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Phosphorylation of Eukaryotic Initiation Factor 2α During Differentiation of Mouse Myoblasts into Myotubes is Mediated by an unknown Kinase

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Abstract: *During differentiation of mouse myoblasts into myotubes, rate of global protein synthesis is decreased to slow down the cell cycle and ultimately cells enter into the quiescent stage. To determine the molecular mechanism of this inhibition of rate of protein synthesis during differentiation, we measured the phosphorylation of eIF2α and its association with p67. Our results show that the phosphorylation of eIF2α during differentiation of C2C12 myoblasts into myotube is increased significantly and is mediated by an unknown kinase, which is not the double-stranded RNA activated kinase, PKR. The level of eIF2α phosphorylation during this differentiation correlates with the inhibition of rates of global protein synthesis and its dissociation from eIF2/p67 complex. Together, our data suggest that dissociation of p67 from eIF2 complex plays an important role to slow down the rates of global protein synthesis during C2C12 myoblasts' differentiation in myotubes.*

Keywords: *C2C12 myoblasts differentiation, eIF2α phosphorylation, eIF2α-specific kinase- PKR or eIF2AK2, and rates of protein synthesis*

1. INTRODUCTION

During normal cellular growth and development, myoblasts differentiate into myotubes and ultimately form myofibers (1). Myotubes are multinucleated; they have withdrawn from the cell cycle, become mitotically quiescent, and arrested at the G_0-phase of the cell cycle, although they are viable (2-4). At the later phase of the cell cycle, there is no DNA replication and very low levels of transcription and translation (5). In addition, several growth promoting cell-signaling pathways are also shut down or slowed down in the myotubes (6).

Earlier, we have demonstrated that the levels of eukaryotic initiation factor 2 (eIF2)-associated glycoprotein, p67, which is also known as MetAP2, increases while myoblasts were differentiated into myotubes in differentiation medium (7-8). Its increased level associates with the mitogen-activated protein (MAP) kinases, extra-cellular-signal regulated kinases (ERK1/2). Subsequently, this causes the inhibition of activation and activity of ERK1/2 MAP kinases and possibly in part the inhibition of cell cycle (8). We wondered what happens to the rates of global protein synthesis while myoblasts are differentiating into myotubes. The rate of global protein synthesis largely depends upon the phosphorylation of the smallest subunit of eukaryotic initiation factor 2 (eIF2α). Once it is phosphorylated by its specific kinase(s), it sequesters the cellular limiting factor, eukaryotic initiation factor 2B (eIF2B) and recycling of eIF2 is inhibited (9). Recycling of eIF2 is essential for the next round of initiation of protein synthesis (9). Consequence of this is the inhibition of the rate of global protein synthesis. There are at least four kinases known to be specific to eIF2α and these kinases are activated at different cellular stress conditions (10). For example, the double-stranded RNA-dependent kinase, PKR or eIF2AK2 is active during viral infection (11-12), heme controlled repressor (HCR or HRI) is active during heme deficiency (13), PKR-like kinase at the endoplasmic reticulum (ER), PERK is active in response to overload of unfolded proteins in ER (14), and the yeast homolog of GCN2 is activated in response to amino-acid starvation (15). Kinases that phosphorylate eIF2α during other cellular stress conditions such differentiation, tumorigenesis, apoptosis, or growth factor deficiency are largely unknown (7). During normal cellular growth conditions, p67 is always bound to eIF2 and prevents it from

phosphorylation by the active kinases (16). Once it is dissociated from eIF2, the active kinase(s) phosphorylates eIF2α and inhibits protein synthesis initiation (7-8). The dissociated form of p67 associates with ERK1/2 MAP kinases and inhibits cell signaling mediated by these kinases (8), providing a mutual communication between cell cycle and protein synthesis machinery.

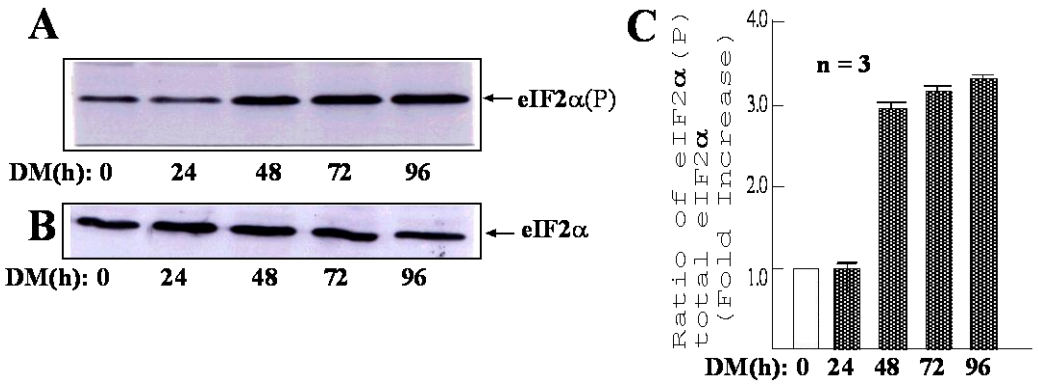
In this study, we have examined (i) the level of eIF2α phosphorylation during differentiation of mouse C2C12 myoblasts into myotubes, (ii) rates of global protein synthesis, (iii) association and dissociation of p67 with eIF2, and (iv) the levels of PKR during differentiation of mouse myoblasts into myotubes. During these differentiation conditions, our results show that eIF2α phosphorylation is increased, rate of global protein synthesis is decreased, p67 dissociates from eIF2α, and PKR level fluctuates. The later results suggest that PKR is not the kinase that phosphorylates eIF2α during differentiation of mouse myoblasts into myotubes in *ex-vivo* conditions.

2. MATERIALS AND METHODS

All materials including chemicals, cells, and other reagents used in this study are essentially described (8). Mouse PKR monoclonal antibody was obtained from Santa Cruz Biotechnology (Sc-6282) and mono-specific antibodies against the phosphorylated form of eIF2α were from Research Genetics (Huntsville, AL). Preparation of polyclonal antibodies specific to p67 or eIF2α was described (16). Cell growth and differentiation conditions of mouse C2C12 myoblasts, preparation of cell extracts, and [^{35}S]Methionine labeling of C2C12 myoblasts during differentiation at different time intervals (0h-96h) were essentially the same as described (8). Procedures for Western blotting and immunoprecipitation assays were also described (8).

3. RESULTS AND DISCUSSION

Previously, we have shown that levels of p67 were increased while C2C12 myoblasts were differentiating into myotubes and it associates with ERK1/2 MAP kinases to inhibit their activation and activity (8). To examine the levels of eIF2α phosphorylation during the above differentiating conditions, we collected total cell extracts from different time intervals (0h to 96h) of differentiation and examined its level via Western blots (Fig. 1A). We also examined the levels of total eIF2α in these cell extracts via Western blots (Fig. 1B). Determination of the ratios of the phosphorylated form of eIF2α and its total amount showed that there is no change between 0h and 24h of differentiation (compare lane 0h with lane 24h in Fig. 1A), a 3-fold increase at 48h of differentiation, and this ratio maximizes to 3.5-fold at 96h of differentiation (Fig. 1A).

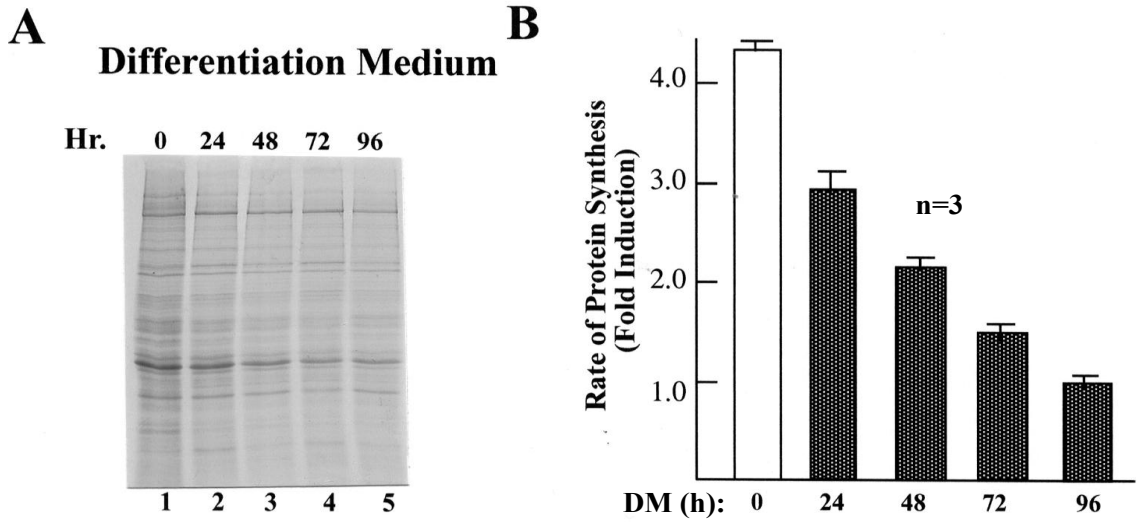


Datta B. and Datta R. Fig. 1

Fig1. *During differentiation of C2C12 myoblasts in differentiation medium (DM), cell extracts were collected at different time intervals (0h – 96h), and immunoblotting experiments were performed using antibodies specific to the phosphorylated form of eIF2α (A) and total eIF2α (B). The ratios of the phosphorylated form of eIF2α and total eIF2α were calculated after scanning the appropriate protein bands and plotted in a graph (C).*

Since levels of eIF2α phosphorylation correlate with the rates of global protein synthesis (7), we therefore examined the rates of protein synthesis by metabolically labeling myoblasts differentiating into myotubes at different time intervals (Fig. 2). Newly synthesized [^{35}S] Methionine labeled proteins were analyzed via fluorography (Fig. 2A) and by scintillation counter

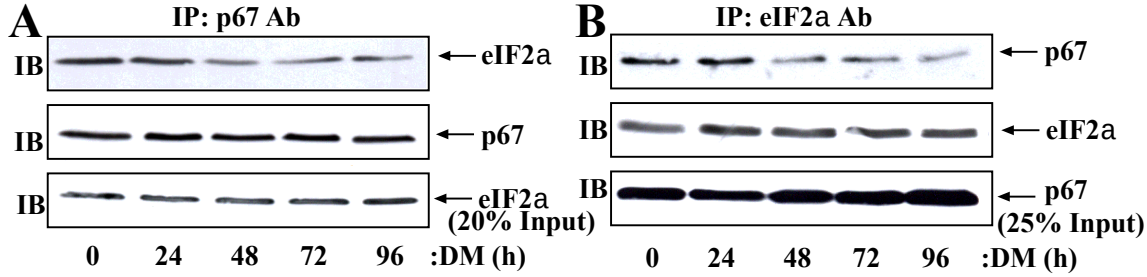
(Fig. 2B). Our results show that there is a gradual decrease in protein synthesis rate while C2C12 myoblasts were differentiating into myotubes and this goes down to 4-fold of the rates of protein synthesis from the beginning of the differentiation, 0h to96h of differentiation (compare lane 1 to lane 5 in Fig. 2A and 0h to 96h in Fig. 2B). These results thus suggest that indeed there is a drop of rates of protein synthesis while C2C12 myoblasts differentiate into myotubes and this is in part due to the increased phosphorylation of the eIF2α by its specific kinase(s).



Datta B. and Datta R. Fig. 2

Fig 2. *During differentiation of C2C12 myoblasts in differentiation medium, cells were metabolically labeled with [^{35}S]Methionine, cells were harvested, and cell extracts were prepared at different time intervals (0h-96h). 25μg of total proteins were analyzed on a 15% SDS-PAGE followed by fluorography (A). 2.5μg of total proteins were used for counting in a scintillation counter and data (cpm/μg of protein) were plotted on a graph (B).*

Phosphorylation of eIF2α by its kinases depends upon its association and dissociation with p67. Once p67 is dissociated from eIF2, eIF2α is phosphorylated (7-8). We therefore tested whether this is true when myoblasts were differentiating into myotubes. First, we immunoprecipitated p67 from total cell extracts collected at different time intervals of differentiation (0h-96h) and immunoblotted with eIF2α polyclonal antibodies (Fig. 3A). Similar experiments were performed except immunoprecipitations were done with antibodies specific to eIF2α and immunoblottings were done with p67 antibodies (Fig. 3B). Since both antibodies either against p67 or eIF2α precipitated almost equal amounts of p67 (Fig. 3A, middle panel) or eIF2α (Fig. 3B, middle panel), and there are different amounts of p67 or eIF2α precipitated by their reciprocal antibodies during differentiation of myoblasts starting at 48h and continued until 96h of differentiation (compare lanes 0h to 96h of differentiation in Figs. 3A&B), it is thus conceivable that there is a dissociation between p67 and eIF2α starting from 48h to 96h of differentiation of C2C12 myoblasts into myotubes. As a consequence of this dissociation, eIF2α is phosphorylated by its kinase(s) during these time intervals of differentiation (Fig. 1A).

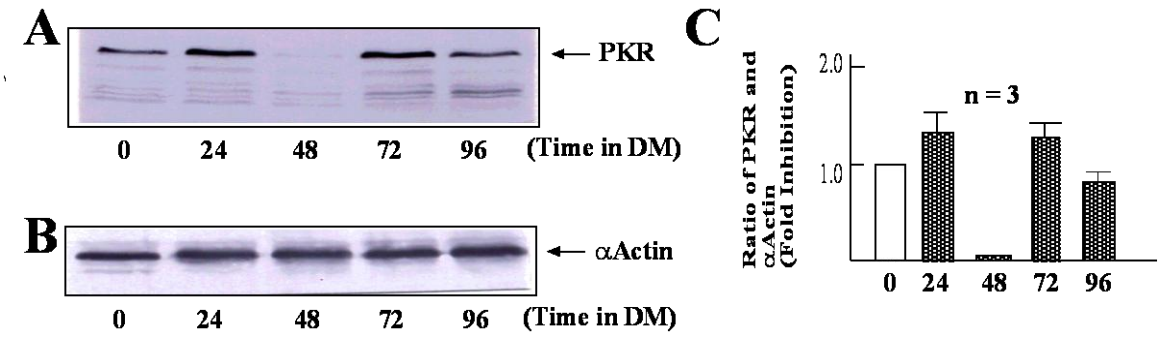


Datta B. and Datta R. Fig. 3

Fig. 3 *Cell extracts from different time intervals (0h - 96h) during differentiation of C2C12 myoblasts were used for co-immunoprecipitation assays using antibodies specific to either p67 A) or eIF2α (B). The*

immunoprecipitates were analyzed on Western blots using antibodies specific to eIF2α (A, upper panel), p67 (A, middle panel), and p67 (B, upper panel) and eIF2α (B, middle panel). The input eIF2α (A, lower panel) and p67 (B, lower panel) were also analyzed on Western blots using their respective polyclonal antibodies. IP, immunoprecipitation; IB, immunoblotting; and DM, differentiation medium.

There are at least four know eIF2α-specific kinases that can phosphorylate this protein (10). In our *ex-vivo* conditions for differentiation of C2C12 myoblasts into myotubes, there is not need for heme and therefore HRI may not be involved in phosphorylating eIF2α. Likewise, during C2C12 differentiation there is no deficiency of the amino acids and that's why homolog of yeast GCN2cannot be involved in phosphorylating eIF2α. In addition, during C2C12 differentiation in *ex-vivo* conditions, rate of global protein synthesis is slowed down significantly and these conditions therefore cannot overload endoplasmic reticulum to activate unfolded protein response (UPR), and PERK may not be the kinase that phosphorylates eIF2α at these conditions. This leaves us to suspect that PKR could be the kinase that phosphorylates eIF2α during C2C12 differentiation into myotubes. We therefore examined the levels of PKR (Fig. 4A) and αActin (Fig. 4B) as a loading control via Western blots in cell extracts taken from different time intervals of C2C12 differentiation. We calculated the ratios of PKR to αActin (Fig. 4C) and our quantification data show that there is a significant fluctuation of PKR level during these time intervals of differentiation. For example, there is a slight increase of its level at 24h and 72h of differentiation while its level nearly undetectable at 48h of differentiation and a slight decrease at 96h as compared to 0h of differentiation (Fig. 4). On the other hand, the level of eIF2α phosphorylation is increased steadily from 48h to 96h of C2C12 differentiation into myotubes (Fig. 1). These results thus suggest that PKR is not the kinase that phosphorylates eIF2α during C2C12 myoblasts differentiation into myotubes.



Datta B. and Datta R. Fig. 4

Fig 4. *Similar experiments were performed as mentioned in legend to figure 1 except the immunoblotting experiments were performed using antibodies specific to PKR (A) and αActin as a control (B). The ratios of PKR and αActin were measured after scanning the appropriate protein bands and plotted on a graph (C).*

We have demonstrated earlier that the levels of p67 is increased during differentiation of C2C12 myoblasts into myotubes in *ex-vivo* conditions and this increased level is due to the low turnover rate of this protein (8). The increased p67 level showed increased binding to ERK1/2 MAP kinases during differentiation and it inhibits the phosphorylation of ERK1/2 MAP kinases (8). The phosphorylation of ERK1/2 MAP kinases is essential for cell signaling to the nucleus to drive the cell cycle at different phases during normal growth conditions of mammalian cells (17). The inhibition of ERK1/2 phosphorylation by p67 is therefore important in part for driving these C2C12 myoblasts into the G_0-phase of the cell cycle. In addition, lowering the activity of the protein synthesis machinery during the above differentiation conditions is necessary to stop the synthesis of mRNAs especially for growth promoting proteins. In this study, we show that there is an increased phosphorylation of eIF2α (Fig. 1), reduced levels of rates of protein synthesis (Fig. 2), and this is possibly due to the weaker interactions between p67 and eIF2αduring differentiation of C2C12 myoblasts into myotubes (Fig. 3). In addition, our data also show that the phosphorylation of eIF2α during these differentiation conditions is not due to PKR (Fig. 4). Collectively, our data suggest that during physiological conditions like myoblasts' growth and

differentiation, there are dynamic interactions within p67, eIF2α, and ERK1/2 and these interactions possibly determine in part the state of these cells' physiology.

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