Cellular Signalling 36 (2017) 212–221

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Direct and indirect activation of eukaryotic elongation factor 2 kinase by AMP-activated protein kinase

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ARTICLE INFO

Keywords:
AMPK
mTORC1
eEF2K
eEF2
Compound 991
Translation

ABSTRACT

Background: Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) is a key regulator of protein synthesis in mammalian cells. It phosphorylates and inhibits eEF2, the translation factor necessary for peptide translocation during the elongation phase of protein synthesis. When cellular energy demand outweighs energy supply, AMP-activated protein kinase (AMPK) and eEF2K become activated, leading to eEF2 phosphorylation, which reduces the rate of protein synthesis, a process that consumes a large proportion of cellular energy under optimal conditions.

Aim: The goal of the present study was to elucidate the mechanisms by which AMPK activation leads to increased eEF2 phosphorylation to decrease protein synthesis.

Methods: Using genetically modified mouse embryo fibroblasts (MEFs), effects of treatments with commonly used AMPK activators to increase eEF2 phosphorylation were compared with that of the novel compound 991. Bacterially expressed recombinant eEF2K was phosphorylated *in vitro* by recombinant activated AMPK for phosphorylation site-identification by mass spectrometry followed by site-directed mutagenesis of the identified sites to alanine residues to study effects on the kinetic properties of eEF2K. Wild-type eEF2K and a Ser491/Ser492 mutant were retrovirally re-introduced in eEF2K-deficient MEFs and effects of 991 treatment on eEF2 phosphorylation and protein synthesis rates were studied in these cells.

Results & conclusions: AMPK activation leads to increased eEF2 phosphorylation in MEFs mainly by direct activation of eEF2K and partly by inhibition of mammalian target of rapamycin complex 1 (mTORC1) signaling. Treatment of MEFs with AMPK activators can also lead to eEF2K activation independently of AMPK probably *via* a rise in intracellular Ca^{2+}. AMPK activates eEF2K by multi-site phosphorylation and the newly identified Ser491/Ser492 is important for activation, leading to mTOR-independent inhibition of protein synthesis. Our study provides new insights into the control of eEF2K by AMPK, with implications for linking metabolic stress to decreased protein synthesis to conserve energy reserves, a pathway that is of major importance in cancer cell survival.

1. Introduction

eEF2K is a highly conserved Ser/Thr kinase and member of the atypical alpha kinase family [1–3]. eEF2K is a highly regulated protein kinase and its activity is almost entirely dependent on Ca^{2+}/calmodulin (CaM), which binds to an amino-terminal regulatory region [4,5]. eEF2K activity is also controlled by multi-site phosphorylation, which modulates CaM-binding, kinetic properties and proteasomal degradation [6–9]. Besides control by protein kinases from various signaling pathways, such as inactivation by p70 ribosomal S6 kinase (p70S6K) downstream of mammalian target of rapamycin complex-1 (mTORC1) [10,11] and activation by AMPK [12] or cAMP-dependent protein kinase (PKA) [13,14], eEF2K extensively autophosphorylates at several sites in the presence of Ca^{2+}/CaM [15,16] to acquire maximal activity. Once activated, eEF2K phosphorylates eEF2 on Thr56 [17,18], preventing its binding to the ribosome, which leads to ribosomal stalling and reversibly halts protein synthesis elongation. It is noteworthy that eEF2K is the only protein kinase known to phosphorylate eEF2 at Thr56.

AMPK is a key regulator of cellular energy homeostasis becoming

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http://dx.doi.org/10.1016/j.cellsig.2017.05.010
Received 12 December 2016; Received in revised form 27 April 2017; Accepted 10 May 2017
Available online 11 May 2017

activated during metabolic stress *via* a rise in AMP:ATP ratio. AMPK phosphorylates eEF2K *in vitro* and phosphorylation at Ser398 was proposed to cause eEF2K activation [12]. The inhibition of protein synthesis by AMPK at peptide elongation is crucial for survival under energy-depleting conditions and logical, since this step of protein synthesis is the most costly in terms of ATP equivalents consumed [19]. It is therefore not surprising that AMPK activation leads to the phosphorylation of eEF2 [20], thereby decreasing the rate of protein synthesis. AMPK activation also inhibits protein synthesis initiation by decreasing PKB/mTORC1 signaling at different levels [21–23].

Several cellular stresses, most of which activate AMPK, have been shown to increase eEF2 Thr56 phosphorylation, namely skeletal muscle contraction [24–26], ischemia [27], hypoxia [28], increasing cell density [29], nutrient deprivation [30], growth factor retrieval [31], genotoxic agents [32], endoplasmic reticulum stress [33,34], ribosomal stress [35], oxidative stress [29,36], osmotic stress [37], chemical stress [29], alcohol [38], and changes in pH [39,40] or temperature [41]. Also, treatment of cells with AMPK activators such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) [12], 2-deoxy-D-glucose (2DG) [12], the "Abbott compound" A-769662 [42], metformin/phenformin [43] or oligomycin [20] leads to increased eEF2 phosphorylation. Most of these chemical treatments activate AMPK indirectly by causing ATP depletion (2DG [44], metformin [45], oligomycin [46]) and thus lack specificity. On the other hand, A-769622 and a small-molecule benzimidazole derivative called "991" activate AMPK by binding directly to the AMPKβ subunit [47]. Compound 991 is the more potent of the two direct AMPK activators and A-769622 seems only to target AMPKβ1. Incubation of skeletal muscles with 991 led to activation of both AMPKβ1- and AMPKβ2-containing complexes to increase glucose-uptake [48,49] and 991 treatment of hepatocytes antagonized glucagon signaling [50], both in an AMPK-dependent manner.

In the present study, we used 991 to activate AMPK in genetically modified mouse embryonic fibroblast (MEF) cell lines deficient either for the two AMPK catalytic subunits, for tuberin of the tuberous sclerosis complex (TSC2), a negative regulator of mTORC1 signaling, or for eEF2K to monitor eEF2 phosphorylation. In parallel, we identified Ser491/Ser492 as a new key *in vitro* phosphorylation site for AMPK in eEF2K and studied eEF2 phosphorylation by 991 treatment and effects on protein synthesis in eEF2K-*null* MEFs in which wild-type eEF2K or a S491A/S492A mutant had been re-introduced by viral transfection. Our data provide new insights into the mechanisms by which AMPK activation leads to increased eEF2 phosphorylation with implications for protein synthesis inhibition in response to cellular stresses.

2. Material and methods

2.1. Reagents and materials

Compound 991 (previously referred to as ex229 [47] from patent application WO2010036613, Merck Sharp & Dohme Corp., Metabasis Therapeutics, Inc. Novel cyclic benzimidazole derivatives useful anti-diabetic agents, 2010) was kindly provided by AstraZeneca, Mölndal, Sweden. All other reagents were from Sigma Aldrich. Cell culture reagents were from Life Technologies. Oligonucleotides were from Integrated DNA Technologies (IDT). [$\gamma-{}^{32}$P] ATP was from Perkin Elmer. Anti-total ACC (Merck Millipore, Catalogue No. 04–322), anti-P-Ser79-ACC (Merck-Millipore, Catalogue No. 07–303), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Merck-Millipore, Catalogue No. MAB374), anti-total eEF2 (Santa Cruz Biotechnology, Catalogue No. 13003), anti-P-Thr56-eEF2 (Cell Signaling Technologies, Catalogue No. 2331), anti-total p70S6K (Cell Signaling Technologies, Catalogue No. 9202), anti-P-Thr389-p70S6K (Cell Signaling Technologies, Catalogue No. 9234), anti-P-Thr172-AMPKα (Cell Signaling Technologies, Catalogue No. 2535) and anti-total eEF2K (Cell Signaling Technologies, Catalogue No. 3692) antibodies were from the sources cited. Sheep polyclonal anti-total AMPKα1/2 antibodies were kindly provided by Prof. G. Hardie (Dundee, UK). HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. A peptide surrounding Ser491/Ser492 of eEF2K (CKWNLLNSSRLHLPI) was synthesized with or without Ser491 phosphorylated and with a N-terminal Cys for coupling to keyhole limpet haemocyanin (KLH) or bovine serum albumin (BSA) (Imject maleimide-activated KLH/BSA kit, Thermo Fisher Scientific). The KLH-coupled phosphopeptide was injected in rabbits (Thermo Fisher Scientific) and the serum was affinity purified on both BSA-coupled phosphopeptide and non-phosphopeptide linked to CH-activated Sepharose 4B (GE Healthcare). Catalytic subunits of PKA from bovine heart and recombinant bacterially expressed AMPK (AMPKα2β1γ1 or AMPKα2β1γ1 complexes) activated by recombinant bacterially expressed LKB1-MO25-STRAD complex were prepared as described [51]. Peptides were synthesized by Vincent Stroobant (LICR, Brussels, BE). The pMSCV-neo vector was provided by Jean-Bernard Beaudry (de Duve Institute, Brussels).

2.2. Cell culture & immortalization

MEFs were maintained in classic culture medium (DMEM containing 4.5 g·l^{-1} glucose, 4 mM glutamine, 1 mM pyruvate, penicillin/streptomycin and 10% (*v/v*) FBS) under a humid atmosphere containing 5% CO_2. Typically, cells were seeded in 6-well-plates (2 ml per well) and grown overnight to subconfluence. Prior to stimulation, the cells were washed in warm PBS and the medium was changed to 1 ml of stimulation medium (DMEM with or without $CaCl_2$ containing 4.5 g·l^{-1} glucose, 4 mM glutamine, 1 mM pyruvate). All calcium-free media were supplemented with 0.5 mM EGTA. Primary MEF cells from eEF2K$^{-/-}$ mice and MEF cells deleted for TSC2 were generated as described [52]. Primary eEF2K$^{-/-}$ MEFs were immortalized using a retrovirus coding for BMI1 as described [53]. Wild-type or S491A/S492A mutant Nt-FLAG-eEF2K was retrovirally introduced into the genome of eEF2K$^{-/-}$ MEFs using the murine stem cell virus (MSCV) approach (Clontech) and neomycin resistance carried by the viral genome was selected. SV40-T-transformed MEF cells deficient for both AMPK catalytic α subunits or for LKB1 were kindly provided by Benoit Viollet (INSERM and Cochin Institute, Paris).

2.3. Cell lysis & immunoblotting

Following cell incubation, media were removed and the cells were washed in cold PBS before lysis in buffer containing 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5% (*v/v*) 2-mercaptoethanol, 50 mM NaF, 5 mM $Na_4P_2O_7$, 5 mM sodium β-glycerophosphate, 1 mM $NaVO_3$, 1 mM dithiothreitol, 0.1% (*w/v*) Triton X-100 and Complete™ protease inhibitor cocktail (150 μl of lysis buffer per well). Extracts were centrifuged (20,000*g* × 5 min at 4 °C) and protein concentrations were measured using the Bio-Rad Protein Assay with BSA as a standard. For immunoblotting, extracts (10 μg of sample protein) were first boiled in Laemmli buffer (60 mM TRIS-HCl pH 6.8, 2% (*w/v*) SDS, 10% (*v/v*) glycerol, 5% (*v/v*) beta-mercaptoethanol, and 0.01% (*w/v*) bromophenol blue) and loaded on 7.5% or 10% (*w/v*) polyacrylamide gels. Following SDS-PAGE, proteins were transferred onto PVDF membranes, which were then blocked in TRIS-buffered saline (TBS) containing 0.1% (*v/v*) TWEEN and 5% (*w/v*) BSA. Membranes were incubated overnight at 4 °C with the indicated primary antibodies diluted in blocking buffer, then washed extensively with TBS containing 0.1% (*v/v*) TWEEN before and after incubation for 1 h with HRP-conjugated secondary antibodies. All antibodies were used at a dilution of 1:1000, except anti-total eEF2, anti-total AMPK, anti-P-eEF2 and anti-GAPDH antibodies which were used at dilutions of 1:5000, 1:10,000, 1:20,000 and 1:40,000, respectively. Secondary antibodies were used at a dilution of 1:20,000. Immunodetection was by ECL Classico substrate (Merck Millipore). Immunoblots were quantified by scanning densitometry using ImageJ

software.

2.4. Protein kinase assays

Purified recombinant activated AMPK and purified PKA were assayed for 10 min at 30 °C in phosphorylation buffer (10 mM MOPS, pH 7.0, 0.5 mM EDTA, 10 mM magnesium acetate, 5 mM dithiothreitol, 100 μM [$\gamma-^{32}$P] ATP (specific radioactivity 1000 cpm/pmol) by measuring ^{32}P incorporation into 200 μM AMARA peptide (AMARAASAAALRRR) for AMPK and 200 μM PFK-2 peptide (PVRMRRNSFT) for PKA as described [54]. Recombinant eEF2K was assayed in phosphorylation buffer without EDTA containing 10 μg/ml calmodulin and the indicated concentrations of free Ca^{2+} obtained with 2 mM EGTA and up to 2 mM $CaCl_2$ calculated with Maxchelator (Stanford University). Assays were for 4 min at 30 °C using 0.5 mM MHC1 peptide (RKKFGESEKTKTKEFL) as substrate. One unit of protein kinase activity catalyzes the incorporation of 1 nmol $\cdot min^{-1}$ of ^{32}P into the substrate peptide under the assay conditions.

2.5. Expression and purification of recombinant eEF2K

The pGEX6p1 vector containing wild-type human eEF2K cDNA was generated as described [55]. Site-directed mutagenesis was carried out using the Herculase II Fusion DNA polymerase (Stratagen) with the QuikChange (Agilent) protocol. GST-tagged eEF2K expressed in BL21 *E. coli* cells was purified as described [15]. Briefly, centrifuged cell lysate was subjected to chromatography first on DEAE-Sepharose and then on GSH-Sepharose. Fractions containing eEF2K activity were pooled and concentrated using a 100 kD ultra-filtration unit (Amicon) while changing the buffer to enzyme storage buffer (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 0.1% (*v/v*) 2-mercaptoethanol, 10% (*v/v*) glycerol).

2.6. In vitro phosphorylation

Recombinant eEF2K (1 μg) was phosphorylated with 0.4 units of purified activated recombinant bacterially expressed AMPK or purified PKA catalytic subunits in 20 μl of kinase assay buffer containing 200 μM AMP (for AMPK only) and 100 μM [$\gamma-^{32}$P] ATP (specific radioactivity 1000 cpm/pmol). For kinetic studies, incubations contained non-radioactive ATP at the same concentration. After 1 h at 30 °C, reactions were stopped on ice and 10 μl of mixture was taken for further analysis. For kinetic studies, samples were diluted 5-fold in enzyme storage buffer prior to eEF2K assay. For measurements of the stoichiometry of phosphorylation, proteins were separated by SDS-PAGE in 7.5% (*w/v*) polyacrylamide gels. Gels were stained using PageBlueTM (Thermo Fisher Scientific) for protein band quantification by OdysseyTM infrared imaging. Bands were then excised from the gels for measurement of ^{32}P-incorporation by liquid scintillation as described previously [51].

2.7. Phosphorylation site identification

Recombinant GST-eEF2K (3 μg) was phosphorylated as described above with 1 unit of activated AMPK in a final volume of 60 μl at 30 °C. After 1 h, the reaction was stopped on ice, 20 μg of BSA was added as carrier and proteins were precipitated with a final concentration of 10% (*w/v*) trichloroacetic acid for 45 min on ice. Precipitated proteins were collected by centrifugation (20,000*g* × 10 min at 4 °C), washed in acetone, vacuum-dried and resuspended in 50 μl of 50 mM NH_4HCO_3 pH 8.0 for overnight digestion with trypsin at 30 °C. Peptides were separated by reverse-phase narrow-bore HPLC and radioactive peaks were analyzed by LC-MS/MS as described [56]. Multi Stage Activation (MSA) was enabled for phosphate neutral loss of 98, 49 or 32.66 with respect to the precursor *m/z*. Peak lists were generated and searched using SequestHT and PhosphoRS 3.1 within the Proteome Discoverer 1.4 software package (Thermo Fisher Scientific) against a home-made protein database containing the human eEF2K sequence obtained from Uniprot and sequences of the different mutants used in this study. Phosphorylation site identification was performed as described [56] and validated manually.

2.8. Measurement of protein synthesis

Protein synthesis rates were determined by [2,3,4,5,6-^{3}H]-Phe incorporation into tricholoroacetic acid-precipitated material as described [57]. Cells grown to ~50% confluence were incubated for 30 min with AMPK activators and/or Torin prior to incubation for 4 h with [2,3,4,5,6-^{3}H]-Phe at 2 μCi/ml with 0.4 mM of cold Phe coming from the medium.

2.9. Statistical analysis

The results are expressed as means ± S.E.M. of at least three separate experiments. Multiple comparisons were tested for statistically significant differences by 2-way ANOVA followed by Bonferroni's *post-hoc* test using the GraphPad Prism software and $P < 0.05$ was considered significant. Otherwise, a paired 2-sided Student's *t*-test was used.

3. Results

3.1. Increased eEF2 phosphorylation by AMPK activators requires Ca^{2+} and is partly dependent on AMPK

Several studies have reported that both physiological and pharmacological AMPK activation leads to increased eEF2 phosphorylation, most likely *via* eEF2K activation. Accordingly, in immortalized MEF cells lacking eEF2K, no eEF2 Thr56 phosphorylation was seen under conditions that led to AMPK activation monitored by AMPKα Thr172 phosphorylation (Supplemental Fig. S1). We therefore investigated whether eEF2 phosphorylation was also strictly dependent on AMPK. In wild-type MEF cells incubated under normal conditions with Ca^{2+} present, eEF2 phosphorylation was seen in the basal condition, and increased in response to treatment of the cells with AMPK activators phenformin, AICAR, A769662 and compound 991 (Fig. 1A,B). To our surprise, when MEFs deleted for both AMPK catalytic subunits were incubated under the same conditions, eEF2 phosphorylation persisted in basal conditions as well as upon treatment with AMPK activators, albeit mostly at reduced levels (Fig. 1A). An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can activate eEF2K, as proposed in exercise/contracting skeletal muscle before AMPK becomes activated [24,26], and off-target effects of the AMPK-activating compounds might increase $[Ca^{2+}]_i$. Therefore, we performed the same incubations in Ca^{2+}-free media. In wild-type MEFs incubated without Ca^{2+}, eEF2 phosphorylation still increased upon treatment with AMPK activators, however the effect was totally abolished in cells lacking AMPKα1/α2 (Fig. 1A). Interestingly, the 991-mediated increase in eEF2 phosphorylation was also strongly reduced in cells lacking the major AMPK activating kinase LKB1 (Supplemental Fig. S2). Taken together, the results suggest that AMPK activation is partly responsible for increased eEF2 phosphorylation under these conditions and that Ca^{2+} is needed for full eEF2 phosphorylation in cells treated with AMPK activators. In the series of experiments that follow, incubations were carried out in Ca^{2+}-free media to reduce high levels of basal eEF2 phosphorylation, thereby focusing on AMPK-dependent eEF2 phosphorylation.

3.2. eEF2 phosphorylation due to AMPK activation is mainly mTORC1-independent

In intact cells, AMPK activation could lead to increased eEF2 phosphorylation by at least two distinct mechanisms: direct activation of eEF2K by AMPK or indirect inhibition of mTOR signaling as a result

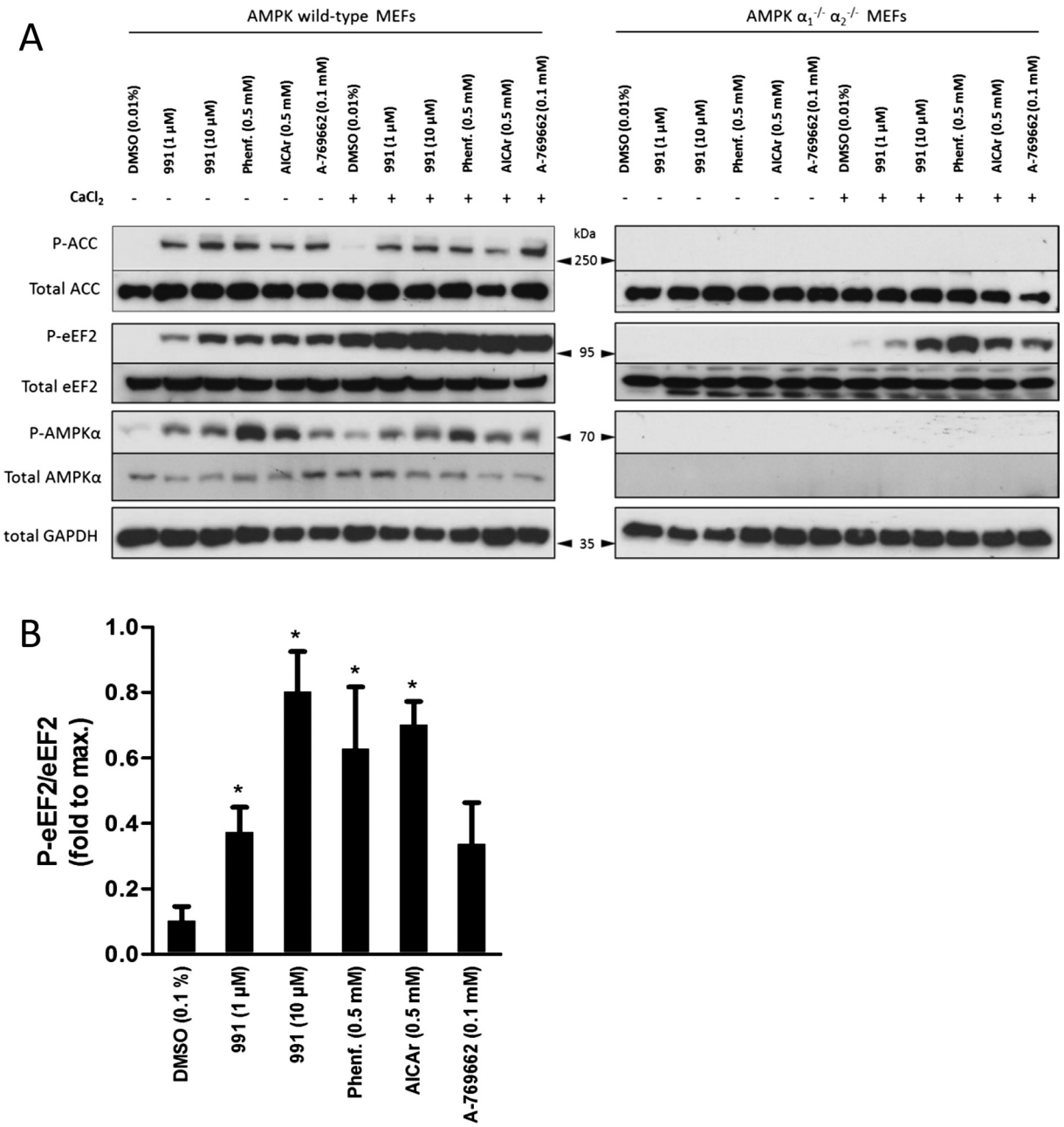


Fig. 1. Role of AMPK and Ca^{2+} in increasing eEF2 phosphorylation in MEFs treated with AMPK activators. Immortalized wild-type MEFs or MEFs lacking both catalytic subunits of AMPK ($\alpha_1^{-/-}$ $\alpha_2^{-/-}$) were incubated for 1 h in serum-free medium with (+) or without (−) $CaCl_2$ (the latter containing 0.5 mM EGTA) and DMSO (vehicle) or AMPK activators at the indicated concentrations. Cell extracts were prepared for immunoblotting with the indicated antibodies. Panel (A) shows one representative experiment in which incubations of wild-type and AMPK $\alpha_1^{-/-}$ $\alpha_2^{-/-}$ MEFs were performed in parallel. In (B), for wild-type MEFs incubated without $CaCl_2$, band intensities relative to total eEF2 as loading control were determined by densitometric scanning and normalized to the maximal value. Data are means ± S.E.M. of 3 separate experiments and * indicates a significant difference ($P < 0.05$) compared to the control (DMSO).

of AMPK-induced phosphorylation of TSC2 and Raptor, leading to reduced activity of p70S6K which phosphorylates and inactivates eEF2K. In growing cells with sufficient nutrients, mTORC1 signaling is active and continuously inhibits eEF2K *via* multiple inputs, allowing high rates of protein synthesis [58]. To distinguish between mTOR-dependent and mTOR-independent effects of AMPK activation on eEF2 phosphorylation, we used a potent specific inhibitor of mTOR kinase, Torin, which inhibits mTORC1 and mTORC2, in MEFs deleted for TSC2. As a consequence, these cells display elevated mTORC1 signaling, as reflected by high p70S6K phosphorylation (Fig. 2A,B), which was resistant to AMPK activation by 991. Basal eEF2 phosphorylation was undetectable in these cells, but treatment with 991 was able to cause an increase in eEF2 phosphorylation regardless of the lack of effect on mTORC1 signaling. Furthermore, pre-treatment of the cells with Torin completely abolished p70S6K phosphorylation and increased eEF2 phosphorylation, which could be further increased on incubation with 991 (Fig. 2A,B). Taken together, the data suggest that the effect of AMPK activation by 991 to increase eEF2 phosphorylation was mainly independent of mTORC1 inhibition.

3.3. Phosphorylation-induced eEF2K activation by AMPK

Since purified recombinant wild-type eEF2K undergoes multi-site autophosphorylation in the presence of Ca^{2+}/CaM [15,16], masking phosphorylation by other protein kinases such as AMPK (Fig. 3A), we investigated whether purified recombinant kinase-inactive (K170M)

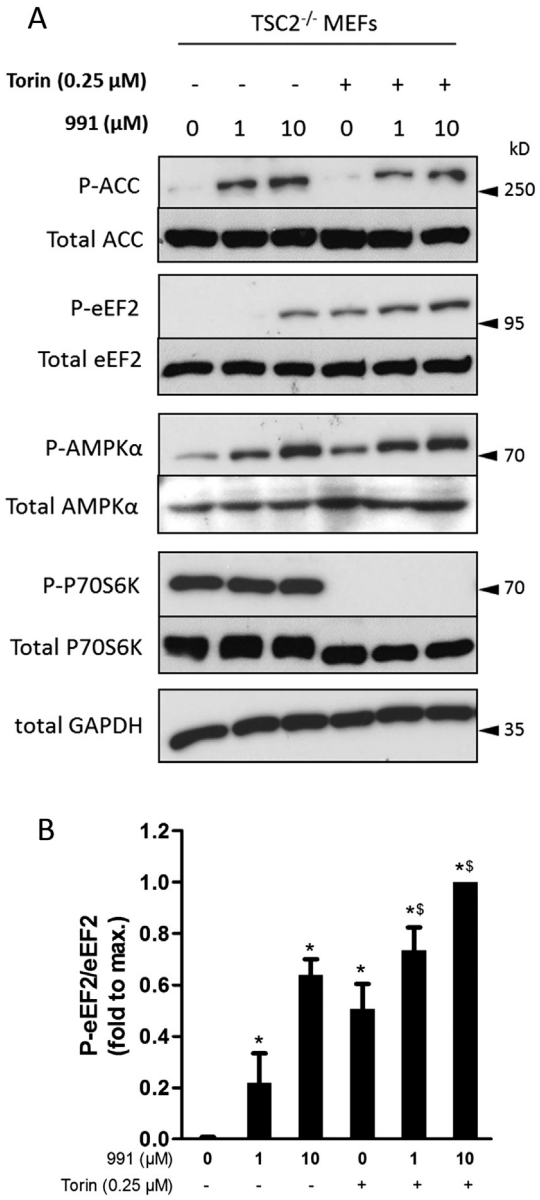


Fig. 2. Role of mTOR-inhibition in the AMPK-induced increase in eEF2 phosphorylation by 991 treatment. Immortalized MEFs lacking TSC2 (TSC2$^{-/-}$) were incubated for 30 min in serum-free medium without $CaCl_2$ (with 0.5 mM EGTA) containing 0.25 μM Torin (or DMSO as vehicle) prior to incubation with the indicated concentrations of 991 (or DMSO as vehicle). Cell extracts were prepared for immunoblotting with the indicated antibodies. Panel (A) shows one representative experiment. In (B), band intensities relative to total eEF2 as loading control were determined by densitometric scanning and normalized to the maximal value. Data are means ± S.E.M. of 3 separate experiments and significant differences ($P < 0.05$) are indicated compared to control (DMSO) conditions in the absence (*) or presence ($) of Torin.

eEF2K [15] was phosphorylated by purified recombinant activated AMPK *in vitro*. However, phosphorylation of this eEF2K mutant by AMPK was barely detectable, even though the kinase-inactive mutant could be phosphorylated by PKA on previously identified sites [14] (Ser366, Ser500) and a novel site, Ser71 (Fig. 3B). We therefore turned to the use of purified recombinant wild-type eEF2K as a substrate, but incubated in the absence of Ca^{2+}/CaM to largely reduce autophosphorylation, which indeed fell from 8 mol ^{32}P incorporated per mol of protein to 0.7 mol ^{32}P incorporated per mol of protein. However, these stoichiometries do not necessarily reflect the number of sites phosphorylated because a proportion of the expressed recombinant protein might not be in the native conformation for phosphorylation. Under these conditions, significant AMPK-dependent ^{32}P-incorporation from [γ−^{32}P]ATP into purified recombinant eEF2K was seen (4.4 mol ^{32}P incorporated per mol of protein, Fig. 3A), suggesting that some eEF2K activity/autophosphorylation is required for eEF2K phosphorylation by AMPK. Five sites (Ser18, Ser61, Ser64, Ser66 and Thr348) were identified in peaks whose labelling was unaffected by AMPK (Fig. 3C), suggesting Ca^{2+}/CaM-independent autophosphorylation at these residues. Following trypsin digestion and peptide separation by HPLC, LC-MS/MS analysis allowed the identification of 7 phosphorylation sites in the major peaks whose radioactive labelling was increased by AMPK (Ser18, Ser71, Ser78, Ser366, Ser398, Ser491, Ser500) (Fig. 3C), three of which (Ser18, Ser491, Ser500) had not been identified in a previous study [12]. Interestingly, Ser71, Ser366 and Ser500 were identified when wild-type eEF2K was phosphorylated from [γ−^{32}P]ATP by PKA under identical conditions (Supplemental Fig. S4). In fact, Ser500 phosphorylation has previously been implicated in Ca^{2+}-independent eEF2K activation by PKA [14]. When purified recombinant wild-type eEF2K was phosphorylated by AMPK without Ca^{2+}/CaM in the presence of protein kinase inhibitors, AMPK-dependent radiolabelling of the HPLC peaks was substantially reduced by Compound C (selective AMPK inhibitor *in vitro*) but labelling of the peaks seen with AMPK was largely preserved in the presence of A484954 (a selective inhibitor of eEF2K *in vitro* and in intact cells) (Fig. 3D). When identical incubations were carried out with AMPK and nonradioactive ATP, eEF2K activity increased by ~50% and AMPK-induced eEF2K activation was still seen in the presence of A484954 (to inhibit autophosphorylation of eEF2K), but not in the presence of Compound C (Fig. 3E), suggesting that autophosphorylation is not crucially involved in the mechanism of eEF2K activation by AMPK. To further investigate AMPK-induced eEF2K activation, purified recombinant wild-type eEF2K was assayed by ^{32}P-incorporation into MHC1 peptide [4] from [γ−^{32}P]ATP with increasing free Ca^{2+} concentrations. Treatment with AMPK led to an increase in V_{max} of eEF2K at saturating Ca^{2+} concentrations with no change in Ca^{2+} affinity (K_a = ~98 nM and ~95 nM with or without treatment with AMPK, respectively) (Fig. 3F). Interestingly, the effect of AMPK to increase the V_{max} of eEF2K was somewhat less when recombinant activated AMPKα1-containing trimers rather than activated AMPKα2-containing trimers were used (Supplemental Fig. S3). Furthermore, eEF2K activation by PKA was seen only at low free Ca^{2+} concentrations, whereas a decrease in V_{max} was seen at high (μM) free Ca^{2+} (Supplemental Fig. S4). Both effects were largely abolished in the S500A eEF2K mutant treated with ATP and PKA but this mutant was still activated by incubation with AMPK (Supplemental Fig. S4).

3.4. Ser491/Ser492 phosphorylation is important for eEF2K activation by AMPK

In a previous study, phosphorylation of Ser398 was implicated in mediating eEF2K activation by partially purified AMPK [12]. However, the profile of eEF2K activation of purified recombinant eEF2K S398A mutant by AMPK with increasing free Ca^{2+} concentrations was not different from that seen with purified recombinant wild-type eEF2K (compare Fig. 4A and Fig. 3F). AMPK-induced activation of purified recombinant eEF2K mutants with non-phosphorylatable Ala residues at each of the AMPK-dependent Ser phosphorylation sites identified by MS was thus investigated. Most of the mutations did not drastically affect basal eEF2K activity, except the Ser366 to Ala mutation, as reported previously [15] (Fig. 4B and legend). For the Ser to Ala eEF2K phosphorylation site mutants, incubation with AMPK led to significant increases in eEF2K activity compared with the wild-type, except for the eEF2K S491A/S492A mutant (Fig. 4B). In fact, incubation of the single mutant eEF2K S491A with [γ−^{32}P]ATP and AMPK followed by trypsin digestion and MS analysis of a radiolabelled peak purified by HPLC indicated phosphorylation of the adjacent Ser492 residue (Supplemental Fig. S6). We therefore continued using an eEF2K S491A/S492A double mutant for which V_{max} was not increased after incubation with

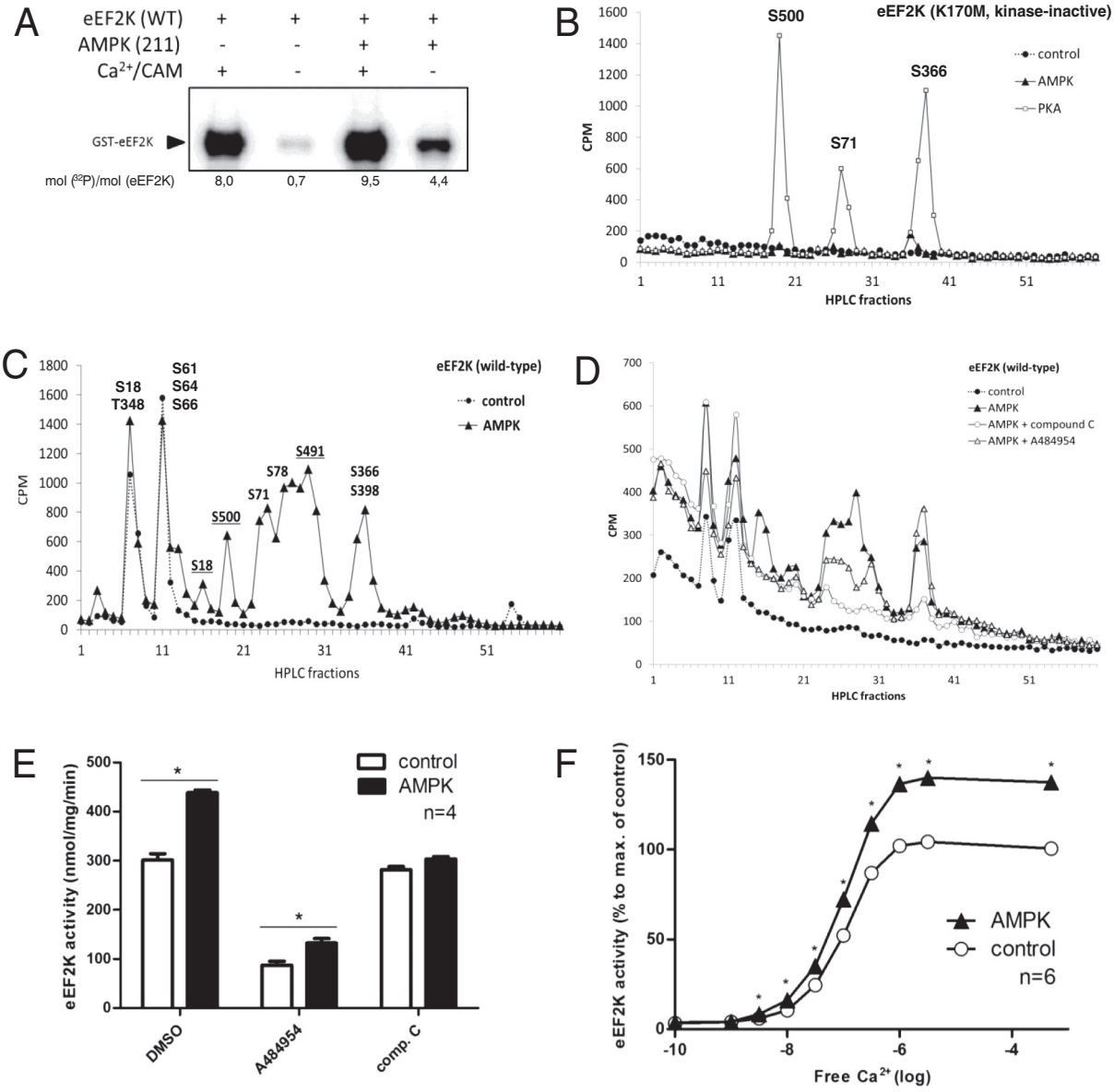
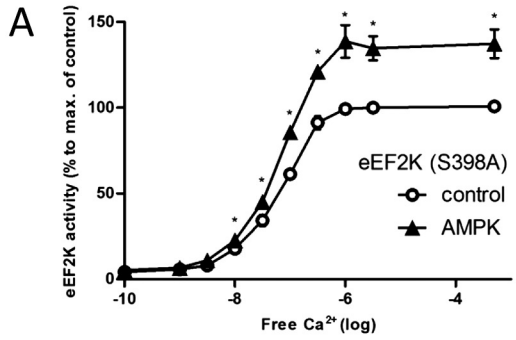
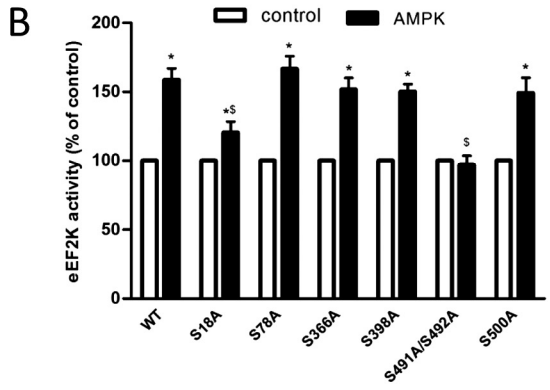


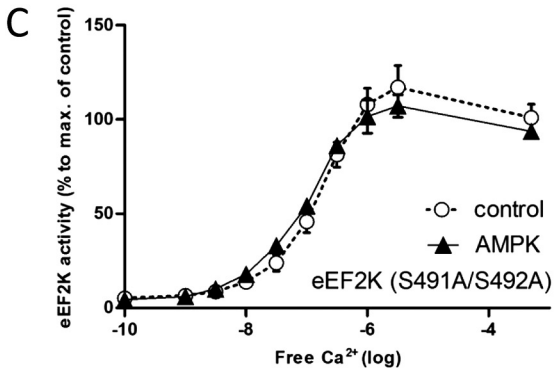
Fig. 3. Phosphorylation-induced eEF2K activation by AMPK *in vitro*. In (A, C, D), purified recombinant eEF2K (wild-type (WT)) was incubated for 1 h with purified recombinant activated AMPKα2β1γ1 (211) in the presence of [γ−^{32}P]ATP for analysis of ^{32}P-incorporation by SDS-PAGE and phosphorimaging (A) or for trypsin digestion and phosphopeptide separation by HPLC (C, D) and phosphosite identification by LC-MS (C). In (B) kinase-inactive (K170M) eEF2K was incubated for 1 h with purified recombinant activated AMPK or purified PKA in the presence of [γ−^{32}P]ATP for trypsin digestion and phosphopeptide separation by HPLC. In E-F, eEF2K was incubated as above but with non-radioactive ATP prior to eEF2K assay with MHC1 peptide as substrate and with saturating Ca^{2+} (E) or with increasing free Ca^{2+} concentrations using a $CaCl_2$-EGTA buffer system (F). In D, E, 100 μM eEF2K inhibitor A484954 or 10 μM AMPK inhibitor compound C (or DMSO as vehicle) were included for labelling. Data are from one representative experiment (A-D) or the means ± S.E.M. of at least 3 separate experiments (E-F) and * indicates a significant difference ($P < 0.05$) compared to control conditions.

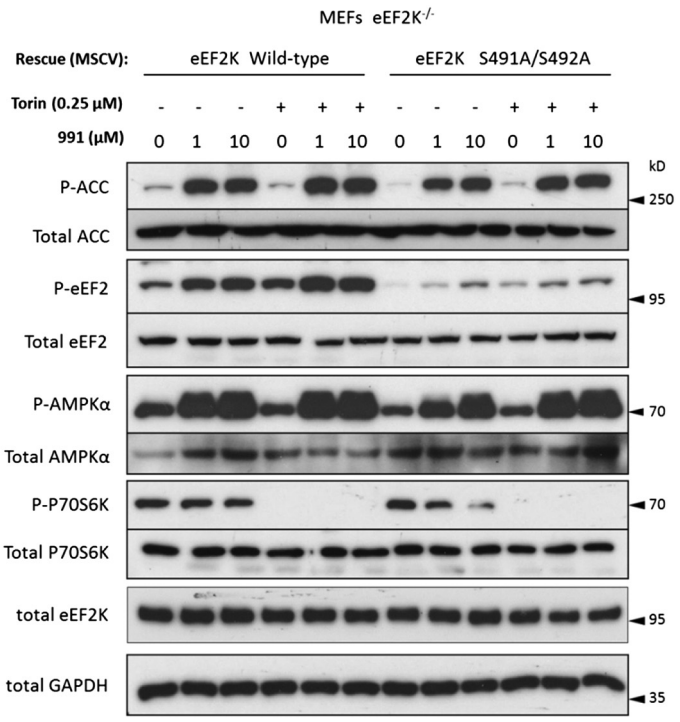
AMPK when eEF2K was assayed at saturating free Ca^{2+} concentrations (Fig. 4C).

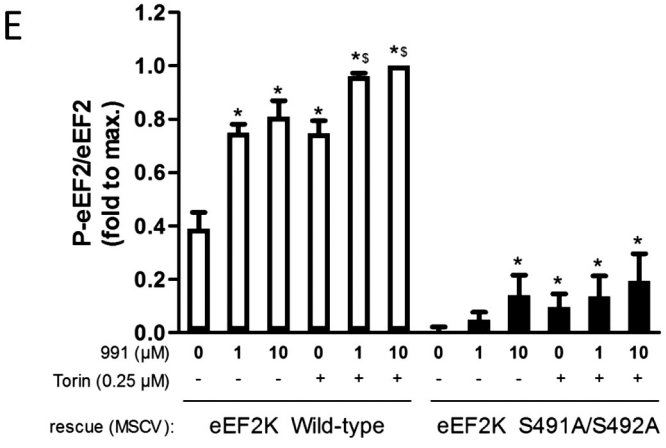
To extend our study to intact cells, immortalized MEF cells obtained from mice bearing a global deletion of eEF2K [59] were used (Supplemental Fig. S1). The wild-type or S491A/S492A eEF2K coding sequence was introduced into these cells by retroviral (MSCV) infection in order to achieve equal and stable expression of both wild-type and mutant eEF2K, albeit at low levels. In cells expressing wild-type eEF2K incubated with 991, a dose-dependent increase in eEF2 phosphorylation was observed, indicative of eEF2K activation as a result of AMPK activation as reflected by dose-dependent increases in AMPKα-Thr172 and ACC-Ser79 phosphorylation (Fig. 4D). However, in cells expressing S491A/S492A eEF2K, 991-induced eEF2 phosphorylation was markedly reduced while dose-dependent AMPK and ACC phosphorylation were still evident (Fig. 4D). Furthermore, in cells expressing wild-type eEF2K, inhibition of mTOR by Torin led to an increase in basal eEF2 phosphorylation, which was further increased upon incubation with 991 (Fig. 4D,E), whereas AMPK and ACC phosphorylation in response to 991 treatment was unaffected (Fig. 4D). In cells expressing eEF2K S491A/S492A and incubated with Torin, only a slight dose-dependent increase in eEF2 phosphorylation was apparent with 991 (Fig. 4D,E) and again 991-induced AMPK and ACC phosphorylation was seen in the presence of Torin. A dose-dependent decrease in p70S6K Thr389 phosphorylation was seen in both wild-type and eEF2K S491A/S492A expressing cells incubated with 991 that was completely lost on incubation with Torin (Fig. 4D). We did try to produce an anti-phospho Ser491 peptide eEF2K antibody (see Methods) but the reagent was not phosphospecific enough to be used to monitor eEF2K Ser491 phosphorylation in cell lysates by immunoblotting, even after immunoprecipitation of eEF2K (data not shown). Taken together though, the results

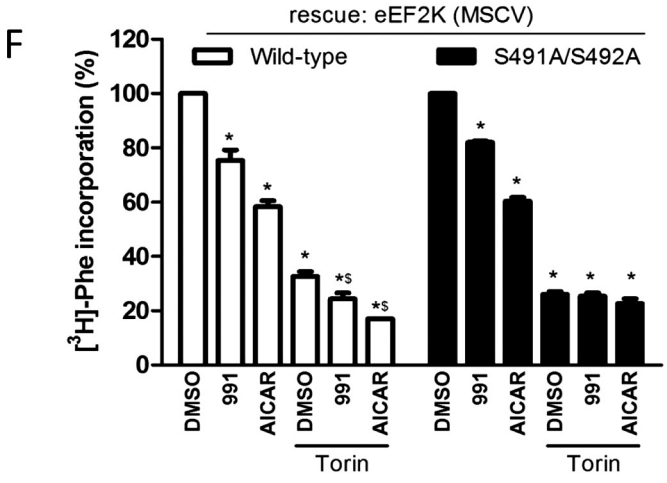












(caption on next page)

Fig. 4. Ser491/Ser492 phosphorylation is important for eEF2K activation by AMPK. Purified recombinant eEF2K, wild-type (WT) or the indicated non-phosphorylatable Ser to Ala mutants, were incubated for 1 h with purified recombinant activated AMPK and ATP for eEF2K assay using MHC1 peptide as substrate and with increasing concentrations of free Ca^{2+} using a $CaCl_2$-EGTA buffer system (A, C) or with saturating Ca^{2+} (B). Basal activities were 492 ± 20 U/mg of protein (WT), 481 ± 20 U/mg of protein (S18A), 528 ± 47 U/mg of protein (S78A), 13 ± 1 U/mg of protein (S366A), 371 ± 21 U/mg of protein (S398A), 357 ± 28 U/mg of protein (S491A/S492A) and 266 ± 28 U/mg of protein (S500A) and * indicates a significant difference compared to control incubations without AMPK, $ significant compared to activation of the wild-type by AMPK. In D and E, immortalized MEFs from $eEF2K^{-/-}$ mice were retrovirally (MSCV) transfected to reintroduce either wild-type or S491A/S492A eEF2K. The cells were cultured overnight and then incubated for 30 min in serum-free medium without $CaCl_2$ (with 0.5 mM EGTA) containing 0.25 μM Torin (or DMSO as vehicle) prior to incubation with the indicated concentrations of 991 (or DMSO as vehicle). Cell extracts were prepared for immunoblotting with the indicated antibodies. Panel D shows one representative experiment. In E, band intensities relative to total eEF2 as loading control were determined by densitometric scanning and normalized to the maximal value. In F, MEFs were incubated for 30 min with 0.25 μM Torin, 10 μM 991 or 0.5 mM AICAR (or with DMSO as vehicle control) in $CaCl_2$-containing medium, prior to addition of [^{3}H]-Phe and further incubation of 4 h. Incorporation of [^{3}H]-Phe into TCA-precipitable material was measured as described in the Methods section. Control values of protein synthesis rates in cells rescued with wild-type or S491A/S492A eEF2K were not significantly different and corresponded to roughly 7000 cpm (~1.3 pmol of Phe) per hour per mg of total cell proteins. Data are means ± S.E.M. of 3 separate experiments and significant differences ($P < 0.05$) are indicated compared to control conditions in the absence (*) or presence ($) of Torin.

suggest that the increase in eEF2 phosphorylation following AMPK activation by 991 is mainly due to eEF2K activation involving phosphorylation at Ser491/Ser492, but that reduced mTORC1 activity by AMPK also plays a minor role, at least in this cell type.

3.5. eEF2K Ser491/Ser492 phosphorylation is important for mTORC1-independent regulation of protein synthesis by AMPK

Protein synthesis rates were compared in eEF2K-*null* cells rescued with either wild-type or S491A/S492A eEF2K (Fig. 4F). Protein synthesis was decreased to the same extent in both cell lines by ~25% following AMPK activation by 991 and by ~40% when AICAR was used to activate AMPK as positive control. Complete inhibition of mTORC1 with Torin (as confirmed by undetectable p70S6K phosphorylation in Fig. 4D) reduced protein synthesis by over 60% in both cell lines, which could be further decreased by treatment with 991 or AICAR, but only in cells expressing wild-type and not S491A/S492A eEF2K. These results were consistent with eEF2 phosphorylation observed in these cells (Fig. 4D,E), although it should be pointed out that inhibition of mTORC1 affects both translation elongation and initiation, explaining the pronounced effect of Torin treatment on protein synthesis. Taken together, the results indicate that eEF2K Ser491/Ser492 phosphorylation contributes to mTORC1-independent inhibition of protein synthesis by AMPK.

4. Discussion

eEF2K is a complicated multi-modulated protein kinase and the control of its activity integrates inputs from many cellular signaling pathways. In addition, eEF2K activity is controlled by $[Ca^{2+}]_i$ and pH, such that an increase in $[Ca^{2+}]_i$ *via* CaM leads to increased eEF2K autophosphorylation/activity and eEF2K displays optimal activity at pH 6.4. Multi-site phosphorylation by protein kinases of different signaling pathways can lead to eEF2K activation or inactivation, change CaM affinity/Ca^{2+}-dependence or affect stability towards proteasomal degradation. Increased eEF2K activity caused by increases in $[Ca^{2+}]_i$ leading to increased eEF2 phosphorylation is probably important during skeletal muscle contraction and neuronal signaling, while eEF2K activation by a fall in intracellular pH might be important during anoxia and hypoxia to slow down protein synthesis [7,60]. Growth factors that induce cell proliferation tend to decrease eEF2K affinity for CaM and eEF2K activity *via* phosphorylation of Ser78 and Ser359/Ser366 mediated by mitogen-activated protein kinases (MAPKs) and various inputs from mTORC1 signaling (directly or *via* p70S6K) [58]. On the other hand, cAMP signaling through phosphorylation of Ser500 by PKA increases Ca^{2+}-independent eEF2K activity. Together, these multiple inputs would allow fine-tuning of eEF2K activity to control protein synthesis *via* downstream eEF2 phosphorylation, in both a quantitative and qualitative way *via* translational rewiring towards specific mRNA subsets through unexplained mechanisms. Phosphorylation of eEF2 at Thr56 impairs ribosome binding and arrests protein synthesis elongation. This residue is extremely highly conserved in all eukaryotes including protists, yeast, plants and insects although in fungi, plants and arthropods, eEF2K orthologues are absent, suggesting that eEF2 Thr56 might be phosphorylated by alternative kinase(s) in these organisms [7,60,61]. Also surprising is the fact that eEF2K deletion is not lethal, either in mice or *C. elegans*, although oocyte quality and embryonic viability were compromised in both species [62].

In the present study using AMPK-*null* and TSC2-*null* MEFs combined with TOR inhibition by Torin, we show that pharmacological AMPK activation by 991 led to eEF2 phosphorylation (Fig. 1,2), which was mainly mTORC1-independent, but dependent on the presence of both AMPK and eEF2K as shown by using the two kinase gene-deficient MEF cell lines (Figs. 1,S1). It is noteworthy that treatment of MEFs with commonly used pharmacological AMPK activators, whether acting directly (991, A769662) or indirectly (AICAR, phenformin), led to an increase in eEF2 phosphorylation that was, to some extent, independent of AMPK as shown in cells lacking both AMPK catalytic subunits (Fig. 1). However, these effects were observed only in presence of Ca^{2+} in the medium, suggesting that eEF2K activation might have been partly due to a rise in $[Ca^{2+}]_i$ by these AMPK activators [63,64]. Indeed, using the fluorescent probe FURA-2, we were able to detect a significant rise in $[Ca^{2+}]_i$ in MEFs treated with A769662 but not with the other AMPK activators at the doses used in this study (data not shown). When MEF incubations were carried out in Ca^{2+}-free media to solely focus on AMPK-dependent effects, direct activation of eEF2K by AMPK was more important than indirect eEF2K activation *via* AMPK-induced reduction in mTORC1/p70S6K activity. However the contributions of direct *versus* indirect eEF2K activation might well differ across different cell types.

In the absence of Ca^{2+}/CaM, recombinant AMPK phosphorylated recombinant wild-type eEF2K on at least 7 different sites (Fig. 3C) and *in vitro* phosphorylation of eEF2K by AMPK led to an increase in the V_{max} of the kinase without affecting Ca^{2+} sensitivity in the presence of CaM (Fig. 3F). By site-directed mutagenesis, Ser491/Ser492 was shown to be important for AMPK-induced eEF2K activation (Fig. 4B). Some of the phosphorylation sites identified are also autophosphorylation sites and by site-directed mutagenesis to Ala, Ser491 was found to substantially reduce eEF2K autophosphorylation at other sites [15]. Kinase-inactive eEF2K was a very poor substrate for AMPK *in vitro* (Fig. 3B), probably because some (Ca^{2+}-independent) autophosphorylation would be required to induce structural changes in eEF2K [40] allowing phosphorylation by AMPK. Comparison of the sequences surrounding the identified AMPK phosphorylation sites revealed that none perfectly fits the AMPK consensus [65] (Supplemental Fig. S5), but recent evidence suggests that there might be flexibility in this regard [66]. Phosphorylation at Ser491/492 was found to be important for eEF2K activation by AMPK *in vitro* (Fig. 4C) and in MEFs eEF2K Ser491/492 phosphorylation contributes to increased eEF2 phosphorylation (Fig. 4D,E) and protein synthesis inhibition (Fig. 4F) in response to 991 treatment. *In vitro* phosphorylation of eEF2K by AMPK and rescue in eEF2K-*null* MEFs was based on the use of human eEF2K. However, since mutation of Ser491 to Ala resulted in Ser492 phosphorylation of human eEF2K by AMPK *in vitro* (Supplemental Fig. S6), we resorted to the use of the S491A/S492A double mutant when human eEF2K was

stably reintroduced in eEF2K-*null* cells to be sure of abolishing AMPK-induced eEF2K activation (Fig. 4C). Supplemental Fig. S7 shows that in mouse and rat, the Ser491 site is not present but the equivalent of the adjacent Ser492 in human eEF2K is conserved (Ser491 in the mouse and rat sequences). Thus in normal MEFs, it is likely that effects of AMPK activation are mediated by phosphorylation at the endogenous eEF2K Ser491 site. The Ser492 site in human eEF2K might well be the functionally important site for eEF2K activation as it lies in a more favourable consensus for AMPK with a hydrophobic residue at the + 4 position (Fig. S5) which is conserved across vertebrates (Fig. S7).

An important role of eEF2K is to protect cells against nutrient deprivation and hypoxia with implications for cancer cell survival [30]. eEF2K as well as its substrate eEF2 appear to be highly expressed in various malignant tissues and cancer cell lines, including breast carcinoma and glioblastoma [7,60]. eEF2K has been implicated in other physiological processes such as autophagy, cell cycle control, cell migration and invasion, angiogenesis, vascular inflammation, glycolysis and cognitive learning/depression [7,60]. Although eEF2K is considered as a dedicated protein kinase, an *in vitro* phosphoproteomics study identified some other targets and AMPKα1 was proposed [67]. Whether any of these are *bona fide in vivo* eEF2K substrates remains to be confirmed. Both AMPK and eEF2K seem to have dual and probably contextual roles towards tumor establishment and persistence: on the one hand by decreasing cancer cell proliferation through inhibition of protein synthesis and regulation of cell cycle progression, and on the other hand by favoring cancer cell survival during nutrient deprivation and anti-cancer treatments. Specific eEF2K inhibitors therefore have therapeutic potential for cancer treatment.

5. Conclusions

Activation of AMPK using pharmacological activators leads to increased phosphorylation of eEF2 through different mechanisms, including increased cytosolic $[Ca^{2+}]_i$, inhibition of mTORC1 by AMPK and direct activation of eEF2K by AMPK-mediated multi-site phosphorylation. Direct activation of eEF2K is the major mechanism, increasing the V_{max} of the kinase and implicating phosphorylation of the newly identified Ser491/Ser492 in human eEF2K.

Acknowledgements

We thank Benoît Viollet and Marc Foretz (INSERM and Cochin Institute, Paris) for kindly providing AMPK- and LKB1-deficient MEFs, Nicolas Tajeddine for help with intracellular $[Ca^{2+}]_i$ measurements and Melissa Drappier for help with production of retroviruses and infection of cells. M.J. was supported by the Fund for Scientific Research in Industry and Agriculture (FRIA). Funding was from the Interuniversity Poles of Attraction Belgian Science Policy (P7/13), the Directorate General Higher Education and Scientific Research, French Community of Belgium, the Fund for Medical Scientific Research (FNRS, Belgium) grant numbers 3.4518.11 and T.0008.15 and a Programme Grant to C.G.P. from the Wellcome Trust (reference 086688).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2017.05.010.

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