Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398

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Abstract:

Protein synthesis consumes a high proportion of the metabolic energy of mammalian cells, and most of this is due to peptide-chain elongation. An important regulator of energy supply and demand in eukaryotic cells is the AMP-activated protein kinase (AMPK). The rate of peptide-chain elongation can be modulated through the phosphorylation of eukaryotic elongation factor (eEF) 2, which inhibits its activity and is catalysed by a specific calcium/calmodulin-dependent protein kinase termed eEF2 kinase. Here we show that AMPK directly phosphorylates eEF2 kinase and we identify the major site of phosphorylation as Ser398 in a regulatory domain of eEF2 kinase. AMPK also phosphorylates two other sites (Ser78, Ser366) in eEF2 kinase in vitro. We develop appropriate phosphospecific antisera and show that phosphorylation of Ser398 in eEF2 kinase is enhanced in intact cells under a range of conditions that activate AMPK and increase the phosphorylation of eEF2. Ser78 and Ser366 do not appear to be phosphorylated by AMPK within cells. Although cardiomyocytes appear to contain a distinct isoform of eEF2 kinase, it contains a site corresponding to Ser398 that is phosphorylated by AMPK in vitro. Stimuli that activate AMPK and increase eEF2 phosphorylation within cells increase the activity of eEF2 kinase. Thus, AMPK and eEF2 kinase may provide a key link between cellular energy status and the inhibition of protein synthesis, a major consumer of metabolic energy.
**Abbreviations:** 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; ACC, acetyl-CoA carboxylase; AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; ARVC, adult rat ventricular cardiomyocytes; CaM, calmodulin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; 2-DOG, 2-deoxy-D-glucose; eEF, eukaryotic elongation factor; GST, glutathione S-transferase; mTOR, the mammalian target of rapamycin; S6K1, the 70 kDa ribosomal protein S6 kinase, isoform 1; TFP, trifluoperazine.
Introduction:

Protein synthesis is a process of fundamental importance for all living cells. It also places substantial demands on the cell in terms of requirements for precursors, viz., amino acids and metabolic energy. Recent work has shown that amino acids regulate the translational machinery in a number of ways, e.g., through control of the mTOR signaling pathway (1-3). Protein synthesis is a major consumer of cellular energy with estimates of 30-50% of total energy being used in this process. The vast majority of this is consumed by peptide elongation, either directly as GTP (two are hydrolysed for each amino acid added) or indirectly during amino acyl-tRNA charging, one ATP being hydrolysed to AMP for each amino acid charging event. Despite this, little is known of the mechanisms by which cells couple the rate of protein synthesis to the availability of energy, i.e., ATP.

In mammalian cells, peptide-chain elongation requires two main elongation factors, eEF1A and eEF2. The latter mediates the translocation step of elongation in which the ribosome moves by the equivalent of one codon relative to the mRNA and the peptidyl-tRNA shifts from the A- into the P-site on the ribosome (4). eEF2 is a phosphoprotein: phosphorylation at a single site, Thr56, renders it unable to bind ribosomes thereby inactivating it (5-7). Phosphorylation is catalysed by a specific and very unusual kinase, eEF2 kinase (8;9). Phosphorylation of eEF2 is decreased by insulin and certain other stimuli through signaling events that require the mammalian target of rapamycin, mTOR (reviewed in (7)), and involves inactivation of eEF2 kinase. One link in the regulation of eEF2 kinase by mTOR is the phosphorylation of eEF2 kinase by the 70kDa ribosomal protein S6 kinase, S6K1. S6K1 is regulated through mTOR and phosphorylates eEF2 kinase at Ser366, which decreases its activity at low Ca$^{2+}$ concentrations (10).
We recently observed that, in cardiomyocytes and other cell types, conditions that cause depletion of ATP lead to increased phosphorylation of eEF2 (11,12). This is associated with inhibition of protein synthesis and increased activity of eEF2 kinase (11). Furthermore, eEF2 phosphorylation is also increased following treatment of cells with a compound that activates the AMP-activated protein kinase, AMPK (5-aminoimidazole-4-carboxamide ribonucleoside; AICAR). Yan et al. (13) have recently shown that AICAR increases the phosphorylation of eEF2 in pancreatic islets or β-cell lines. AMPK is a key player in cellular energy homeostasis (14,15): depletion of ATP leads (through the action of adenylate kinase) to a rise in AMP levels, which activates AMPK. AMPK is known to phosphorylate a range of target proteins to decrease energy consumption and increase energy supply.

In this report, we show that AMPK directly phosphorylates eEF2 kinase and identify the major site at which this occurs as Ser398. We show that phosphorylation of Ser398 is enhanced by treating cells with agents that deplete cellular ATP levels and/or activate AMPK. We also show that these effects are not exerted through impairment of mTOR signaling, which has been reported to occur when ATP levels are severely depleted (16). Lastly, we show that agents or treatments that activate AMPK and increase eEF2 phosphorylation also increase the phosphorylation of eEF2 kinase at Ser398 and stimulate its activity. These data provide important new insights into the ways in which cells coordinate the rate of protein synthesis, a major consumer of energy, with the metabolic status of the cell.
Experimental procedures:

Materials

\[^{\gamma-32}P\]ATP and materials for protein purification were obtained from Amersham Pharmacia Biotech, UK. Unlabeled ATP was from Roche Molecular Biochemicals (Lewes, UK), cell culture media from Gibco (Paisley, UK), microcystin LR, and rapamycin from Calbiochem (Nottingham, UK), and Immobilon P membranes from Millipore (Bedford, UK). Other chemicals were of the highest purity available and were purchased from Merck (Poole, UK) or Sigma-Aldrich (Poole, UK). \[^{\gamma-32}P\]ATP and ECL reagents were purchased from Amersham Pharmacia Biotech. Bovine serum albumin (fatty acid-free) was from Roche Molecular Biochemicals. The AMARA peptide was a gift from Professor D. G. Hardie, University of Dundee. AICAR was obtained from Sigma (this product has now been discontinued) or Toronto Research Chemicals. Unless otherwise indicated, all reagents were obtained from Sigma or Merck.

Antisera

Antibodies for eEF2 kinase phosphorylated at Ser 359, Ser 366, Ser 377 and Ser 396 have been described previously (17, 18) and were provided by Dr Jane Leitch, Division of Signal Transduction Therapy, University of Dundee. Antibodies to the \(\alpha_1\) and \(\alpha_2\) subunits of AMPK and to acetyl CoA carboxylase (ACC) phosphorylated at Ser 79 were kindly provided by Professor D. G. Hardie, University of Dundee. Antisera for 4E-BP1, S6K1, eEF2 phosphorylated at Thr 56, eEF2 and ribosomal protein S6 phosphorylated at Ser 235 were as described previously (19). Rabbit anti-sheep IgG and goat anti-rabbit IgG antibodies, both conjugated to peroxidase, were obtained from Perbio Science Ltd (Tattenhall, UK).
Antisera for the Ser 78 and Ser 398 phosphorylated forms of eEF2 kinase were raised in rabbits using the peptides GSPANSpFHFKEC and LPSSPSpSATPHC (where Sp indicates phosphoserine), respectively. Peptides were synthesized by Dr Graham Bloomberg (University of Bristol, UK), coupled to keyhole limpet hemocyanin and injected into rabbits at Diagnostics Scotland (Edinburgh, UK). Antibodies were affinity purified on phosphopeptide antigen–Sepharose columns and were used at a concentration of 0.5 μg/ml in the presence of 10 μg/ml of the unphosphorylated peptide antigen. Antibodies against total eEF2 kinase were raised against bacterially expressed GST-eEF2 kinase in rabbits at Diagnostics Scotland (Edinburgh, UK) and then affinity purified on maltose-binding protein-eEF2 kinase–Sepharose to eliminate anti-GST antibodies. Pre-immune IgG was purified from serum from the pre-immune bleed of the rabbit used to raise the anti (P)Ser398 eEF2 kinase antibody using protein G Sepharose.

Isolation, culture, treatment and lysis of cells

Adult rat ventricular cardiomyocytes (ARVC) were isolated and cultured overnight exactly as described (20). KB cells (human oral epidermoid carcinoma cells) were cultivated in Dulbecco’s modified Eagle medium containing 10% (v/v) fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. When they had reached 60% confluence, KB cells were placed in low glucose medium before the experiment (see legends for details). The medium used for the ARVC (M199 medium) contains a low concentration of glucose (5.6 mM) so no change of medium was necessary. Cells were then treated with AICAR, CCCP, 2-deoxyglucose or rapamycin at the final concentrations, and for the times, indicated in the figures. Control cells received an appropriate dose of the relevant vehicle.
For western blot analyses, cells were extracted into buffer comprising 50 mM β-glycerophosphate pH 7.5; 1 mM EGTA; 1 mM EDTA; 1% (v/v) Triton X-100; 1 mM Na₃VO₄; 100nM microcystin-LR; 0.1% (v/v) β-mercaptoethanol and protease inhibitors (leupeptin, pepstatin and antipain, each 1 µg/ml) and phenylmethylsulfonyl fluoride (200 µM). Lysates were centrifuged at 13,000 rpm to remove debris. Protein concentrations in the resulting supernatants were determined as described (21). For immunoprecipitations, supernatants were removed to fresh tubes and then rotated for 1h at 4°C in the presence of protein G Sepharose pre-bound with anti-FLAG or anti-total eEF2 kinase antibody. Immune complexes were washed three times with extraction buffer and resuspended in SDS–PAGE sample buffer or in eEF2 kinase assay buffer.

Gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis and western (immuno) blotting were performed as described earlier (21). Blots were visualised using the enhanced chemiluminescence method.

AMP-activated protein kinase assay.

To determine the activity of AMPK from KB cell lysates the AMPK was immunoprecipitated using 10µl of antibody coupled beads (0.2µg anti α1/α2 AMPK per µl protein G-Sepharose beads) from 100µg of lysate protein. Immune complexes were then washed 3 times with lysis buffer and once with AMPK assay buffer (50mM HEPES pH7.5; 0.02%(w/v) Brij –35; 0.1% (v/v) β-mercaptoethanol) and then incubated with AMPK assay buffer plus 200µM ATP (1000 cpm/pmol [γ-32P]ATP) and 15nM MgCl₂ plus 200µM AMARA substrate peptide (AMARAASAAALARRR). After 10 min, the reactions were terminated by spotting 0.02 ml aliquots onto 2 x 2 cm squares of Whatman P81 phosphocellulose paper, followed by
immersion in 75 mM phosphoric acid. After washing four times with 75 mM phosphoric acid and then once with acetone, the papers were dried and analysed by Čerenkov counting. One unit (U) of kinase activity is the amount of enzyme that phosphorylates 1 nmol of peptide in 1 min.

**Phosphorylation of eEF2 kinase by AMPK and protein chemical analyses**

The AMPK used was an extensively-purified preparation (5U/ml) from rat liver, purified through five steps as far as the gel filtration column, and was kindly provided by Professor D. G. Hardie, University of Dundee (22). This material was then further immunopurified as indicated using antibodies raised against the α1 and α2 catalytic subunits of AMPK coupled to protein G Sepharose and used to phosphorylate GST-eEF2 kinase as described below.

Phosphorylation of bacterially expressed GST-eEF2 kinase (1µg fusion protein per reaction) was carried out using 5mU of immunopurified AMPK in AMPK assay buffer plus 200µM ATP (1000cpm/pmol [γ−32P]ATP), where indicated, 200µM AMP (where indicated), and 15nM MgCl₂. For eEF2 kinase autophosphorylation reactions the immunopurified AMPK and AMP were omitted from the reaction and 0.5 µg/ml calmodulin plus 1mM CaCl₂ added to the reaction mixture. Incubations were at 30°C for 15 min and were stopped by addition of SDS-PAGE loading buffer and heating to 96 °C for five min. Incorporation of phosphate into GST-eEF2 kinase was determined following electrophoresis of samples on a SDS-PAGE gel by autoradiography or following western transfer to Immobilon-P membrane by immunoblotting using phosphorylated residue specific antibodies.

To map the sites on eEF2 kinase phosphorylated by AMPK GST–eEF2 kinase was incubated with immunopurified AMPK as described above except that the reaction was
performed for 60 min and a 10-fold higher specific radioactivity of [γ-32P]ATP was used. Reactions were terminated by adding 1% (w/v) SDS and 1% mM β-mercaptoethanol, and heating at 100°C for 1 min. The sample was subjected to electrophoresis on a SDS-PAGE gel, and the 32P-labeled band corresponding to GST–eEF2 kinase was excised and cut into smaller pieces. For analysis of polypeptides resolved on SDS-PAGE, fragments of gel chip were washed sequentially for 15 min on a vibrating platform with 1 ml of the following: water, a 1:1 mixture of water and CH3CN, 0.1 M NH4HCO3, a 1:1 mixture of 0.2 M NH4HCO3 and CH3CN, and finally CH3CN. The gel pieces were dried by rotary evaporation and incubated in 0.3 ml of 50 mM NH4HCO3, 0.05% (w/v) Zwittergent 3-16 containing 2 µg of alkylated trypsin. After 16 h the supernatant was removed, the gel pieces washed for 10 min in a further 0.3 ml of 50 mM NH4HCO3, 0.05% (w/v) Zwittergent 3-16 and 0.1% (v/v) trifluoroacetic acid (TFA); the combined supernatants containing >90% of the 32P-radioactivity were then analysed by reverse-phase HPLC as described in the legend to Fig. 1.

The tryptic peptides contained in peaks P1 and P2 were analysed by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Elite-STR mass spectrometer using α-cyanocinnamic acid as the matrix. Spectra were obtained in both the linear and reflector mode. The mass spectrum was acquired and was internally mass-calibrated. The tryptic peptide ions obtained were scanned against the SwissProt and Genpep databases using the MS-FIT programme of Protein Prospector. The sequence of these peptides was also confirmed by solid phase Edman sequencing on an Applied Biosystems 476A sequenator. Tryptic peptide P2 was subjected to additional Asp-N cleavage prior to Edman sequencing. The site of phosphorylation was determined by solid-phase Edman degradation of the peptide coupled to Sequelon-AA membrane
(Milligen) as described previously (23). Phosphoamino acid analysis was used to confirm the type of residue phosphorylated in each tryptic peptide.

**Two-dimensional peptide mapping**

Trypsin and Asp-N digested AMPK phosphorylated GST-(WT)eEF2 kinase and GST-(S398A)eEF2 kinase phosphopeptide samples were analyzed by two-dimensional peptide mapping, as described previously (24) at pH 1.9 (electrophoresis) and 3.5 (chromatography). The thin-layer cellulose plates were allowed to dry and then subjected to autoradiography.

**Expression and mutagenesis of GST-eEF2 kinase**

A modified cDNA encoding human eEF2 kinase with an N-terminal FLAG tag cloned between the BamHI sites of the vector pGEX-4T was kindly provided by Dr Maria Deak of the Division of Signal Transduction Therapy, University of Dundee. For expression of GST-eEF2 kinase in *E. coli* BL21 cells were transformed with this vector and induced with isopropyl β-thiogalactoside (100 µM) for 3 h at 26°C. Cells were lysed (by sonication) into 50 mM Tris pH 7.5, 150 mM NaCl, 0.03% Brij-35, 1 mM EDTA, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol and proteinase inhibitors (1 mM each of phenylmethylsulfonyl fluoride, leupeptin, benzamidine and pepstatin), and extracts were prepared. GST-eEF2 kinase was isolated by chromatography on glutathione-Sepharose and eluted using 100 mM glutathione. Purified protein was dialysed overnight at 4°C against 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50% (v/v) glycerol, 1 mM EDTA and 0.1% (v/v) β-mercaptoethanol and then stored at -20°C. For expression as a maltose binding protein fusion, human eEF2 kinase was subcloned into the modified pMal-HA vector (kindly provided by Dr Jane Leitch, Division of Signal Transduction...
Therapy, University of Dundee) using the BamH1 restriction sites. Expression and purification were as described above except that chromatography was on amylose resin and elution with 20 mM maltose.

Mutagenesis of the Ser78 phosphorylation site in human eEF2 kinase to alanine was performed by PCR using ‘QuikChange’ (Stratagene). The forward primer was 5’-CGGCAAAACGCTTCCACTTCAAGGAAGCC -3’ and the reverse primer was 5’-GGCTTCTTGAAGTGAAGGCGGTTTGCCG -3’. For the mutation of serine 398 of human eEF2 kinase to alanine the forward primer was 5’-CTCTCCCTTCTTTCCAGCTAGCGCCACACCACACCACAGGC-3’ and the reverse primer was 5’-GGCTGTGTGTGCTGCTAGCTGGGGAAAGAGGAGGAG-3’. In both cases the template used was the pGEX-4T-eEF2 kinase vector. For transfection of KB cells wild type or mutant FLAG tagged human eEF2 kinase was subcloned into pcDNA3.1 using the BamH1 sites and transfection was carried out using the GenePORTER™ 2 transfection system (Cambridge BioSciences) according to the manufacturer’s instructions using 10µg of plasmid DNA per 10 cm cell culture dish.

Reproducibility

All experiments were performed at least three times, with similar outcomes. In the case of Western blots, data from a typical experiment is shown.

Assays for eEF2 kinase

eEF2 kinase was assayed as described earlier (25) with the following modifications: The cells were harvested in high Ca²⁺ buffer (50 mM HEPES pH 7.4; 50 mM NaCl; 50 mM β-
glycerophosphate; 0.3% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (“CHAPS”) and 1 mM CaCl₂.

Immunoprecipitation of eEF2 kinase was performed as above. Immunoprecipitated eEF2 kinase was washed three times with 20 mM 3-(N-morpholino)propanesulfonic acid (pH 7.0) containing 5 mM MgCl₂; 2 mM EDTA; 1 mM dithiothreitol; 2% (v/v) glycerol; 4.14 mM CaCl₂/5 mM EGTA (free Ca²⁺ = 1 µM at pH 7). The washed immunoprecipitated eEF2 kinase was assayed for activity for 30 min at 30°C in the presence of purified eEF2 (2 µg) and (where added) 0.1 µg CaM in a final volume of 30 µl.
Results:

AMPK phosphorylates eEF2 kinase

We and others have shown previously that conditions that activate the AMP-activated protein kinase (AMPK) lead to increased phosphorylation of eEF2 and activate eEF2 kinase (11-13). We therefore studied whether eEF2 kinase was a substrate for AMPK in vitro. Recombinant human eEF2 kinase was made as a GST-fusion protein in *E. coli*. It was then incubated with a highly purified preparation of AMPK and \[^{32}P\]ATP as described in the Experimental procedures. As shown in Fig. 1A, this resulted in the incorporation of radiolabel into eEF2 kinase which was markedly stimulated by addition of AMP to the reaction, confirming that phosphorylation is indeed due to AMPK. As an alternative, we used immunopurified AMPK, which also catalysed efficient incorporation of radiolabel into GST-eEF2 kinase. However, no such incorporation was seen when we tested the pellet from a mock immunopurification in which pre-immune serum rather than anti-AMPK was used (Fig. 1A). To identify the site(s) phosphorylated by AMPK, eEF2 kinase was radiolabeled by AMPK and then subjected to tryptic digestion. The resulting peptides were resolved by reverse-phase HPLC: two main peaks of radioactive material were observed (Fig. 1B). Further analysis by MALDI mass spectrometry revealed that they corresponded to the peptides containing residues 69 - 82 (peak 1) or 364 – 406 of eEF2 kinase (peak 2; Fig. 1C,D).

Solid-phase Edman degradation revealed that the peptide in peak 1 was phosphorylated at position 10, indicating that it is YSSSGSPANSpFHFK, where the phosphorylated residue is Ser78 of eEF2 kinase (Fig. 1C). Peak 2 contains a very large peptide and, to facilitate further analysis, it was subdigested with Asp-N prior to Edman sequencing. This sample showed release of label at three cycles – 1, 3 and 8 (Fig. 1D). Inspection of the sequences of the predicted
products of Asp-N digestion shows that the release at cycle 3 could be due to Ser366 or Thr389. However, since phosphoamino acid analysis revealed only P-Ser and no P-Thr (data not shown), the Thr389 can be ruled out. The only possible explanation for release at cycle 8 is that it corresponds to Ser398 in the most C-terminal of the Asp-N fragments derived from the original tryptic peptide. The radioactivity at cycle 1 is likely due to leaching from the matrix of some of this peptide that is coupled via the side chain of its N-terminal Asp residue rather than the C-terminal \( \alpha \)-carboxy group. Each Edman sequencing run proceeded through sufficient cycles to go beyond the end of the peptide: thus, there can be no further sites of phosphorylation within these peptides. These data indicate that, \textit{in vitro}, AMPK phosphorylates three sites, Ser78, Ser366 and Ser398, in eEF2 kinase. Other data suggest that Ser398 is the major site phosphorylated by AMPK (see below).

Ser366 and Ser398 lie C-terminal to the catalytic domain of eEF2 kinase (Fig. 1E) in a region that contains several known sites of phosphorylation (12,17,18). Ser398 has not previously been identified as a phosphorylation site in eEF2 kinase. Ser78 is also a novel site: it lies N-terminal to the catalytic domain, immediately adjacent to the CaM binding site (26,27) (Fig. 1E).

To confirm the identity of the three major sites of \textit{in vitro} AMPK phosphorylation in eEF2 kinase and for use as tools for investigating the regulation of the \textit{in vivo} phosphorylation of these sites we generated phosphospecific antisera for each of the two novel sites (Ser78, Ser398) by immunising rabbits with appropriate phosphopeptides. Antibodies were purified as described in the Experimental procedures. Fig. 2A-C shows data for their characterisation. When GST-eEF2 kinase was incubated with AMPK and cold ATP and subsequently analysed by SDS-PAGE and western blotting, clear signals were seen using the anti-(P)Ser78 and anti-(P)Ser398 antisera, as well as for a phosphospecific antiserum for Ser366 that has previously been developed by us.
No signal was observed for the anti-(P)Ser78 and (P)Ser398 antisera when mutants of eEF2 kinase were used in which these sites had been individually mutated to alanines, thus demonstrating the specificity of the phosphospecific antisera for their intended epitopes. eEF2 kinase undergoes extensive autophosphorylation in the presence of its activators, Ca-ions and CaM (28,29). The data in Fig. 2A also show that neither Ser78 nor Ser366 is a site of autophosphorylation, since neither becomes phosphorylated when eEF2 kinase is incubated with Ca\(^{2+}\)-ions and CaM in the absence of AMPK.

Although the AMPK used here was obtained by specific immunoprecipitation from an already highly purified preparation of enzyme, it was theoretically possible that co-immunoprecipitating kinases might contribute to the observed phosphorylation of eEF2 kinase. To confirm that these three sites are indeed phosphorylated by AMPK rather than any possible co-immunoprecipitating kinases, we performed phosphorylation reactions using the immunopurified AMPK in absence or presence of AMP. As shown in Fig. 2D, AMP very markedly increased the phosphorylation of all three sites. This strongly suggests that their phosphorylation is indeed catalysed by AMPK.

Serine 398 is the major site of AMPK phosphorylation in eEF2 kinase.

In order to determine which sites in eEF2 kinase were preferentially phosphorylated by AMPK we again radiolabeled eEF2 kinase in vitro using AMPK and subjected the labeled protein to digestion successively with trypsin and the proteinase Asp-N. The resulting peptides were then analysed by two-dimensional mapping followed by autoradiography to locate radiolabeled species. As shown in Fig. 3A, three radiolabeled peptides were observed. These comprise two minor phosphopeptides (labeled ‘a’ and ‘b’ in Fig. 3A) and one major species (‘c’
in Fig. 3A). To identify which of these are due to phosphorylation at Ser398, we performed a similar analysis using a mutant of eEF2 kinase in which Ser398 is mutated to alanine. As shown in Fig. 3A, the major species (‘c’) seen in the map from wildtype eEF2 kinase is entirely absent from the map derived from the Ser398Ala mutant strongly indicating that Ser398 is the major site in eEF2 kinase that is phosphorylated by AMPK \textit{in vitro}. The phosphopeptides ‘a’ and ‘b’ were identified in a similar manner as the Ser366 and Ser78 phosphopeptides respectively (GJB, unpublished data). Ser398 and adjacent amino acids are completely conserved in the known sequences of eEF2 kinases from mammals.

eEF2 kinase undergoes extensive autophosphorylation, at least \textit{in vitro} (see, e.g., (28,29)), and this can affect its activity, e.g., render it partially independent of Ca\(^{2+}/\text{CaM}\) (29). It was therefore important to establish whether Ser398 was a site of autophosphorylation. We therefore incubated GST-eEF2 kinase with either AMPK or Ca\(^{2+}\)-ions and CaM in the presence of cold ATP. Although a clear signal was seen using the anti-Ser398 phosphospecific antibody when eEF2 kinase was pretreated with AMPK, none was seen under autophosphorylation conditions (Fig. 3B) showing that Ser398 is not a site for autophosphorylation in eEF2 kinase.

We have previously shown that ATP depletion or treatment of cardiomyocytes with an agent that activates AMPK (AICAR) leads to increased phosphorylation of eEF2 (12). There is some evidence that cardiac myocytes may possess a distinct form of eEF2 kinase (12) although this enzyme has neither been purified nor cloned. To examine whether cardiac eEF2 kinase contained a phosphorylation site corresponding to Ser398, we subjected a cardiac extract to chromatography on FPLC (Mono Q Column) as described earlier (12), in order to partially purify eEF2 kinase. Fractions were monitored for eEF2 kinase activity. The material in the peak fraction was subjected to immunoprecipitation with anti-eEF2 kinase antibodies and the
immunoprecipitate was incubated with AMPK and unlabeled ATP. The reaction products were analysed by SDS-PAGE followed by immunoblot analysis using the anti-Ser398 phosphospecific antibody. As shown in Fig. 3C, following treatment of this material with AMPK and cold ATP, a signal was detected with the anti-Ser398 antiserum, indicating that eEF2 kinase from cardiac tissue contains a site corresponding to Ser398 that is phosphorylated by AMPK. A reaction using GST-human eEF2 kinase was performed as a positive control. To be certain that this represents eEF2 kinase from cardiomyocytes, rather than other cell types present in the heart, we immunoprecipitated eEF2 kinase from isolated cardiomyocytes and again treated this with AMPK, followed by analysis by SDS-PAGE and western blotting. As shown in Fig. 3D, a clear signal was again observed for the Ser398 phosphospecific antibody, confirming that a site corresponding to Ser398 is a substrate for AMPK in eEF2 kinase from cardiomyocytes.

*Ser78 and Ser398 are both phosphorylated in intact cells*

Ser366 has previously been shown to be a substrate for S6K1 and p90RSK (10) and phosphorylation here inhibits the activity of eEF2 kinase. It was therefore surprising to find that AMPK phosphorylates this site, given that conditions that activate AMPK increase, rather than decrease, the phosphorylation of eEF2. It was therefore important to study whether Ser78, Ser366 and Ser398 are phosphorylated within cells in a manner consistent with their being physiological targets of AMPK i.e. under conditions where AMPK is activated. To test this, eEF2 kinase was immunoprecipitated either from lysates of serum-fed KB cells or from lysates of cells that had been pretreated with relatively low concentrations of 2-DOG, which depletes cellular ATP leading to a rise in AMP levels. We measured the activity of AMPK using a peptide substrate to confirm that 2-DOG treatment does indeed stimulate it in KB cells (Fig. 4A).
To study the phosphorylation of eEF2 kinase, it was also immunoprecipitated from the cell lysates, and then analysed by SDS-PAGE and western blotting. In serum-fed cells, a clear signal was seen with the anti-(P)Ser78 antibody indicating that Ser78 is indeed phosphorylated within cells (Fig. 4B). However, under these conditions no signal was seen with the anti-(P)Ser398 antiserum. Treatment of cells with 2-DOG led to increased phosphorylation of Ser398 (Fig. 4B), consistent with it being an in vivo target for AMPK. However, 2-DOG treatment actually decreased and, at higher concentrations, abolished the phosphorylation of eEF2 kinase at Ser78 and Ser366 (Fig. 4B). This strongly suggests that Ser78 and Ser366 are not targets for AMPK within cells since, if they were, 2-DOG treatment would be expected to increase their phosphorylation.

Immunoblotting of samples of cell lysate with the phosphospecific (anti-[P]Thr56) antibody for eEF2 itself revealed that 2-deoxyglucose increased the phosphorylation of eEF2 itself (Fig. 4C), under conditions where AMPK is activated as shown by the increased phosphorylation of ACC at Ser79 (Fig. 4C). This is consistent with our earlier findings for the effects of ATP-depletion on eEF2 phosphorylation in other cell types (11,12).

The above data suggest that Ser398 in eEF2 kinase may be a direct target for AMPK within cells, but strongly imply that Ser78 and Ser366 are not. It was thus possible that Ser78 and Ser366 are only phosphorylated by AMPK in vitro and not physiologically, perhaps because phosphorylation at these sites by AMPK is relatively inefficient compared to that of Ser398.

We also studied whether 2-DOG treatment affected the phosphorylation of other sites that have recently been identified in eEF2 kinase, i.e., Ser359, Ser377 and Ser396, for which phosphospecific antisera are also available (17,18). In the case of Ser359, 2-DOG treatment resulted in a decrease in phosphorylation. For Ser377, a marked increase was observed, while for
Ser396 no change was seen (Fig. 4B). Ser377 has previously been reported to be phosphorylated by MAP kinase-activated protein kinase 2 (18). This enzyme lies downstream of the SB203580-sensitive p38 MAP kinase α/β pathway. We therefore tested whether SB203580 affected the 2-DOG glucose-induced phosphorylation of this site. As shown in Fig. 4D, SB203580 completely eliminated the ability of 2-DOG to increase the phosphorylation of this site, suggesting its phosphorylation is mediated through the p38 MAP kinase α/β pathway, not via AMPK. Phosphorylation of Ser398 in eEF2 kinase and the AMPK substrate ACC was not affected by SB203580, indicating that this drug does not interfere with the activity or activation of AMPK. Furthermore, in vitro experiments in which eEF2 kinase was incubated with AMPK and samples were then analysed by SDS-PAGE/western blotting using anti-(P)Ser377 antisera showed that this site cannot be phosphorylated by AMPK in vitro. Thus the increased phosphorylation of Ser377 seen in response to 2-DOG is mediated via activation of the stress-regulated p38 MAP kinase pathway rather than through AMPK.

Ser398 is phosphorylated in cells under conditions where AMPK is activated and eEF2 phosphorylation increases.

Since the data described above suggest that Ser78 and Ser366 are very unlikely to be intracellular targets for AMPK, the remainder of this study focuses on Ser398, the main site that is phosphorylated by AMPK in vitro and one whose phosphorylation does increase in response to AMPK activation induced by 2-DOG.

However, it was conceivable that the effects of 2-DOG on the phosphorylation of eEF2 kinase might be due to consequences of ATP depletion other than the activation of AMPK. For example, severe ATP depletion, induced by very high concentrations of 2-DOG, has been
reported to impair mTOR signaling (16), which in turn is known to modulate the phosphorylation state of eEF2 (reviewed in (7)). To assess whether this was the case in KB cells and at the low concentrations of 2-DOG used here, we studied the regulation of S6K1, a well-known target for mTOR signaling. This analysis makes use of the facts that activation of S6K1 involves its phosphorylation at multiple sites and that this causes a marked retardation of its mobility on SDS-PAGE. In untreated cells, S6K1 migrated as a ladder of bands that differ in their states of phosphorylation indicating that the enzyme is at least partially activated under this condition (Fig. 5A). Treatment of the cells with rapamycin, which blocks mTOR function, causes a complete collapse of the ladder to a single major rapidly-migrating species indicative of dephosphorylation of S6K1. 2-DOG caused only a modest dephosphorylation of S6K1, as indicated by the loss of the slowest migrating, most highly phosphorylated, species. 2-DOG treatment may thus partially inhibit mTOR signaling, although the effect is clearly very much smaller than that of rapamycin or of the very high concentrations of 2-DOG used by Dennis et al. (16).

To study this further, we also tested the effects of AICAR, which following entry to the cell, is converted into a compound that specifically activates AMPK (30,31). In contrast to the situation for 2-DOG, upon treatment of cells with AICAR, there was no shift in the pattern of bands observed for S6K1 (Fig. 5A) indicating that activation of AMPK per se does not interfere with mTOR signaling. Nonetheless, treatment of KB cells with AICAR increased the level of phosphorylation of eEF2 at Thr56, as reported earlier by us for primary rat hepatocytes, Chinese hamster ovary (CHO)-K1 cells and cardiac myocytes (11,12) (Fig. 5B).

Interestingly, AICAR, 2-DOG and rapamycin raised the phosphorylation of eEF2 to similar extents (Fig. 5A; middle section), suggesting that effects other than inhibition of mTOR
signaling may underlie the increase in eEF2 phosphorylation elicited by 2-DOG and in particular by AICAR. All these treatments increased the phosphorylation of ACC, with 2-DOG having the most marked effect (Fig. 5A: bottom section).

However there was a robust increase in ACC phosphorylation with 2-DOG and a slight activation with AICAR. Given that all three treatments, 2-DOG, AICAR and rapamycin, have similar effects on the phosphorylation of eEF2 (Fig. 5A: middle section), while the first two have little or no effect on mTOR signaling, it is clear that 2-DOG, and in particular AICAR, increase eEF2 phosphorylation through events that are independent of the mTOR pathway.

A further, and potent, way of activating AMPK, is to treat cells with the mitochondrial uncoupler, CCCP. This induces a marked increase in the phosphorylation of eEF2 (Fig. 5B). CCCP treatment also increased the phosphorylation of eEF2 kinase to a similar extent to that observed with AICAR (Fig. 5C). Treatment of KB cells with AICAR or 2-DOG increased the phosphorylation of Ser398 in eEF2 kinase (Fig. 5D). However, rapamycin did not induce an increase in the phosphorylation of this site (data not shown), indicating that it is not a consequence of inhibition of mTOR signaling.

The data obtained using AICAR provide several important pieces of information. Firstly, they give strong evidence that Ser398 in eEF2 kinase is a target for AMPK within living cells. Secondly, they show that the increase in phosphorylation of eEF2 kinase at Ser398 is unlikely to be a secondary consequence of inhibition of mTOR signaling. Thirdly, they also show that the increase in eEF2 phosphorylation cannot be attributed to impairment of mTOR signaling (which can under conditions have this effect), and they thus imply that it is likely to be a direct consequence of activation of AMPK and perhaps of the phosphorylation of eEF2 kinase at Ser398. Taken together, our data are consistent with the conclusion that phosphorylation of
Ser398 is a consequence of the activation of AMPK, probably because this site is a direct substrate for this enzyme.

Several other phosphorylation sites have been identified within eEF2 kinase. These include Ser366 and Ser78, which are also targets for AMPK *in vitro* (see above). It was of interest to ascertain whether agents that affect eEF2 kinase activity and activate AMPK, affected their phosphorylation. As shown in Fig. 4B, the phosphorylation of eEF2 kinase at Ser78 or Ser366 decreased following treatment of KB cells with 2-DOG. Although treatment of cells with AICAR, 2-DOG or CCCP caused a marked increase in the phosphorylation of eEF2 itself, none of these treatments affected the phosphorylation of Ser366 in eEF2 kinase, as revealed by western blot analysis of immunoprecipitates from KB cells pretreated with these agents (Fig. 5E).

*Conditions that stimulate AMPK lead to increased activity of eEF2 kinase*

The observation that ATP depletion or AICAR leads to increased phosphorylation of eEF2 suggested that such conditions were also likely to activate eEF2 kinase. The activity of eEF2 kinase is normally completely dependent on Ca$^{2+}$ and calmodulin (28,32,33). There are therefore, in principle, several ways in which such activation might come about. It could involve, for example, an increase in the maximal activity of eEF2 kinase (i.e., at saturating Ca$^{2+}$/CaM) or an increase in the affinity of eEF2 kinase for CaM.

To study the possible effects of AICAR or 2-DOG treatment on the activity of eEF2 kinase, the enzyme was immunoprecipitated from lysates of KB cells or ARVC that had been subjected to various treatments and harvested in the presence of Ca$^{2+}$ to ensure that any tightly bound CaM remained associated with the eEF2 kinase. Kinase assays were then performed in the presence of a low concentration of Ca$^{2+}$ (1 μM) and in the presence or absence of added CaM.
Under the latter conditions, eEF2 kinase activity is dependent upon the CaM that remains bound to eEF2 kinase during the IP. To verify the specificity and efficiency of the immunoprecipitation, we also performed immunoprecipitations using pre-immune IgG as well as anti-eEF2 kinase, and measured eEF2 kinase activity in the pellets, and immunoblotted the supernatant for residual eEF2 kinase. As shown in Fig. 6A, the anti-eEF2 kinase antiserum completely immunoprecipitated the eEF2 kinase from the cell lysate, as judged either by immunoblot or kinase assay. In the case of the pre-immune IgG, both kinase protein and activity remained in the supernatant. Thus, no kinase activity against eEF2 is immunoprecipitated non-specifically. To further verify that the activity against eEF2 shows the characteristics expected for eEF2 kinase, we checked its dependence on Ca\(^{2+}\) and CaM. In anti-eEF2 kinase immunoprecipitates from ARVC, kinase activity against eEF2 was completely dependent upon added Ca\(^{2+}/CaM\) (Fig. 6B; left side). In lysates from KB cells, a trace of activity was still seen in the absence of added Ca\(^{2+}/CaM\) but this was eliminated by addition of the CaM antagonist trifluoperazine (TFP; Fig. 6B, right side) suggesting the trace basal activity was due to endogenous CaM or contaminating Ca\(^{2+}\). Thus, the kinase activity against eEF2 behaves exactly as expected for the known eEF2 kinase indicating that there is unlikely to be any contribution from other (unknown) enzymes.

In assays in which additional CaM was added, no effect of pretreatment of the KB cells with AICAR or 2-DOG on eEF2 kinase activity was evident (Fig. 6C). In contrast, rapamycin did elicit a marked increase in eEF2 kinase activity, when measured in this way, consistent with our earlier conclusion that AMPK activation and inhibition of mTOR signaling affect eEF2 kinase activity in distinct ways. However, when the assays of eEF2 kinase from KB cells were performed without added CaM, it was clear that pretreatment of KB cells with AICAR or 2-DOG caused a significant and reproducible increase in eEF2 kinase activity (Fig. 6D). Data from
multiple experiments are analysed in Fig. 6E. It is clear that treatment of KB cells with any of several treatments that activate AMPK – AICAR, 2-DOG or CCCP – activates eEF2 kinase. This likely explains their common ability to increase the phosphorylation of eEF2. It is also evident from these data that rapamycin also elicits a marked activation of eEF2 kinase as measured in this way. The observation that the effects of agents that activate AMPK are only seen when measured without addition of CaM to the assay could be interpreted to indicate that such agents, and perhaps the phosphorylation of eEF2 kinase at Ser398, increase the binding of eEF2 kinase to CaM, so that more of it co-immunoprecipitates with eEF2 kinase and is therefore able to activate eEF2 kinase in the assay.

In the case of ARVC, a different picture emerged, in that an increase in eEF2 kinase activity was seen even under conditions where CaM was added to the assay (Fig. 6F), while in the absence of CaM a similar pattern was observed although the overall activity was, as expected, lower (data not shown). This could suggest that agents that activate AMPK lead to increased maximal activity of eEF2 kinase in cardiomyocytes. This accords with our earlier conclusions (12) that cardiomyocytes may contain a different isoform of eEF2 kinase and that its regulation, in particular the modulation of its control by Ca$^{2+}$-ions, differs from that of the well-characterised isoform found in many other cell types.
Discussion

The present data are of considerable potential importance for understanding the links between cellular energy metabolism and the regulation of protein synthesis. Given that protein synthesis uses a high proportion of cellular energy, it is perhaps surprising that so little work has previously been devoted to understanding how cells might co-ordinate the rate of protein synthesis with the availability of metabolic energy. Our data thus provide new insights into the poorly understood links between energy status and the rate of protein synthesis. It has been previously noted that low oxygen *per se*, i.e., in the absence of ATP depletion, does not affect protein synthesis rates, while depletion of ATP does lead to a fall in the rate of translation (34). This fits well with our findings which suggest that AMPK is a key link between cellular status and the regulation of protein synthesis: AMPK would be activated under the latter condition, but not under the former.

In this study, we identify a new target of AMPK, i.e., eEF2 kinase. We show that AMPK can phosphorylate three sites in eEF2 kinase *in vitro*. Of these, Ser398 appears to be more efficiently phosphorylated than either Ser78 or Ser366. Using an antibody that we have developed for eEF2 kinase phosphorylated at Ser398, we show that its phosphorylation is enhanced in response to a range of treatments designed to activate AMPK. These include treatment of cells with 2-DOG, with mitochondrial poisons such as CCCP or with the specific AMPK activator, AICAR. Phosphorylation of Ser78 or Ser366 does not increase under such decreases, and in some cases even falls (e.g., Ser78 in cells treated with 2-DOG). Thus, it is likely that Ser398 is a physiological target for AMPK, while Ser78 and Ser366 are almost certainly not.
Our data show that Ser398 is the main site in eEF2 kinase whose phosphorylation is increased under conditions of ATP depletion and the major site in eEF2 kinase that is phosphorylated by AMPK in vitro. Phosphorylation at Ser398 in eEF2 kinase is increased by a range of conditions that activate AMPK and correlates well with phosphorylation of the substrate, eEF2 itself. These conditions all result in activation of eEF2 kinase as measured against eEF2 in vitro suggesting that phosphorylation of eEF2 kinase at Ser398 leads to an increase in its activity. The fact that these effects are smaller than those of rapamycin likely reflects the facts that rapamycin also affects the phosphorylation of other sites in eEF2 kinase, e.g., Ser359 and Ser366 (10,17) which results in additional activation of eEF2 kinase. Rapamycin can decrease the phosphorylation of both sites, and since phosphorylation of either exerts an inhibitory effect on eEF2 kinase activity, this likely contributes to the relatively larger increase in eEF2 kinase activity seen following rapamycin treatment.

As phosphorylation of Ser366 serves to decrease the activity of eEF2 kinase (10), it would make little sense for its phosphorylation to be enhanced under conditions of ATP depletion where eEF2 phosphorylation actually increases. Very recent work in this laboratory suggests that phosphorylation at Ser78 may also decrease the activity of eEF2 kinase and a similar argument would thus also apply to this site (GJB, unpublished findings).

2-DOG treatment also increased the phosphorylation of eEF2 kinase at Ser377, and, as expected, this was mediated through the SB203580-sensitive p38 MAP kinase pathway (18). Since phosphorylation here is not thought to affect the activity of eEF2 kinase, this modification is unlikely to contribute to its activation following 2-DOG treatment.

Recent work from Rider’s group shows that in anoxic cardiomyocytes AMPK activation was associated with an increase in eEF2 phosphorylation and an inhibition of protein synthesis
These authors identified Ser78 in eEF2 kinase as a potential site for phosphorylation by AMPK \textit{in vitro}. Our data corroborate this finding but reveal that, although Ser78 is phosphorylated \textit{in vivo}, its phosphorylation does not change in a manner consistent with it being a site for phosphorylation by AMPK in living cells.

In principle, one could test the effect of phosphorylation of eEF2 kinase at Ser398 by incubating purified eEF2 kinase with AMPK \textit{in vitro} and then measuring its activity or ability to bind CaM. However, this possible approach is made complicated by the fact that AMPK also phosphorylates at least two other sites in eEF2 kinase, Ser78/366, and by the fact that phosphorylation of at least, possibly both, of these sites also affects eEF2 kinase activity (10 and unpublished data).

Recent data from Yan \textit{et al.} (13) showed that glucose depletion increases the level of phosphorylation of eEF2 in primary pancreatic islets and in β-cell lines. Their study also provided evidence that these effects involve AMPK and changes in the activity of eEF2 kinase.

Our data suggest that, while mTOR signaling may be impaired under conditions of severe ATP depletion, it is not affected very much by modest ATP depletion, e.g., by low concentrations of 2-DOG. Nor is it influenced by the activation of AMPK by treatment of cells with AICAR. This suggests that mTOR signaling is not a direct target for inhibition by AMPK, at least in the cells we have studied, and that modest impairment of ATP production does not markedly impair mTOR signaling. It is important to note that in the study by Dennis \textit{et al.} (16), where inhibition of mTOR signaling was observed, very high concentrations of 2-DOG were used (100 mM).

Our data show that AICAR and, at low concentrations, 2-DOG, have little or no effect on the phosphorylation of proteins involved in the initiation stage of mRNA translation (S6K1 (Fig. 5), eIF2 and 4E-BP1 (11). Thus, it seems likely that modest ATP depletion may primarily affect
translation elongation rather than initiation. This does seem logical, as far more energy is consumed during elongation that during initiation (for the assembly of a typical protein, >99% of the energy is used in elongation). Furthermore, inhibition of elongation will retard the rate of progress of ribosomes along the mRNA, thus saving energy, but without causing polysomes to dissociate (in fact, there would likely accumulate). This would therefore allow translation to resume fully and quickly once cellular energy levels had recovered and eEF2 has been dephosphorylated, without any need to reassemble polysomes. This regulatory mechanism may be of considerable importance in matching the rate of translation elongation to fluctuations in cellular energy status.
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**Figure legends**

**Fig. 1.** Identification of sites within eEF2 kinase that are phosphorylated by AMPK. (A) GST-eEF2 kinase was incubated with extensively purified AMPK (‘AMPK prep’) or immunoprecipitates obtained from this material using pre-immune IgG or anti-AMPK. Incubations were performed in the presence of $[\gamma^{-32}P]$ATP and phosphorylation was analyzed as described in Experimental procedures. Samples were then subjected to SDS-PAGE. The figure is an autoradiograph of the fixed gel. (B) GST-eEF2 kinase was incubated with immunopurified AMPK in the presence of $[\gamma^{-32}P]$ATP, subjected to digestion by trypsin and the resulting peptides were analyzed by reverse phase (C$_{18}$) HPLC. The dotted line indicates the percentage (v/v) of CH$_3$CN. (C, D) The radioactive fractions corresponding to peaks 1 and 2 were subjected to solid phase sequencing, and the release of radioactivity was determined at each cycle. The material in peak 2 had been subjected to further digestion with Asp-N prior to the solid phase sequencing shown here. The sequences of the peptides are displayed at the top of each panel. The phosphorylated serines are indicated in large bold text (S) and Asp-N cleavage sites are shown by arrows in Panel D. (E) Diagram showing the location of residues in eEF2 kinase phosphorylated by AMPK *in vitro* in relation to known functional domains. The black box denotes the catalytic domain, the hatched area indicates the CaM-binding site and the stippled area signifies the substrate (eEF2) binding domain.

**Fig. 2.** Validation of phosphospecific antibodies for phosphorylation sites in eEF2 kinase. 

(A-C) Samples (1µg) of GST-eEF2 kinase or the S78A, S366A and S398A mutants of GST-eEF2 kinase were incubated with MgATP in the presence of either 10mU of immunopurified AMPK or in some cases (A) with Ca$^{2+}$/calmodulin for 10 min. Samples were analyzed by SDS-
PAGE and western blotting using antibodies specific for eEF2 kinase phosphorylated at residues Ser78, Ser366 and Ser398 as described in the Experimental procedures. The loading of eEF2 kinase was confirmed by Coomassie staining (A) or by probing a parallel blot with anti-eEF2 kinase (B-C). In (D), GST-eEF2 kinase (1 µg) was incubated with MgATP and 1mU of immunopurified AMPK in the presence or absence of 200 µM AMP. Samples were analyzed by SDS-PAGE and western blotting using antibodies specific for eEF2 kinase phosphorylated at residues Ser78, Ser366 or Ser398 and total eEF2 kinase as described in the Experimental procedures. Immunoblotting with antibodies to the α1 and α2 forms of AMPK confirmed equal amounts of immunopurified AMPK were present in each assay. The position of the heavy chain of the IgG (arising from the immunoprecipitation of the AMPK) is also indicated.

**Fig. 3.** Serine 398 is the major site within eEF2 kinase that is phosphorylated by AMPK. (A) Wild type GST-eEF2 kinase (GST-(WT)eEF2 kinase) or GST-kinase in which Ser398 had been mutated to Ala (GST-(S398A)eEF2 kinase) were incubated with immunopurified AMPK in the presence of [γ-32P]ATP, subjected to digestion by trypsin followed by Asp-N and the resulting phosphopeptides were analysed by two-dimensional peptide mapping and autoradiography. (B) GST-eEF2 kinase was incubated with MgATP in the presence of either 10mU of immunopurified AMPK or of Ca2+/calmodulin for 10 min. Samples were analyzed by SDS-PAGE and western blotting using antibodies specific for eEF2 kinase phosphorylated at Ser398 or total eEF2 kinase. (C) GST-eEF2 kinase or eEF2 kinase isolated by FPLC from lysates of isolated ARVC, and then further purified by immunoprecipitation from the peak FPLC fraction, were incubated with MgATP in the presence of 10mU of immunopurified AMPK for 60 min. Samples were analyzed by SDS-PAGE and western blotting using an antibody specific for Ser398 phosphorylated eEF2.
kinase. (D) GST-eEF2 kinase or cardiac eEF2 kinase immunoprecipitated from lysates from isolated adult rat cardiac myocytes were incubated with MgATP in the presence of 10mU of immunopurified AMPK for 60 min. Samples were analyzed by SDS-PAGE and western blotting using an antibody specific for eEF2 kinase phosphorylated at Ser398.

Fig. 4. Analysis of the phosphorylation of eEF2 kinase in cells. (A-C) KB cells (70 % confluent) were incubated in DMEM (containing 1g/l D-glucose) plus fetal calf serum and treated with 5 or 25mM 2-DOG for 30 min. (A) AMPK was immunoprecipitated from 100 µg of cell lysate protein and assayed for activity in the presence of [γ-³²P]ATP against a peptide substrate as described in the Experimental procedures. Results are the mean of two independent experiments ±SEM. The AMPK immunoprecipitates were also subjected to SDS-PAGE and western blotted for α1/α2 AMPK (top section) to show that the amounts of AMPK were similar in all cases. (B) eEF2 kinase was immunoprecipitated from 100µg of cell lysate protein and subjected to SDS-PAGE before western blotting for phosphorylated or total eEF2 kinase, as indicated. KB cells were treated with the indicated concentrations of 2-DOG for 30 min. In (C), equal amounts of cell lysate (20µg protein) were analysed directly by SDS-PAGE and western blotting for ACC phosphorylated at Ser79 or eEF2 phosphorylated at Thr56, or, as a loading control, for total eEF2. (D) KB cells (70 % confluent) were incubated in DMEM (containing 1g/l D-glucose) plus fetal calf serum and then pretreated with 10µM SB203580 or DMSO as control for 30 min before being incubated in the presence or absence of 5 mM 2-DOG for 30 min. eEF2 kinase was immunoprecipititated from 100µg of cell lysate protein and subjected to SDS-PAGE before western blotting for phosphorylated or total eEF2 kinase, as indicated. Equal amounts of cell
lysate (20µg by protein) were analysed directly by SDS-PAGE and western blotting for ACC phosphorylated at Ser79.

**Fig. 5.** Analysis of the phosphorylation of eEF2 kinase in cells. (A-G) KB cells (70 % confluent) were incubated in DMEM (containing 1g/l D-glucose) plus fetal calf serum and treated as indicated above each lane. (A,B) lysates were subjected to SDS-PAGE and western blotted for the following: (A) total S6K1 (the band marked with a * is a non specific band which has been reported previously), eEF2 phosphorylated Thr56 eEF2 and ACC phosphorylated at Ser79 or (B) eEF2 phosphorylated at Thr56 and total eEF2. In some cases (C-F), eEF2 kinase (eEF2k) was first immunoprecipitated from 100µg of cell lysate protein and then subjected to SDS-PAGE before western blotting for eEF2 kinase phosphorylated at the indicated site(s) or total eEF2 kinase (as a loading control), as indicated. Western blots were developed with the following antisera: (C) eEF2 kinase phosphorylated at Ser398 or total eEF2 kinase, (D) eEF2 kinase phosphorylated at Ser398 and total eEF2 kinase, and (E) eEF2 kinase phosphorylated at Ser366, total eEF2 kinase and eEF2 phosphorylated at Thr56. The positions of the corresponding bands are indicated by labeled arrows.

**Fig. 6.** Analysis of changes in eEF2 kinase activity upon treatment of cells with agents that activate AMPK. (A) KB cells (70% confluent) were starved of serum overnight, lysed and the cell lysates incubated with equal amounts (1µg per IP) of either pre-immune IgG or anti-eEF2 kinase antibody coupled to protein G Sepharose. The whole lysate and the pre-immune and anti-eEF2 kinase immune pellet and supernatant (sup.) fractions were either subjected to SDS-PAGE and immunoblotting for eEF2 kinase (top section) or assayed *in vitro* for kinase activity against
purified eEF2 (lower section position of radiolabeled eEF2 is indicated). (B) Lysates of ARVC (left side) were subjected to IP with anti-eEF2 kinase and the pellets were assayed for kinase activity against eEF2 under the conditions indicated. Right side, KB cell lysates were assayed directly for eEF2 kinase activity under the conditions shown (eEF2 was omitted from the reaction where indicated ‘-‘). The positions of the radiolabeled eEF2 and of a non-specific band that becomes labeled in KB cell lysates are shown. (C-E) KB cells (70% confluent) that had been starved of serum overnight were incubated in DMEM (containing 1g/l D-glucose) containing fetal calf serum. eEF2 kinase was immunoprecipitated from 100µg of cell lysate and washed three times in low Ca\(^{2+}\) kinase buffer before assaying for eEF2 kinase activity in the absence (D) or presence (C) of additional calmodulin. (E) Data from multiple experiments of the type shown in (D) was quantified by PhosphorImager analysis, the number of replicates being denoted by the number above the bar. Where no ‘error bar’ is shown, it was too small to be visible. As assessed by Student’s t-test, the changes seen with AICAR, DOG or rapamycin are statistically significant at \(p<0.05\) (DOG; #) or \(<0.01\) (others; *). Only the data for CCCP failed to reach significance. (F) isolated ARVC (cultured overnight as described in Experimental Procedures) were treated as indicated and harvested in high Ca\(^{2+}\) extraction buffer. After IP with anti-eEF2 kinase antibodies, pellets were assayed for kinase activity against purified eEF2 in the presence of CaM. Panels B-D and F show autoradiographs of the corresponding stained SDS-gels.
**Fig. 1**

**A**

![Diagram](https://via.placeholder.com/150)

**B**

![Graph](https://via.placeholder.com/150)

**C**

![Graph](https://via.placeholder.com/150)

**D**

![Graph](https://via.placeholder.com/150)

**E**

![Diagram](https://via.placeholder.com/150)
Fig. 2

A

Antiserum

(P) Ser78 -eEF2k

(P)Ser366 -eEF2k

(Coomassie stained)

AMPK

Ca²⁺/calmodulin

GST-(WT)eEF2k

GST-(S78A)eEF2k

B

Antiserum

(P)Ser366 -eEF2k

(P) Ser78 -eEF2k

Total eEF2k

AMPK

GST-(WT)eEF2k

GST-(S366A)eEF2k

C

Antiserum

(P)Ser398 -eEF2k

(P)Ser78 -eEF2k

Total eEF2k

AMPK

GST-(S398A)eEF2k

GST-(WT)eEF2k

D

Antiserum

(P)Ser78 -eEF2k

(P)Ser366 -eEF2k

(P)Ser398 -eEF2k

Total eEF2k

AMPK

IgG

GST-(WT)eEF2k

0 5 10 0 5 10

Time (minutes)

200 µM AMP
Fig. 3

A

GST-(WT)eEF2k + AMPK (Trypsin + Asp-N)

(a) [Antiserum]

(b) [Total eEF2k]

(c) [AMPK]

B

Antiserum

- [AMPK]

- [Ca\(^{2+}\)/calmodulin]

C

FPLC purified heart eEF2k

-GST-(human)eEF2K

- [FPLC purified heart eEF2k]

- [GST-(human)eEF2K]

D

Immunopurified cardiomyocyte eEF2k

-GST-(human)eEF2k

- [Immunopurified cardiomyocyte eEF2k]

- [GST-(human)eEF2k]
Fig. 4

A

AMPK activity (mU/100mg protein).

H2O control 5mM 2-DOG 25mM 2-DOG

B

Antiserum for WB

(P)Ser78 eEF2k (P)Ser366 eEF2k (P)Ser398 eEF2k (P)Ser359 eEF2k (P)Ser377 eEF2k (P)Ser396 eEF2k Total eEF2k

H2O control 5 mM 2-DOG 25 mM 2-DOG

C

Antiserum for WB

(P)Ser79 ACC (P)Thr56 eEF2 Total eEF2

H2O control 5 mM 2-DOG 25 mM 2-DOG

D

eEF2k IP

lysate

(DMSO) 10µM SB203580

(P)Ser377 eEF2k (P)Ser398 eEF2k Total eEF2k (P)Ser79 ACC

5mM 2-DOG
Fig. 5

A

|          | AICAR 1mM | AICAR 1mM | Rap 50nM | DOG 10mM |
|----------|-----------|-----------|----------|-----------|
| Con 15 min |           |           |          |           |
| Con 30 min |           |           |          |           |
| Con 60 min |           |           |          |           |
| Con 30 min |           |           |          |           |

Antiserum

Lysate

S6K1

(P)Thr56 eEF2

(P)Ser79 ACC

B

|          | CCCP, 10µM (min) | AICAR, 1mM (min) | DOG 10mM |
|----------|------------------|------------------|----------|
| Con 0.5  |                  |                  |          |
| Con 1    |                  |                  |          |
| Con 2    |                  |                  |          |
| Con 5    |                  |                  |          |
| Con 10   |                  |                  |          |
| Con 20   |                  |                  |          |
| Con 30   |                  |                  |          |

Antiserum

Lysate

(P)Thr56 eEF2

eEF2

C

|          | CCCP, 10µM (min) |
|----------|------------------|
| Con 1    |                  |
| Con 2    |                  |
| Con 5    |                  |

Antiserum

eEF2k

Lysate

(P)Ser366 eEF2k

Pellet

eEF2k

D

|          | AICAR, 1mM (min) | DOG 10mM |
|----------|------------------|----------|
| Con 15   |                  |          |
| Con 30   |                  |          |

Antiserum

eEF2k

Pellet

eEF2k

E

|          | AICAR 1mM | DOG 10mM | Rap 50nM | CCCP 10µM |
|----------|-----------|-----------|----------|-----------|
| Con 30 min |           |           |          |           |
| Con 30 min |           |           |          |           |
| Con 60 min |           |           |          |           |
| Con 1 min  |           |           |          |           |

Antiserum

eEF2k

Lysate

(P)Thr56 eEF2

(P)Ser366 eEF2k

Pellet
Fig. 6

A  KB cells, added CaM

B  ARVC (IP) kinase assay

C  KB cells (IP), kinase assay, added CaM

D  KB cells (IP), kinase assay, no added CaM

E  KB cells (IP), kinase assay, no added CaM

F  ARVC (IP), kinase assay, added CaM
Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398

Gareth J. Browne, Stephen G. Finn and Christopher G. Proud

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