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

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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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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