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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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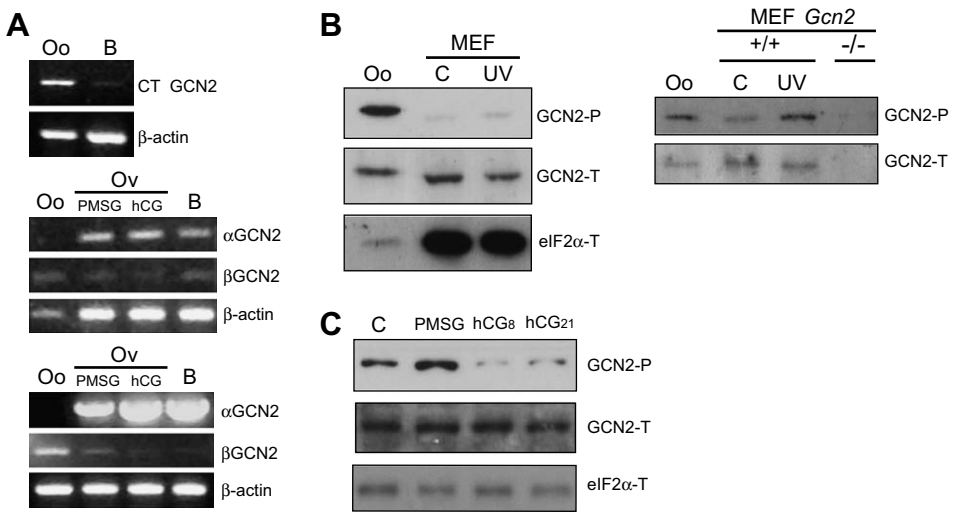

Fig. 1. GCN2 in mouse stage MII oocytes. (A) The GCN2 mRNA is abundant in oocytes. *Upper panel:* Amplification of the segment encoding the C-terminus of GCN2 by RT-PCR from ovulated oocytes (Oo), compared to mouse brain (B); β-Actin was used as control. *Middle panel:* RT-PCR amplification of *GCN2* 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-

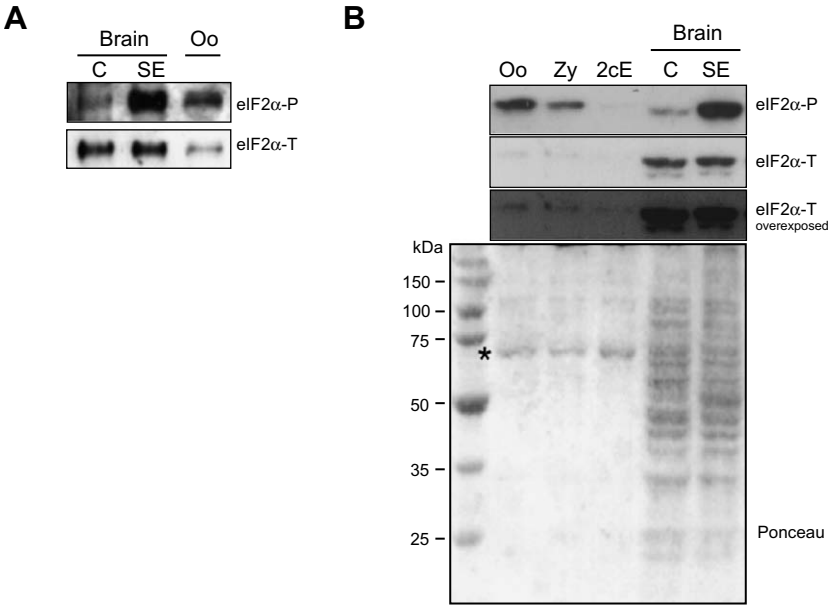

Fig. 2. Phosphorylated eIF2α in oocytes. (A) eIF2α is hyperphosphorylated in mature oocytes. Immunoblots of oocytes (120 cells) and protein extracts (10 μg) from the brain cortex of control mice (C) and mice subjected to 30 min *status epilepticus* (SE), using anti-eIF2α-P antibodies followed by anti-eIF2α-T antibodies. (B) eIF2α phosphorylation decreases after fertilization. Immunoblot of oocytes (66), zygotes (55) (Zy) and 2-cell embryos (50) (2cE) with anti-eIF2α-P and anti-eIF2α-T antibodies. The Ponceau stained membrane is shown in the lower panel; the asterisks indicates the ZP protein.

els in the oocyte samples can be estimated by comparison with Fig. 2B (see below).Thus, GCN2 is much more abundant in ovulated oocytes than in other cell types examined thus far. Moreover, GCN2 is activated in oocytes, as determined by using antibodies that recognize the phosphorylated form of GCN2 (GCN2-P) (Fig. 1B). As control for the specificity of these antibodies, wild type MEF cells irradiated with UV, a known activator of GCN2, as well as *Gcn2*$^{-/-}$ MEFs were used (Fig. 1B). Further indication of extensive phosphorylation of GCN2 in oocytes is the slower migration of the oocyte protein, evident in the left panel of Fig. 1B.

The levels of GCN2 and its phosphorylation state were also analyzed in ovaries following administration of PMSG or of PMSG followed by hCG (Fig. 2C). An increase in GCN2-P is observed in the PMSG ovaries relative to control ovaries. Ovulation resulted in a marked decrease in GCN2-P in the ovaries, indicating that the majority of the GCN2-P was in the released oocytes. The amount of total GCN2 also seems to increase after PMSG treatment, and to decrease after ovulation, when normalized for eIF2α.

eIF2α is highly phosphorylated in ovulated mouse oocytes

The presence of elevated amounts of phosphorylated GCN2 in metaphase II arrested oocytes prompt us to analyze eIF2α-P in these cells. Immunoblots of these oocytes indicated very high levels of eIF2α-P (Fig. 2A). As a control, we used extracts from cerebral cortex of control mouse and of a mouse subjected to *status epilepticus* (SE) induced by pilocarpine administration. We have previously shown that in this model of epilepsy, brain eIF2α is highly phosphorylated and protein synthesis is severely inhibited [21]. When normalized to the amount of total eIF2α, it is evident that in oocytes eIF2α phosphorylation is equal or even higher than in the SE brain sample. The level of eIF2α-P in oocytes should thus prevent initiation of translation. Because protein synthesis must resume upon fertilization, we then addressed the phosphorylated state of eIF2α in zygotes and 2-cell embryos. As shown in Fig. 2B, a dramatic decrease in eIF2α-P occurs during this transition, reaching levels that could be compatible with global protein synthesis by the 2-cell stage.

Discussion

We have demonstrated in this work that the βGCN2 transcript and the GCN2 protein are extremely abundant in ovulated oocytes of *Mus musculus*. The finding that this pool of GCN2 is active parallels the high levels of phosphorylated eIF2α in these cells.

Translational control plays a central role in oogenesis and early embryo development in metazoans. From the primary oocytes, arrested in the prophase of meiosis I (MI), hormonal signals lead to a surge in cell growth, with active transcription and translation occurring during the maturation of the oocyte, up to the arrest at metaphase of meiosis II (MII) in the ovulated oocyte. Fertilization triggers the release from MII and maternally synthesized mRNAs and proteins control early embryogenesis until the activation of the zygotic genome. The timely translational activation or translational repression of specific mRNAs is thought to occur by *cis*-acting sequences and *trans*-acting proteins that control the length of the poly(A) tail or the availability of the eIF4F cap-binding complex (reviewed in [23–25]).

The results shown here suggest that phosphorylation of eIF2α, mediated by GCN2, may also be part of the mechanisms controlling translation during oogenesis and early embryogenesis. Recently, the presence of high levels of phosphorylated eIF2α has been described in sea urchin unfertilized eggs [26]. In cell free translation systems of sea urchin MII oocytes, which closely match *in vivo* data, total protein synthesis is strongly impaired, even for exogenously added mRNAs [27,28]. This inhibition was partially overcome by the addition of eIF2B and eIF-4F [28,29]. The relief brought about by eIF2B can be explained in light of the fact that eIF2α-P acts as a competitive inhibitor of eIF2B, the eIF2 GTP exchange factor, which is rate limiting in protein synthesis. In vertebrates, translation in MII stage oocytes seems to be only modestly impaired relative to fertilized eggs. In mice, early reports indicated that there is a modest 45% increase in translation following fertilization, as measured by amino acid incorporation [30]. However, the rate of translation in fertilized mouse eggs, as measured from injected globin mRNA, seems to be severely inhibited when compared to HeLa cells or to reticulocytes [31]. Thus, in both the sea

urchin and in the mouse models the high levels of eIF2α-P found in the MII stage oocyte may contribute to general protein synthesis inhibitory mechanisms.

The abundance of activated GCN2 in the oocytes suggested that it is responsible for the phosphorylation of eIF2α in these cells. Interestingly, a microarray analysis of different developmental stages, from unfertilized eggs to blastula, that also indicated the presence of the GCN2 mRNA in unfertilized eggs, showed that this mRNA decreases to very low levels by the 2-cell embryo stage, at a time point we determined that eIF2α phosphorylation reached levels compatible with efficient translation [32]. *Gcn2*$^{-/-}$ mice have no apparent fertility problems and display normal embryonic development [3]. However, given the many examples of cross-talks among eIF2α kinases, it is likely that another representative of this class of kinases could compensate for the lack of GCN2 in the knock-out animals. One such candidate might be HRI, whose mRNA was detected in unfertilized eggs both in the microarray analysis of Hamatani et al. [32] as well as in the Synatlas array [18]. It will thus be relevant to analyze the oocytes from *Gcn2*$^{-/-}$ mice for the expression of the other kinases and the reproductive capacity of double and triple knock-out animals, which, except for those involving PEK/PERK, should be viable. Alternatively, other compensatory mechanisms could be in place in the *Gcn2*$^{-/-}$ mice. The direct study of fertility of eIF2-Ser51Ala homozygote mutants is not possible since they are not viable [33].

The data shown here indicate that GCN2 is expressed preferentially in maturing oocytes, suggesting that it is activated late in oogenesis, providing an additional mechanism of translational regulation during this process and early embryogenesis, both to down-regulate general protein synthesis as well as to up-regulate translation of specific messages, as exemplified by ATF4 and ATF5 in somatic cells. Our findings implicate GCN2 in additional functions in mammals besides the recently described roles in metabolism and in the central nervous system.

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