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GCN2 activation and eIF2α phosphorylation in the maturation of mouse oocytes

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ABSTRACT

GCN2 is one of the four mammalian kinases that phosphorylate the alpha subunit of the translation initiation factor 2 (eIF2α) in a variety of stress situations, resulting in protein synthesis inhibition. GCN2 is involved in regulating metabolism, feeding behavior and memory in rodents. We show here that, relative to other cells, the beta isoform of the *GCN2* transcript and the GCN2 protein are highly abundant in unfertilized mouse eggs. In addition, GCN2 in these cells is active, resulting in elevated levels of phosphorylated eIF2α. After fertilization, eIF2α phosphorylation decreases drastically. These results suggest that GCN2 mediated translational control may contribute to regulatory mechanisms operating during oocyte maturation.

The translation initiation factor eIF2 delivers the aminoacylated initiator tRNA to the 40S ribosomal subunit at each round of initiation of a polypeptide chain on eukaryotic mRNAs, in the form of a ternary complex eIF2·GTP·Met – $tRNA_i^{Met}$. This heterotrimeric factor is also fundamental in the regulation of translation. Phosphorylation of the Ser51 residue of its alpha subunit (eIF2α) by specific stress-activated kinases impedes translation initiation by inhibiting the GTP exchange reaction catalyzed by eIF2B. Paradoxically, low ternary complex availability augments the translation of specific messages encoding transcriptional factors that activate stress-remedial proteins [1].

Mammals have four eIF2α kinases, activated by different stress conditions [2]. GCN2 is the only kinase that is conserved in all eukaryotic organisms and its mechanism of activation has been well characterized in yeast [1]. It is proposed that uncharged tRNAs that accumulate under amino acid starvation conditions bind to its His-RS-like domain, causing a conformational change that results in the autophosphorylation and consequent activation of the catalytic domain. In mammals, GCN2 is activated by indispensable amino acid deprivation, UV irradiation and proteasome inhibition [3–7]. The other eIF2α kinases in mammals are PEK/PERK, activated by endoplasmic reticulum stress [8,9]; PKR, activated by dsRNA [10]; and HRI, activated by heme deprivation and arsenite stress [11].

GCN2 has been shown to be important in physiological responses in rodents. It is involved in memory formation, in directing animal behavior towards adequate food sources, in controlling lipid metabolism and in immunoregulatory pathways [4,12–16].

The *Gcn2* gene is transcribed in three mRNA isoforms that differ in their 5′ sequences, which encompass the GI domain of the protein, required for binding to its activator, GCN1. The βGCN2 transcript encodes a complete GCN2 protein, the αGCN2 isoform lacks the GI domain, and the gamma isoform contains a truncated GI motif. The βGCN2 mRNA was shown to be similarly expressed in several organs while the other isoforms show expression preferences [3,17].

A mouse microarray database covering a more comprehensive panel of organs/tissues revealed a similar pattern of expression, except for a very high abundance of the *GCN2* mRNA in mature oocytes and in fertilized eggs (http://symatlas.gnf.org) [18].

Given the relevance of GCN2 in mammalian physiology, we then sought to validate and extend these observations, by analyzing GCN2 mRNA isoforms and protein levels in ovulated oocytes and ovaries. We show that the βGCN2 mRNA isoform and the GCN2 protein are found in the ovulated oocytes at amounts that far exceed those found in other cells. In addition, we found that in oocytes GCN2 is active and may be responsible for the observed high level of phosphorylation of eIF2α. These data suggest that GCN2 is also relevant in the maturation of mouse oocytes and early embryogenesis.

Methods

This study was conducted under protocols approved by the Animal Care and Use Ethics Committee of the Universidade Federal de

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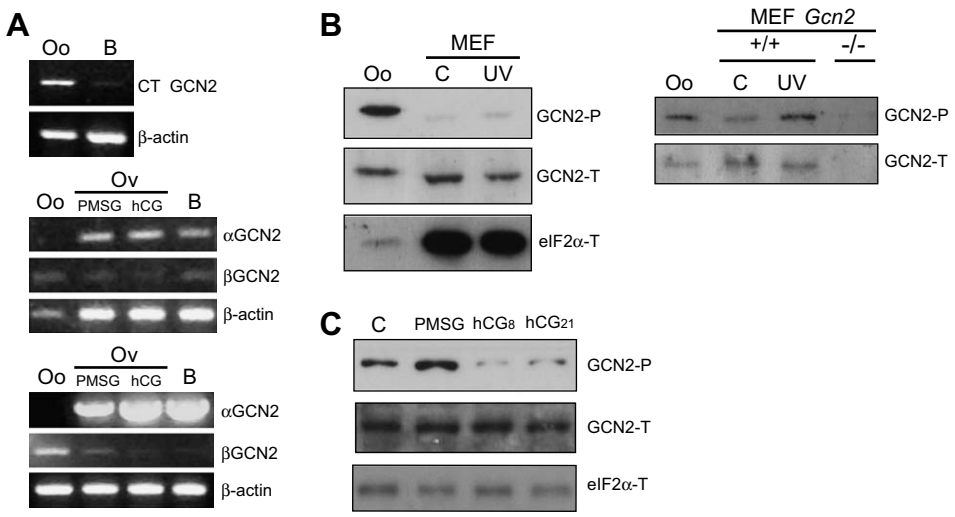

Fig. 1. GCN2 in mouse stage MII oocytes. (A) The GCN2 mRNA is abundant in oocytes. *Upper panel:* Amplification of the segment encoding the C-terminus of GCN2 by RT-PCR from ovulated oocytes (Oo), compared to mouse brain (B); β-Actin was used as control. *Middle panel:* RT-PCR amplification of *GCN2* mRNA isoforms from mouse oocytes (Oo) and ovaries (Ov) colleted 48 h after PMSG administration and 21 hr after hCG. *Lower panels:* PCR over the RT-PCR products shown in the middle panel, after normalization for β-actin. (B) Activated GCN2 protein is abundant in oocytes. Immunoblots of oocytes (150 cells) and of total protein extracts (5 μg) from control MEFs (C) and of UV-irradiated MEFs (UV). *Left panel:* immunoblots of samples subjected to 8% SDS–PAGE using antibodies directed against phosphorylated GCN2 (GCN2-P); after removal of these antibodies, the same membrane was incubated with antibodies against total GCN2 (GCN2-T). For the detection of eIF2α, identical samples were subjected to 10% SDS–PAGE, followed by immunoblot with antibodies against eIF2α-T. *Right panel:* samples were subjected to same procedure as above, including as control MEFs derived from *Gcn2* knock-out mice (−/−). (C) Activated GCN2 is detected in superovulated ovaries. Immunoblots of extracts (10 μg) from ovaries extracted 48 h after injection of PMSG or of saline (C), and after 48 h of PMSG followed by 8 or 21 h after treatment with hGC, using anti-GCN2-P antibodies; after removal of these antibodies, the same membrane was incubated with anti-GCN2-T antibodies.

São Paulo, in accordance with the Guide for Care and Use of Laboratory Animals adopted by the National Institutes of Health.

Oocytes, zygotes and 2-cell embryos isolation. C57BL/6J mice (25-days-old) were superovulated with injection of 5 IU gonadotropin (“Pregnant mare serum gonadotropin”—PMSG, Sigma), followed by human chorionic gonadotropin (hCG, Sigma) [19]. Ovulated MII stage oocytes were collected from the oviducts as described [19]. One-cell embryos (zygotes) and two-cell embryos were collected from the oviducts of superovulated females approximately 16 h or 1.5 days post-coitum, respectively. After cumulus cells elimination in M2 medium with hyaluronidase 30 μg/ml (Sigma), cells were washed in M16 medium (Sigma) and developmental stage determined by morphological characteristics.

RT-PCR. Total RNA from oocytes and brain of C57BL/6J mice was extracted using Trizol reagent (Invitrogen), according to manufactureŕs protocol and subjected to reverse transcription (RT) using ImpromII® reverse transcriptase (Promega) with extension at 37 °C during 1 h, according to the manufactureŕs protocol; cDNAs were used in PCRs with specific primers. The primers for GCN2 isoforms were as described previously [17]. For the region corresponding to the C-terminus of the protein, the primers were: Fw-5′-GGTCGACCTGTCATCAAGGTGCAAAACAAG-3′; Rv-5′-GGCGGCC GCGCAGCCCGACGACGTCCTCGA-3′.

Protein extracts. Immortalized embryonic fibroblasts (MEFs) derived from *Gcn2*$^{+/+}$ or *Gcn2*$^{-/-}$ mice were cultured as previously described [20]. Brain from status epilepticus mice (Swiss albino) were obtained as previously described [21]. Total protein extracts from cells or brain were prepared in 20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 4 μg/ml aprotinin, 2 μg/ml pepstatin, 100 mM NaF and 10 mM tetrasodium pyrophosphate and quantified by the Bradford method. Oocytes, zygotes and 2-cell embryos were ressuspended directly in Laemmli buffer.

Immunoblot analysis. Proteins were transferred to nitrocellulose membranes (Hybond™-C Extra—GE Healthcare). After blocking with 5% non-fat milk, membranes were incubated with anti-eIF2α-P antibodies, followed by HRP-conjugated secondary antibodies and detection with ECL chemiluminescence system (GE Healthcare). The antibodies were stripped from the membranes, as described in [20], and total eIF2α detected in the same membrane, using anti-eIF2α-T antibodies (1:1000) (Biosource). For GCN2, membranes were incubated with anti-GCN2 guinea pig primary antibodies [22] (1:500), followed by protein A-HRP. The phosphorylated form of GCN2 was detected using anti-GCN2-P antibodies (1:1000) (Cell Signaling), followed by anti-rabbit IgG-HRP.

Results

GCN2 is abundant and active in mouse oocytes

The presence of the *GCN2* mRNA in ovulated oocytes was analyzed by RT-PCR. Using primers for the region corresponding to the protein C-terminus, common to the three mRNA isoforms, we observed that *GCN2* mRNA is highly abundant in oocytes when compared with the brain (Fig. 1A, upper panel). Employing primer sets to detect each isoform, we determined that β*GCN2* was the only isoform found in oocytes, whereas α*GCN2* can be found in the ovaries and brain (Fig. 1A, middle panel). Even after a reamplification reaction, the α*GCN2* isoform was not detectable in oocytes (Fig. 1A, bottom panel). In superovulated ovaries, β*GCN2* was clearly detected, above the levels obtained from post-ovulation ovaries. We were not able to detect the γ*GCN2* isoform in these samples (data not shown).

The presence of the GCN2 protein in ovulated stage MII oocytes was then analyzed by immunoblots using affinity-purified antibodies directed to mouse GCN2 (Fig. 1B). The GCN2 protein is easily detectable in oocytes and largely exceeds the levels found in mouse embryonic fibroblasts (MEFs) when normalized against its substrate eIF2α. These MEFs express GCN2 and eIF2α to the same levels found in the brain or in other tissues [22]. Total protein lev-