *C. elegans* eEF-2K is not regulated by PKA, at least on these sites. This does not rule out the possibility that alternative sites of phosphorylation in *C. elegans* eEF-2K could have the same role however. It is of note that these phosphorylation sites do not lie within the CaM-binding domain, as we have mapped this domain to a region N-terminal to the catalytic domain [21].

The findings discussed above show that phosphorylation of Ser-499 induces autonomous eEF-2K activity and therefore represents the first identification of a regulatory phosphorylation site in eEF-2K. This is an important step in the elucidation of the molecular mechanisms involved in the regulation of peptide chain elongation in response to hormonal stimuli.

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