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The channel-kinase TRPM7 regulates phosphorylation of the translational factor eEF2 via eEF2-k

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ABSTRACT

Protein translation is an essential but energetically expensive process, which is carefully regulated in accordance to the cellular nutritional and energy status. Eukaryotic elongation factor 2 (eEF2) is a central regulation point since it mediates ribosomal translocation and can be inhibited by phosphorylation at Thr56. TRPM7 is the unique fusion of an ion channel with a functional Ser/Thr-kinase. While TRPM7's channel function has been implicated in regulating vertebrate Mg^{2+} uptake required for cell growth, the function of its kinase domain remains unclear. Here, we show that under conditions where cell growth is limited by Mg^{2+} availability, TRPM7 via its kinase mediates enhanced Thr56 phosphorylation of eEF2. TRPM7-kinase does not appear to directly phosphorylate eEF2, but rather to influence the amount of eEF2's cognate kinase eEF2-k, involving its phosphorylation at Ser77. These findings suggest that TRPM7's structural duality ensures ideal positioning of its kinase in close proximity to channel-mediated Mg^{2+} uptake, allowing for the adjustment of protein translational rates to the availability of Mg^{2+}.

1. Introduction

Nutritional signaling is at the core of cellular adaptive responses to changing environmental conditions. Given its high metabolic and energy requirements, protein translation is carefully adjusted to the level of cellular resources. The inhibitory phosphorylation of eukaryotic elongation factor 2 (eEF2) thereby plays a central role. The only known kinase of eEF2, eEF2-kinase (eEF2-k), phosphorylates eEF2 at Thr56, leading to its failure to bind ribosomes, and therefore its inactivation [1,2]. The activity of eEF2-k is regulated via phosphorylation at several positions through the mTOR pathway, which is a central signal integration system for cell growth control [3]. eEF2-k belongs to the small atypical family of α-kinases [4,5] that also includes the kinase portion of the TRPM7 "chanzyme" [6].

TRPM7 and its closest relative TRPM6 are the only known channel-kinase fusions, a structural feature unique to the vertebrate TRPM6/7 proteins. TRPM6-deficient patients suffer from hypomagnesemia with **s**econdary hypocalcemia (HSH), leading to lethal seizures [7,8]. Affected individuals live a normal life if provided with a Mg^{2+}-rich diet. Similarly, we have found that a genetically engineered TRPM7$^{-/-}$ DT40 B-cell line displays massive growth impairment and becomes Mg^{2+}-deficient. This phenotype can be completely reversed by supplementation of the media with 5–10 mM $MgCl_2$ or $MgSO_4$, but not with Mn^{2+}, Ca^{2+}, Zn^{2+}, or Ni^{2+} [9,10]. TRPM7 homologues are also involved in Mg^{2+}-homeostasis regulation in other organisms including *C. elegans* [11,12] and zebrafish [13]. The biophysical characterization of TRPM7 has shown that its pore is divalent cation selective and permeable to Ca^{2+} and Mg^{2+} [9]. TRPM7-mediated currents are inhibited by intracellular Mg^{2+} or MgATP [9,14], and native currents with properties resembling recombinant TRPM7 were thus named "Magnesium Inhibited Cation" (MIC), also called MagNuM (magnesium-nucleotide-regulated metal ion currents) [9,14]. Combined with the ubiquitous distribution of TRPM7's gene expression, these findings collectively suggest that one crucial aspect of TRPM7 biology is to ensure adequate levels of Mg^{2+} in a wide variety of cell types. Although these functions might not be all solely related to Mg^{2+} homeostasis, TRPM7 is essential in various biological contexts including cell proliferation [10,15], cytoskeletal organization and cell migration [16,17], as well as embryonic development in mice [18]. Studies with clinical significance have revealed a central role for TRPM7-mediated ion fluxes in the context of anoxic neuronal cell death [19], and that suppression of TRPM7 expression restricted to the neurons of the hippocampus protected these cells from ischemia-associated damage [20]. Furthermore, a genetic TRPM7 variant resulting in a channel with heightened sensitivity to intracellular Mg^{2+} appears to favor the occurrence of human degenerative disorders [21], and a recent genomics approach has shown that TRPM7 is one of only 18 genes that are dysregulated in three different animal disease models (multiple sclerosis, Alzheimer's, and stroke) [22].

Abbreviations: TRPM7, Transient Receptor Potential Melastatin 7; eEF2, eukaryotic elongation factor 2; eEF2-k, eEF2-kinase; WT, wildtype.

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Based on the unique presence of a kinase domain covalently linked to the channel portion of TRPM7 in vertebrates, we hypothesized that TRPM7 might function as a master regulator of cellular Mg^{2+} homeostasis, coordinating cellular functions with the availability of this essential growth factor. The phosphotransferase activity of TRPM7-kinase is neither required for channel activation, nor for its inhibition by intracellular Mg^{2+} [10,23,24]. The involvement of TRPM7 in signaling via the phosphorylation of exogenous substrates, such as annexin I and myosin IIA, has been suggested [17,25]. Because TRPM7-kinase is a relative of eEF2-k, and given that Mg^{2+} is an essential co-factor for protein translation, we investigated whether TRPM7 might be involved in adjusting the rate of protein synthesis to the availability of Mg^{2+}. Here, we show that the inhibitory Thr56 phosphorylation of eEF2 is increased under hypomagnesic conditions, and that this Mg^{2+}-dependent regulation requires TRPM7 with an active kinase. We further found that eEF2 does not appear to be a direct substrate of TRPM7-kinase, but rather, that TRPM7 is regulating the activity of eEF2's cognate kinase eEF2-k. *In vitro* phosphorylation assays revealed that TRPM7 can phosphorylate eEF2-k on Ser77. Using a complementation approach with eEF2-k S77A and S77D mutants in a genetically engineered eEF2-k deficient DT40 cell line, we propose that phosphorylation of eEF2-k on Ser77 improves its protein stability, resulting in higher levels of phosphorylated eEF2, and consequently, reduced rates of protein synthesis. This is the first study supporting the idea that TRPM7 fulfils the role of an environmental sensor, adjusting the level of physiological processes, such as protein synthesis, to the availability of ions.

2. Material and methods

2.1. Cloning and generation of cell lines stably overexpressing proteins of interest

HEK-293 T-Rex cells (Invitrogen) with stable and dox-inducible expression of human TRPM7 wt and kinase mutants have been previously described [10]. For use in *in vitro* phosphorylation assays, a C-Terminal TRPM7 fragment (TRPM7-kinase C3, aa position 1440–1865) and TRPM7-kinase (aa position 1562 to 1865) were cloned into the pcDNA4/TO-Flag plasmid to generate stably expressing HEK-293 cell lines. For the expression of mouse eEF2 wt, its full length cDNA (GenBank™ accession number BC060707) was purchased as an I.M.A.G.E. clone (Invitrogen). Mouse eEF2-k cDNA [26] was cloned into the pcDNA4/TO-Flag and pcDNA5/TO-Flag plasmids, allowing for dox-inducible expression of N-terminally flag-tagged protein in HEK-293 and DT40 T-Rex cells. Mouse eEF2-k cDNA was used to generate eEF2-k mutants S61A, S77A(D), S240A, S444A, and S61A/S240A using site directed mutagenesis (Stratagene). The predicted DNA sequences of all constructs were verified by sequencing.

2.2. Generation of an eEF2-k deficient DT40 B-cell line

Chicken *eEF2-k* genomic DNA and cDNA were obtained by screening NCBI chicken genome and cDNA libraries. The targeting vectors (*eEF2-k-histidinol* (His) and *eEF2-k-puromycin* (Puro) were constructed by replacing the genomic fragment containing exons 8 to 10, which corresponds to a highly conserved region of the catalytic domain, with histidinol or puromycin cassettes. These cassettes were flanked by 3950 bp and 1780 bp of chicken *eEF2-k* genomic sequence on the 5′ and 3′ sides, respectively. The predicted DNA sequences of all constructs were verified by sequencing. The linearized targeting constructs were sequentially introduced into DT40 cells stably expressing the Tet-repressor (similar to the HEK-293 cells described earlier) by electroporation. Cell clones were selected in the presence of puromycin and histidinol.

The integration of the histidinol targeting construct into the *eEF2-k* gene locus was analyzed by Southern blot. To this purpose, genomic DNA was digested with NcoI. Restriction enzyme sites (N, NcoI), probe for Southern blot analysis (white bar), and targeted exons are indicated in Fig. 4A. Targeting of the second allele was indirectly analyzed by detection of the *eEF2-k* mRNA using RT-PCR, and confirmed by Western blot to detect eEF2-phosphorylation levels. The first allele targeting using the *eEF2-k* histidinol construct resulted in homologous recombination with a frequency of 20%. The targeting of the second allele showed a similar success rate using the *eEF2-k* puromycin targeting vector.

The RT-PCR reaction to detect *eEF2-k* transcripts was performed using RNA from potential eEF2-k deficient DT40 cell clones or from DT40 wt cells (positive control). The control PCR was performed with the following chicken specific oligonucleotides for NudT9 (housekeeping enzyme):

ACGTGTCGACATGTTGGCCTTCGCCACTGTGCC and TAGCTACAAACTGCAAGATGTTTTTGCCAGTA.

2.3. Cell culture

Transiently or stably transfected HEK-293 cells were maintained in DMEM medium supplemented with 5% fetal bovine serum and Blasticidin S (5 μg/ml, Invivogen) to maintain stable expression of the Tet-repressor. Zeocin (400 μg/ml, Invitrogen) was added for HEK-293 cell lines transfected with pcDNA4TO constructs and hygromycin (100 μg/ml, Calbiochem) when pcDNA5TO constructs were used. Protein overexpression was induced by adding dox (100–1000 ng/ml) to the growth medium.

The TRPM7-deficient DT40 cell lines complemented with stable overexpression of hTRPM7 wt (cWT TRPM7, also cWT M7) or mutant channels (cKR M7 and cΔK(inase) M7) in an inducible fashion were described previously [10]. These cell lines were cultured in complete, chemically defined media with added 10 mM $MgCl_2$ for normal maintenance. DT40 wt cells or eEF2-$k^{-/-}$ DT40 cells were kept in the same medium without additional Mg^{2+}. To study native eEF2 phosphorylation, complete, chemically defined HyQ CCM1 and customized Mg^{2+}-free HyQ CCM1 media (Hyclone) supplemented with 1% chicken serum (Sigma) were used as indicated.

2.4. In vitro phosphorylation assays of hTRPM7 wt, mutant channels, eEF2 and eEF2-k

TRPM7 wt, TRPM7 mutant channels, a TRPM7-C-terminal fragment (TRPM7-C3), TRPM7-kinase, eEF2, or eEF2-k was immunoprecipitated with anti-flag or anti-HA coated beads. The beads were incubated 20–30 min at 32 °C in a total volume of 40 μl reaction buffer (50 mM Tris–HCl, 0.1 (v/v) β-mercaptoethanol, 10 nM Calyculin A, 10 mM MgAc, 1 mM $MnCl_2$, 0.5 mM $CaCl_2$, 100 mM Mg-ATP and/or 10 μCi γ^{32}P-ATP (specific activity of 3000 Ci/mmol) as indicated. Phosphorylation was analyzed following gel electrophoresis and blotting, either by film and phosphor-imaging (radiolabeling experiments), or anti-P-Thr-/P-Ser immunoblotting. The pH was kept constant in all phosphorylation reactions at 7.2.

2.5. Immunoblotting

$0.1–10\times10^6$ (when specified the larger cell numbers per gel lane were used to detect eEF2 basal phosphorylation) HEK-293 cells, DT40 wt or mutant cells with dox inducible expression of the indicated molecules were plated, and expression induced by adding dox to the growth media for 24–48 h. HEK-293 or DT40 cells were lysed using standard protocols, or 250 ng purified eEF2-k protein was used for (co-)immunoprecipitation experiments. Anti-flag, anti-HA, and anti-GST (all from Sigma) immunoprecipitations were performed from cell lysates; the phosphatase inhibitor Calyculin A (10 nM, Cell Signaling) was added to the lysis buffer for phosphorylation experiments.