Cellular Signalling 28 (2016) 284–293

Contents lists available at ScienceDirect

Cellular Signalling

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

Elongation factor 2 kinase promotes cell survival by inhibiting protein synthesis without inducing autophagy

Claire E.J. Moore [a,b], Xuemin Wang [a,b,c], Jianling Xie [a,b], Jo Pickford [a], John Barron [a], Sergio Regufe da Mota [a,1], Matthias Versele [d], Christopher G. Proud [a,b,c,*]

[a] Centre for Biological Sciences, University of Southampton, Southampton, SO17 1BJ, United Kingdom
[b] South Australian Health & Medical Research Institute, North Terrace, Adelaide, SA5000, Australia
[c] School of Biological Sciences, University of Adelaide, Adelaide SA5005, Australia
[d] Janssen Research & Development, Turnhoutseweg 30, 2340 Beerse, Belgium

ARTICLE INFO

Article history:
Received 11 November 2015
Received in revised form 14 January 2016
Accepted 15 January 2016
Available online 18 January 2016

Keywords:
eEF2K
Translation
Glucose starvation

ABSTRACT

Eukaryotic elongation factor 2 kinase (eEF2K) inhibits the elongation stage of protein synthesis by phosphorylating its only known substrate, eEF2. eEF2K is tightly regulated by nutrient-sensitive signalling pathways. For example, it is inhibited by signalling through mammalian target of rapamycin complex 1 (mTORC1). It is therefore activated under conditions of nutrient deficiency.

Here we show that inhibiting eEF2K or knocking down its expression renders cancer cells sensitive to death under nutrient-starved conditions, and that this is rescued by compounds that block protein synthesis. This implies that eEF2K protects nutrient-deprived cells by inhibiting protein synthesis. Cells in which signalling through mTORC1 is highly active are very sensitive to nutrient withdrawal. Inhibiting mTORC1 protects them. Our data reveal that eEF2K makes a substantial contribution to the cytoprotective effect of mTORC1 inhibition.

eEF2K is also reported to promote another potentially cytoprotective process, autophagy. We have used several approaches to test whether inhibition or loss of eEF2K affects autophagy under a variety of conditions. We find no evidence that eEF2K is involved in the activation of autophagy in the cell types we have studied.

We conclude that eEF2K protects cancer cells against nutrient starvation by inhibiting protein synthesis rather than by activating autophagy.

1. Introduction

Recent work has identified eukaryotic elongation factor 2 kinase (eEF2K) as playing a key cytoprotective role in cancer cells under conditions of nutrient starvation [1], implying that inhibiting its activity may offer a novel therapeutic avenue in oncology. With this in mind, it is crucial to understand how eEF2K exerts this cytoprotective function.

eEF2K phosphorylates and inactivates eEF2, the protein required to help move the ribosome along the mRNA during translation elongation [2]. eEF2K belongs to a small group of atypical protein kinases, termed α-kinases [3]. To date, eEF2 is the only protein, other than eEF2K itself [4,5], that is known to be phosphorylated by eEF2K. Protein synthesis consumes a large amount of energy [6], almost all (>99%) of it in the elongation process. Elongation also uses almost all the amino acids consumed by protein synthesis. Consistent with this, the activity of eEF2K and the phosphorylation of eEF2 are increased under conditions of energy depletion or amino acid starvation [1,2,7].

Energy depletion (such as starvation of cells for glucose) can activate eEF2K via the AMP-activated protein kinase, AMPK [7], while amino acid starvation causes the inhibition of signalling through mammalian target of rapamycin complex 1 (mTORC1), a negative regulator of eEF2K. As noted, recent data show that eEF2K is important for the ability of cancer cells to withstand nutrient deprivation [1], and promotes breast cancer [8]. These recent discoveries may mean that eEF2K is a potential target for therapeutic intervention to tackle poorly-vascularised solid tumours. Since eEF2K slows down the elongation stage of mRNA translation, eEF2K may protect nutrient-deprived cells by decreasing the demands of protein synthesis for nutrients and energy.

Autophagy is a degradative process which can also protect cells during nutrient starvation and, like eEF2K, is negatively regulated by mTORC1 signalling [9]. Autophagy can break down macromolecules into their component building blocks and may therefore help cells to withstand periods of amino acid starvation. Activation of AMPK can also promote autophagy, likely by phosphorylating ULK1, an upstream

* Corresponding author at: Centre for Biological Sciences, University of Southampton, Southampton, SO17 1BJ, United Kingdom
E-mail address: Christopher.Proud@sahmri.com (C.G. Proud).
[1] Present address: Cancer Research UK Centre, Somers Building, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom.

http://dx.doi.org/10.1016/j.cellsig.2016.01.005

regulator of autophagy ([10–13]; see [14] for a review). Signalling through the mammalian target of rapamycin complex 1 (mTORC1) also regulates autophagy, in that case to restrain it [9]. Several studies have suggested that eEF2K may regulate (promote) autophagy in glioma cells [15–17] or mouse embryonic fibroblasts [18], such that it might provide a link between mTORC1 signalling and the regulation of autophagy. Dysregulation of autophagy is one of the hallmarks of cancer, although the role of autophagy in cancer is complex [19]. Autophagy may hinder tumorigenesis initially, but subsequently aid the survival of cancer cells in established tumours [19,20]. However, no mechanism has so far been identified to explain how eEF2K could control autophagy.

The emerging data which show that eEF2K protects cancer cells against nutrient deprivation prompted us to examine how it exerts these cytoprotective effects. Our data provide strong evidence that eEF2K does so by inhibiting protein synthesis, and do not support the idea that eEF2K regulates autophagy, at least in the cell types we have studied. These findings are particularly important given the growing interest in eEF2K as a target for cancer therapy.

2. Experimental

2.1. Materials

Bradford assay reagent (500--0001) was from Bio-Rad. Compounds JAN-384, -452, -613 and -849 were kindly provided Janssen Pharmaceutica NV (Beerse, Belgium). The properties of JAN-384 and the much less active analogue JAN-452 are described in [21]. JAN-613 is inactive against in vitro eEF2K at 30 μM; it was not tested against other kinases. JAN-849 was tested in a 234-kinase panel at Millipore, with following results (IC50): eEF2K, 16 nM; CLK2, 109 nM; GSK3α, 126 nM; GSK3β, 132 nM; CDK7, 269 nM; CDK9, 333 nM; all other kinases >1000 nM. A patent covering these compounds was published in 2015 (WIPO Patent Application WO/2015/150557).

Signalling inhibitors and other compounds were obtained as follows: rapamycin (553211), Calbiochem; AZD8055 (S1555), MK2206 (S1078) and MG132 (S2619), Selleck; cycloheximide (CHX) (01810), Harringtonine (19079), chloroquine (C6628) and bafilomycin A1 (B1793), Sigma-Aldrich.

Primary antibodies: the P-eEF2 Thr56 antisera were custom made by Eurogentec (Belgium). Anti-eEF2K antibody was kindly provided by the Division of Signal Transduction Therapy, College of Life Sciences, University of Dundee, UK. Other antibodies were obtained as follows: eEF2 (catalogue number 2332), P-protein kinase B (PKB, also termed Akt, Ser473, 4060), P-ribosomal protein S6 240/244 (5364), LC3A/B (12741), poly ADP-ribose polymerase (PARP, 5625), all from Cell Signalling Technology; GAPDH (G8795), actin (A5441) and glutathione *S*-transferase (GST, GE27-4577-01) from Sigma-Aldrich.

2.2. Cell culture, transfection and treatment

Mouse embryonic fibroblasts (MEFs) from eEF2K$^{-/-}$ (knockout) mice and matched wild-type counterparts were prepared from embryos at embryonic day 13.5. MEFs from eEF2K (WT) and eEF2K$^{-/-}$ mouse embryos were cultured in Dulbecco's modified Eagle's medium (DMEM (41966); Invitrogen) supplemented with 10% foetal bovine serum (FBS, F2442, Sigma-Aldrich), 100 units/mL penicillin and 0.1 mg/mL streptomycin (15140–122, Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2. Immortalised TSC2$^{-/-}$ MEFs were generously provided by Dr. David Kwiatkowski (Harvard University, Boston). Human colon carcinoma HCT116 cell lines were cultured using standard procedures in a humidified incubator at 37 °C with 5% CO_2 in McCoy's 5A media supplemented with 10% (v/v) FBS, 1.5 mM glutamine (25,030–081, Gibco), 100 units/mL penicillin and 0.1 mg/mL streptomycin. Human lung carcinoma A549 cell line containing an inducible shRNA plasmid directed towards the eEF2K mRNA was generously provided by Janssen Pharmaceutica NV and cells were cultured using standard procedures in a humidified incubator at 37 °C with 5% CO_2 in DMEM media supplemented with 10% (v/v) FBS, 1.5 mM glutamine (25030-081, Gibco), 100 units/mL penicillin and 0.1 mg/mL streptomycin. To induce knockdown of eEF2K, cells were cultured for 5 days with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, I6758, Sigma-Aldrich) prior to experimentation.

After treatment, cells were lysed in ice-cold lysis buffer containing 1% (v/v) Triton X-100 (215680010, Acros Organics), 50 mM Tris (T6066-1KG, Sigma-Aldrich)–HCl (258148-2.5 L, Sigma-Aldrich) pH 7.4, 50 mM NaCl (BP358-212, Fisher Scientific), 0.2 mM EDTA (BP120-500, Fisher Scientific), 0.2 mM EGTA (E1102, Melford), 50 mM β-glycerophosphate (G9422, Sigma-Aldrich), 1 mM Na_3VO_4 (S6508, Sigma-Aldrich), 15 mM β-mercaptoethanol (125472500, Acros Organics) and 1× protease inhibitor cocktail (5056489001, Roche). Lysates were spun at 16,000 × *g* for 10 min at 4 °C; the supernatants were kept and total protein concentration was quantified by Bradford assay following the manufacturer's instructions.

2.3. SDS-PAGE and western blot analysis

These procedures were performed as described previously [22].

2.4. BHMT cleavage assay

A549 cells were transfected using lipofectamine LTX (15338100, Life Technologies) with a GST- betaine homocysteine methyltransferase (BHMT) reporter vector (kindly provided by Carol Mercer, University of Cincinnati, USA). 48 h after transfection cells were treated with AZD8055 (1 μM) for 16 h in the presence of E64d (6 μM, E8640, Sigma-Aldrich) and leupeptin (11 μM, L9783, Sigma-Aldrich). Cells were lysed as above. Total protein concentration was determined by Bradford assay and GST-BHMT was isolated using glutathione-sepharose (GE17-0756-01, Sigma-Aldrich). The precipitated GST-BHMT was washed three times in the ice cold lysis buffer. Precipitates were then boiled in SDS-PAGE sample buffer, resolved by SDS-PAGE and analysed by western blotting using anti-GST antibody.

2.5. Cell survival

Caspase 3/7 assays (G8090, Promega) were performed according to the manufacturer's instructions. Briefly, 10,000 cells/well were plated overnight in a 96-well plate. Cells were treated as described in the figure legends for the indicated time period. To measure caspase 3/7 activity, 50 μL of caspase Glo 3/7 reagent was added to each well for 2 h with constant shaking at room temperature. Luminescence was measured using a BMG Labtech FLUOstar Optimi plate reader. Cytotoxicity was evaluated by CellTox Green® cytotoxicity assay (Promega). Briefly, 10,000 cells/well were plated overnight in a 96-well plate. Cells were treated as described in the figure legends for the indicated time period. CellTox green dye was diluted 1/500 in test media and applied to cells for the times indicated in the figure. Fluorescence was measured at 485–500 nm_{Ex}/520–530 nm_{Em} using a BMG Labtech FLUOstar Optimi plate reader.

2.6. Autophagic flux analysis

A549 cells were transfected with a vector encoding mCherry-EGFP-LC3B, a tandem fluorescent-tagged LC3 (a kind gift from Dr. Terje Johansen, Biochemistry Department, Institute of Medical Biology, University of Tromsø, Norway), using lipofectamine 3000 (L3000001, Life technologies) following manufacturer's instructions. After treatment cells were washed twice in phosphate buffered saline (PBS, 18912-014, Gibco) and then fixed in 4% formaldehyde