

# Regulation of ubiquitous 6-phosphofructo-2-kinase by the ubiquitin-proteasome proteolytic pathway during myogenic C2C12 cell differentiation

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**Abstract** 6-Phosphofructo-2-kinase catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, activator of phosphofructokinase-1 and inhibitor of fructose 1,6-bisphosphatase. These properties confer to this bifunctional enzyme a key role in the control of glycolysis and gluconeogenesis. Several mammalian isozymes generated by alternative splicing from four genes, designated *pfkfb1–4*, have been identified. The results presented in this study demonstrate the expression of the *pfkfb3* gene in C2C12 cells and its downregulation during myogenic cell differentiation. We also show that the decrease of ubiquitous 6-phosphofructo-2-kinase isozyme levels, product of *pfkfb3* gene, is due to its enhanced degradation through the ubiquitin-proteasome proteolytic pathway.

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**Key words:** 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase; Glycolysis; Ubiquitin; Proteasome; Myogenic differentiation

## 1. Introduction

Skeletal muscle differentiation is characterized by terminal withdrawal from the cell cycle, the coordinated activation of muscle-specific gene expression, and the fusion of myoblasts into multinucleated myotubes [1–3]. All these events are coordinated by a family of muscle-specific basic helix-loop-helix (bHLH) transcription factors, belonging to the MyoD family, that include MyoD [4], Myf5 [5], myogenin [6] and MRF4 [7]. Members of the MyoD family heterodimerize with ubiquitously expressed members of the E-protein family and bind to a DNA consensus CANNTG, known as an E-box, found in promoters of several muscle-specific genes [8,9].

6-Phosphofructo-2-kinase [EC 2.7.1.105]/fructose 2,6-bisphosphatase [EC 3.1.3.46] (PFK-2) is a homodimeric bifunctional enzyme that catalyzes the synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6-P<sub>2</sub>) [10]. Fru-2,6-P<sub>2</sub> is the

most powerful activator of phosphofructokinase-1 and an inhibitor of fructose 1,6-bisphosphatase [10–13]. These properties confer to this bifunctional enzyme a key role in the control of glycolysis and gluconeogenesis. Since the discovery of this system in liver, other mammalian tissue-specific bifunctional isozymes have been identified. These isozymes are generated by alternative splicing from four genes, designated *pfkfb1–4* [10], and they differ in physicochemical, immunological and functional properties, as well as in their response to phosphorylation by protein kinases. The expression of *pfkfb* genes is regulated by hormones, growth factors and metabolites, that control tissue-specific isoforms. *Pfkfb3* gene accounts for two reported isoforms, ubiquitous (uPFK-2) and inducible, generated through the alternative splicing of exon 15 [14]. *Pfkfb3* gene product has the highest kinase:phosphatase activity ratio and thus maintains elevated Fru-2,6-P<sub>2</sub> levels, which in turn sustains high glycolytic rates in the cell [15–17]. This gene has been related to proliferation because it is ubiquitously expressed in different tissues and in transformed cell lines and tumors [16–19] and transfection of antisense oligonucleotides against this gene in K562 leukemia cells produces an inhibition of DNA synthesis and cell proliferation [18]. Furthermore, it has been recently found that it is highly induced by hypoxia through the hypoxia-inducible transcription factor-1 complex [20] and modulated by adenosine monophosphate (AMP)-kinase-dependent phosphorylation [21].

The aim of this report is to study *pfkfb3* gene expression and regulation during muscle differentiation of C2C12 cells. Our results show that during myogenesis, the expression of *pfkfb3* decreases and the uPFK-2 gene product is degraded through the ubiquitin-proteasome proteolytic pathway.

## 2. Materials and methods

### 2.1. Plasmid constructs

*Pfkfb3* cDNA encoding the human uPFK-2 was cloned into pcDNA3.1 vector for cellular expression (uPFK-pc3) as described previously [16]. The expression vector pMT107 contains a ubiquitin His-tagged cDNA obtained as described [22]. The glutathione S-transferase (GST)-ubiquitin is a bacterial expression vector for the ubiquitin cDNA fused to the GST protein, and it was generated and described elsewhere [22].

### 2.2. Cell culture and transfection

All cell lines are cultured at 37°C with 95% humidity and 10% CO<sub>2</sub>. C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). Muscle

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**Abbreviations:** PFK-2, 6-phosphofructo-2-kinase [EC 2.7.1.105]/fructose 2,6-bisphosphatase [EC 3.1.3.46]; uPFK-2, ubiquitous 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase; Fru-2,6-P<sub>2</sub>, fructose 2,6-bisphosphate; LLnL, *N*-acetyl-leucyl-leucyl-*N*-norleucinal

differentiation was induced by culturing subconfluent C2C12 cells in DMEM supplemented with 2% FBS. HEK-293T cells were cultured in DMEM supplemented with 10% FBS and 1 mM sodium pyruvate. C2C12 and HEK-293T cells were transiently transfected with the indicated vectors using the calcium phosphate transfection method as described [23]. *N*-acetyl-leucyl-leucyl-*N*-norleucinal (LLnL) (Sigma) (50  $\mu$ M) was added 8 h before cell harvesting. Des(1,2,3)insulin-like growth factor (IGF)-I was from Dra Angelika F. Schutzdeller (Tubingen, Germany) and bone morphogenic protein (BMP)-2 from Genetics Institute (Cambridge, MA, USA).

### 2.3. RNA analysis by Northern blot hybridization and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured C2C12 cells ( $1 \times 10^7$  cells) using an RNA isolation kit following the manufacturer's protocol (Ultraspec RNA Biotech Laboratories). A partial cDNA clone of 0.7 kb, corresponding to the 3'-untranslated region (UTR) of mouse uPFK-2 (accession number AF294617), was used as a probe. The probe was directly labeled by incorporation of [ $^{32}$ P] $\alpha$ -dCTP using standard procedures. RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel and transferred to nylon membranes using capillary transfer. Hybridization was carried out at 42°C, and membranes were then washed three times at 46°C in 0.1% sodium dodecyl sulfate (SDS) and  $0.1 \times$  standard saline citrate (SSC) and subjected to autoradiography by exposing to Kodak MS film for 36 h at  $-80^\circ\text{C}$ . mRNA levels were normalized using ribosomal RNA fluorescent intensity in the agarose-formaldehyde gel.

For RT-PCR conditions: 1  $\mu$ g of total RNA was reverse-transcribed using a Ready-To-Go T-Primed First-Strand Kit from Pharmacia Biotech (Uppsala, Sweden). PCR amplification was carried out using reverse-transcribed RNA and forward primer Mus 1 and reverse primer Mus 3 (5'-GGGCGCTTCCTCAGCCT-3' and 5'-ATCCTT-TAGGGCTGCTAGAG-3', respectively) for uPFK-2, myog1 and myog2 (5'-AGTGAATGCAACTCCACAGCGCCT-3' and 5'-TG-GCTTGTGGCAGCCAGGG-3') for myogenin, and gapdh1 and gapdh2 (5'-CAAAGTGGACATTGTTGCCATC-3' and 5'-GCGC-AGTAGACTCCACGACA-3') for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### 2.4. Western blot analysis

Total cellular protein was obtained from C2C12 cells using 500  $\mu$ l of extraction buffer (0.5 M Tris-HCl at pH 6.8 with 1% SDS) for 5 min at  $95^\circ\text{C}$ . 50  $\mu$ g of total cellular protein were separated on 8% SDS-polyacrylamide gel under reducing conditions and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was probed with a specific antibody against the C-terminus of uPFK2 [17], inducible PFK-2 (Santa Cruz), liver PFK-2 (Santa Cruz), anti-myogenin antibody F5D (Pharmingen), anti- $\beta$ -actin (Sigma) or anti- $\alpha$ -tubulin (Calbiochem). Immunoreactive proteins were then visualized by developing membranes with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1:5000) followed by incubation with electrochemoluminescent (ECL) Western blot reagent (Amersham Pharmacia Biotech, Uppsala, Sweden).

### 2.5. Fru-2,6-P<sub>2</sub>, lactate and creatine kinase assays

Fru-2,6-P<sub>2</sub> was measured by a modification of the method described by Van Schaftingen et al. [24]. Extracts were obtained by washing the cells twice in ice-cold phosphate-buffered saline (PBS) and then scraping the cell layer in 0.4 ml of 100 mM NaOH with 0.1% Triton X-100 and centrifuged at 10000 rpm for 10 min at  $4^\circ\text{C}$ . Creatine kinase activity was measured in C2C12 extracts obtained by washing the cells twice in ice-cold PBS and then scraping the cell layer in 0.4 ml of PBS with 0.1% Triton X-100 and centrifuged at 10000 rpm for 10 min at  $4^\circ\text{C}$ . Supernatants were incubated for 5 min at  $30^\circ\text{C}$  with 1 ml of the reaction mix (23 mM cysteine, 2.3 mM adenosine diphosphate (ADP), 23 mM D-glucose, 2.3 mM nicotinamide adenine dinucleotide phosphate (NADP), 4000 U/l hexokinase, 2300 U/l glucose 6-phosphate dehydrogenase) in reaction buffer (128 mM imidazole, 2.5 mM ethylenediamine tetraacetic acid (EDTA), 12.8 mM magnesium acetate at pH 6.7). The reaction was initiated with creatine phosphate, detecting the change in absorbance at 340 nm. One creatine kinase unit corresponds to the amount of enzyme that catalyzes the synthesis of 1  $\mu$ mol of product per min. Lactate was measured by mixing 1 ml of culture medium with 1 ml of 10% HClO<sub>4</sub>. After 10 min

at  $0^\circ\text{C}$  and centrifugation at  $2000 \times g$  for 10 min, the supernatant was neutralized with 3 M KOH-KHCO<sub>3</sub> and lactate concentration measured as described [25].

### 2.6. Protein stability

uPFK-2 was labeled with [ $^{35}$ S]methionine in an *in vitro* transcription/translation reaction using the manufacturer's protocol (Promega). C2C12 cells at 0 or 3 days after initiation of muscle differentiation were trypsinized and cell pellets were obtained by centrifugation at 1000 rpm for 5 min at  $4^\circ\text{C}$ . Cell pellets were washed twice with ice-cold PBS and resuspended in 100 ml of 10 mM Tris, pH 8.0 for 10 min and sonicated briefly. Cell debris was removed by centrifugation at 10000 rpm for 10 min at  $4^\circ\text{C}$ . The protein concentration of the resultant supernatant was determined by bicinchoninic acid (BCA) protein assay (Pierce). For the stability assay, 150  $\mu$ g of total protein was mixed with 10  $\mu$ l of  $^{35}$ S-labeled uPFK-2, and the volume adjusted to 130  $\mu$ l using lysis buffer. Following incubation at  $30^\circ\text{C}$  for various periods of time, 40  $\mu$ l of the reaction mixture was taken and the reaction stopped by adding  $2 \times$  SDS sample buffer. Samples were boiled for 3 min and separated on a 8% SDS-polyacrylamide gel electrophoresis (PAGE). Following migration, the gel was fixed in 20% methanol, 10% acetic acid for 30 min, dried and exposed to Kodak XR film at  $-80^\circ\text{C}$ . If treatment with LLnL (Sigma) was required, C2C12 cells were differentiated in DMEM with 2% FBS and 8 h before cell collection were treated with 50  $\mu$ M of the proteasome inhibitor LLnL dissolved in dimethyl sulfoxide (DMSO) or with the same volume of DMSO in fresh medium. Following treatment, cells were harvested and uPFK-2 levels quantified by Western blot as described above.

For *in vivo* stability C2C12 cells differentiated for 3 days in 2% FBS or in growing medium (20% FBS) were incubated for 0, 1, 2, 4 or 8 h in the presence of 10  $\mu$ g/ml of the protein synthesis inhibitor cycloheximide. After these times, cells were lysed and uPFK-2 levels quantified by Western blot as described above.

### 2.7. Ubiquitin binding assays

To detect ubiquitinated uPFK-2 *in vivo*, subconfluent cultures of HEK-293T cells were transfected as described above with 2  $\mu$ g of empty vector or uPFK-pc3 expression constructs, in either the presence or absence of 4  $\mu$ g of the plasmid pMT107, which encodes His-tagged human ubiquitin. After 20–24 h, cells were treated with 50  $\mu$ M of the proteasome inhibitor LLnL dissolved in DMSO or with the same volume of DMSO in fresh medium for 8 h. Cell extracts were prepared after washing cells twice in cold PBS by boiling cells for 5 min with 50 mM Tris-HCl, 1% SDS at pH 6.8. Protein concentration was determined using a BCA protein assay (Pierce). Samples were diluted in eight volumes of pull-down buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 20 mM imidazole at pH 7.4) with protease and phosphatase inhibitors as described [22]. Histidine-tagged proteins were purified on nickel-nitrilotriacetic acid (NTA)-agarose (Qiagen) and uPFK-2 was detected by SDS-PAGE and immunoblotting as described above.

For *in vitro* uPFK-2 ubiquitination, *in vitro* translated  $^{35}$ S-labeled human uPFK-2 (25  $\mu$ l) was incubated with 5  $\mu$ g of GST or GST-ubiquitin at  $30^\circ\text{C}$  for 1 h. GST proteins were purified on glutathione-Sepharose (Amersham-Pharmacia) and analyzed by SDS-PAGE. After electrophoresis, gels were stained with Coomassie blue, dried and autoradiographed (Kodak XR film) at  $-80^\circ\text{C}$ .

## 3. Results

### 3.1. Pfkfb3 gene expression during myogenic differentiation of C2C12 cells

As previously described, C2C12 cells with a limited supply of growth factors differentiate into myoblasts, that later fuse into multinucleated myotubes [1–3]. To promote differentiation, subconfluent C2C12 cells were serum-starved for up to 7 days. On microscopic examination of the cultures we observed the expected morphological changes, namely cell alignment and fusion into multinucleated myotubes (data not shown). Total RNA was extracted from C2C12 cells at different days of differentiation to determine the expression of

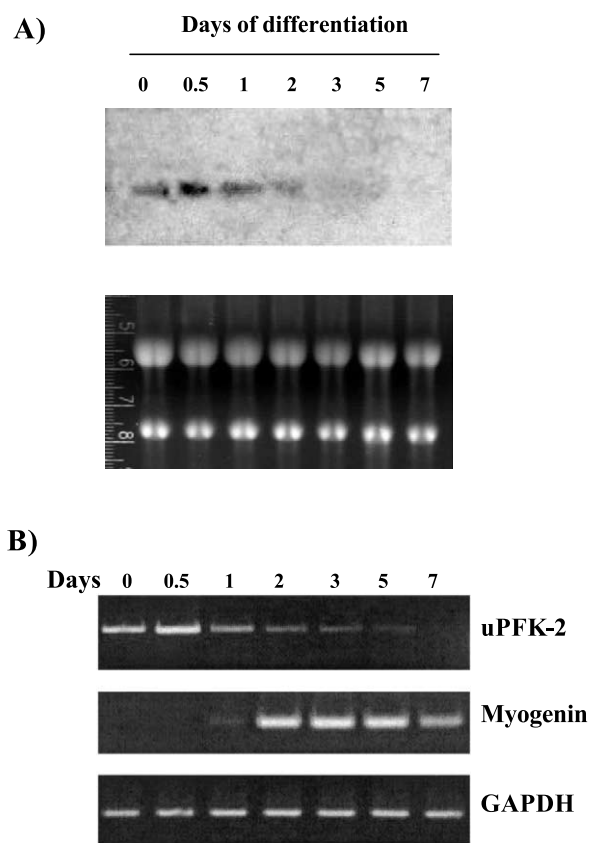


Fig. 1. *Pfkfb3* gene expression during muscle differentiation of C2C12 cells. A: Total RNA was extracted from C2C12 cells at different days of myogenic differentiation and analyzed by Northern blot with a specific probe for mouse ubiquitous *pfkfb3* as described in Section 2. Lower panel shows the levels of RNA loaded in each lane stained with ethidium bromide. B: For RT-PCR analysis, total RNA was reverse-transcribed using a Ready-To-Go T-Primed First-Strand Kit from Pharmacia Biotech (Uppsala, Sweden). PCR amplification was carried out using reverse-transcribed RNA with specific primers, as described in Section 2.

uPFK-2 mRNA by Northern blot (Fig. 1A). Myogenic differentiation of the C2C12 cells produced a progressive decrease of uPFK-2 mRNA levels, showing that *pfkfb3* gene expression was downregulated during this process. These results were confirmed using RT-PCR analysis (Fig. 1B).

### 3.2. Fru-2,6-P<sub>2</sub> and uPFK-2 expression during myogenic differentiation

To examine the effect of myogenic differentiation on the levels of uPFK-2 protein, we performed Western blot analysis of C2C12 protein extracts at different days of differentiation with a specific antibody against the C-terminus of the ubiquitous isozyme that does not recognize the inducible isoform [17] (Fig. 2A). uPFK-2 expression decreased throughout the differentiation process, being barely detectable after 3 days, whereas myogenin, a marker of differentiation, showed a transient increase in expression with a peak at 2 days. Inducible or liver PFK-2, products of the *pfkfb3* and *pfkfb1* gene, respectively, were not detectable during this process (data not shown). Correlating with uPFK-2 levels, the concentration of Fru-2,6-P<sub>2</sub> decreased and a lower lactate was observed in 3 days extracts ( $62.9 \pm 4.2$   $\mu\text{mol}$  lactate/mg protein in 2% FBS cells versus  $80.6 \pm 9.3$   $\mu\text{mol}$  lactate/mg protein in 20% FBS

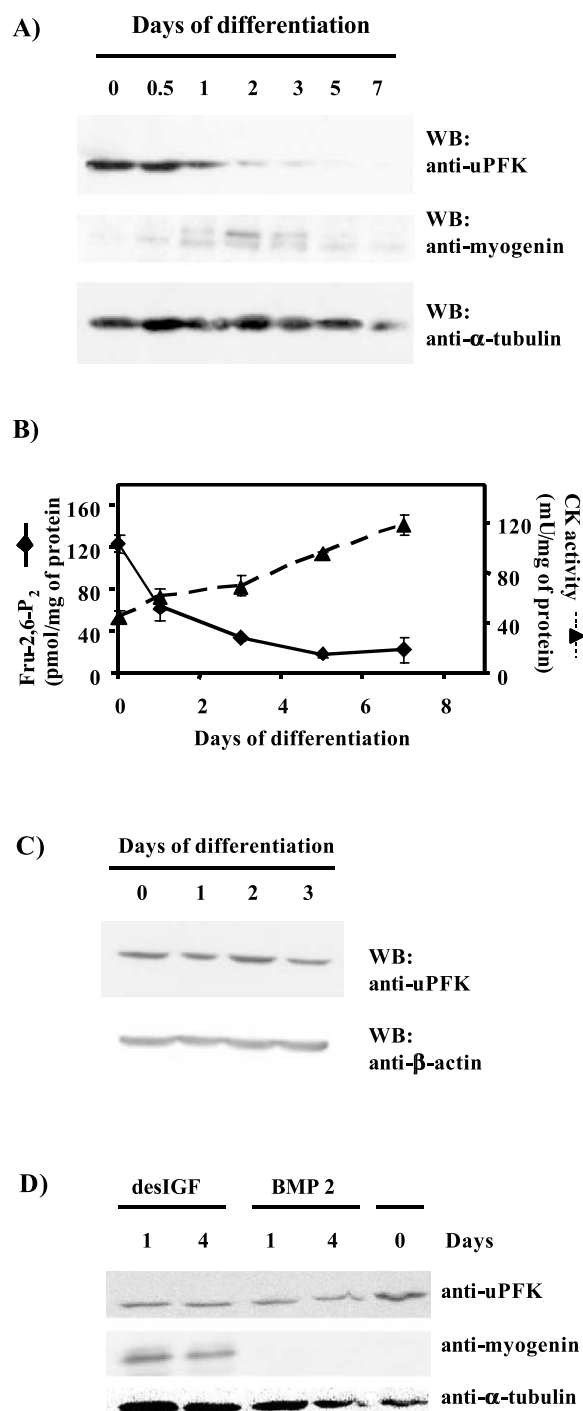


Fig. 2. PFK-2 protein level and Fru-2,6-P<sub>2</sub> concentration. A: 100  $\mu\text{g}$  of whole protein extract from C2C12 cells at different times of myogenic differentiation were resolved by SDS-PAGE and analyzed by Western blot with anti-uPFK-2, with anti-myogenin and with anti- $\alpha$ -tubulin as described in Section 2. B: Determination of the concentration of Fru-2,6-P<sub>2</sub> (pmol/mg of protein,  $\blacklozenge$ ) and creatine kinase activity (mU/mg of protein,  $\blacktriangle$ ). Each point represents the mean  $\pm$  S.E.M. of three independent experiments. C: Western blot against uPFK-2 from NIH3T3 whole extracts incubated for different times with 2% of serum and normalized with  $\beta$ -actin. D: C2C12 cells treated with 2 nM desIGF-I or 2 nM BMP-2 at 2% FBS. Representative experiments are shown.

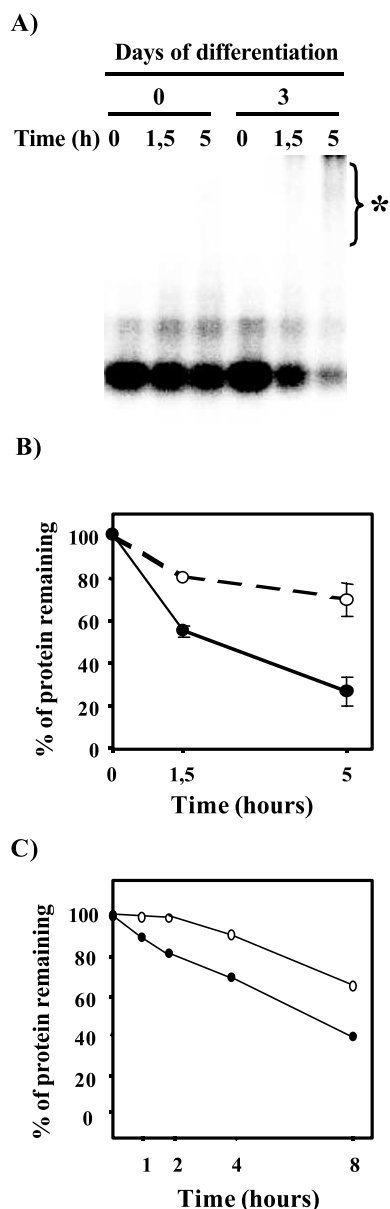


Fig. 3. In vitro degradation of uPFK-2 during myogenic differentiation. A: 10  $\mu$ l of in vitro translated  $^{35}$ S-labeled uPFK-2 was incubated in the presence of 150  $\mu$ g of total protein extracts from C2C12 cells at 0 or 3 days of myogenic differentiation for the indicated periods of time at 30°C. Following electrophoresis, [ $^{35}$ S]uPFK-2 was visualized by autoradiography. Asterisk shows the bands of high molecular weight that appeared in the 3 days extracts. B: Densitometric quantification of the remaining protein with respect to the protein present at 0 h represented as 100%, each point represents the mean  $\pm$  S.E.M. of three independent experiments. C: C2C12 cells at 0 ( $\circ$ ) or 3 days ( $\bullet$ ) of myogenic differentiation were incubated for the periods of time indicated in the presence of 10  $\mu$ g/ml of the protein synthesis inhibitor cycloheximide. Cells were lysed and uPFK-2 was immunodetected by Western blot. Results were quantified by densitometric analysis. Results are the mean of two independent experiments.

cells) whereas creatine kinase activity increased during myogenic differentiation (Fig. 2B). To confirm the specific implication of muscle differentiation and rule out the possible effect on uPFK-2 expression of reduced serum during differentiation, we cultured NIH3T3 cells with 2% of serum in the medium and analyzed the amount of uPFK-2 protein by Western

blot at different times during differentiation. As shown in Fig. 2C, after 3 days of serum starvation we did not detect variations in the uPFK-2 protein level. These results indicate that downregulation in uPFK-2 expression is dependent on the myogenic process.

Furthermore, we induced C2C12 differentiation in low serum concentration using desIGF-I, a known myogenic differentiation factor which increases myogenesis and myogenin in this cell model [26]. The results obtained show a 85% decrease in uPFK-2 protein levels during the first 24 h while the same percentage of reduction using the serum starvation protocol was observed after 3 days (Fig. 2D). In addition, we also found a decrease in the expression of uPFK-2 in C2C12 cells in the presence of BMP-2 (Fig. 2D). The presence of BMP-2 in C2C12 cells cultured with 2% FBS stops the differentiation into myotubes and promotes the differentiation into osteoblasts [27]. Taken together these results suggest that the decrease in uPFK-2 levels is dependent on cell differentiation.

### 3.3. Protein stability of uPFK-2 during myogenic differentiation

To investigate the stability of uPFK-2 during myogenic differentiation, we labeled uPFK-2 using an in vitro transcription and translation method (see Section 2) and incubated it with C2C12 cell extracts from 0 or 3 days of differentiation and the amount of uPFK-2 was analyzed (Fig. 3A). The results obtained showed a faster disappearance of the ubiquitous protein when incubated with the C2C12 extracts from 3 days of differentiation (Fig. 3B) as compared to the levels obtained when the protein was incubated with extracts from

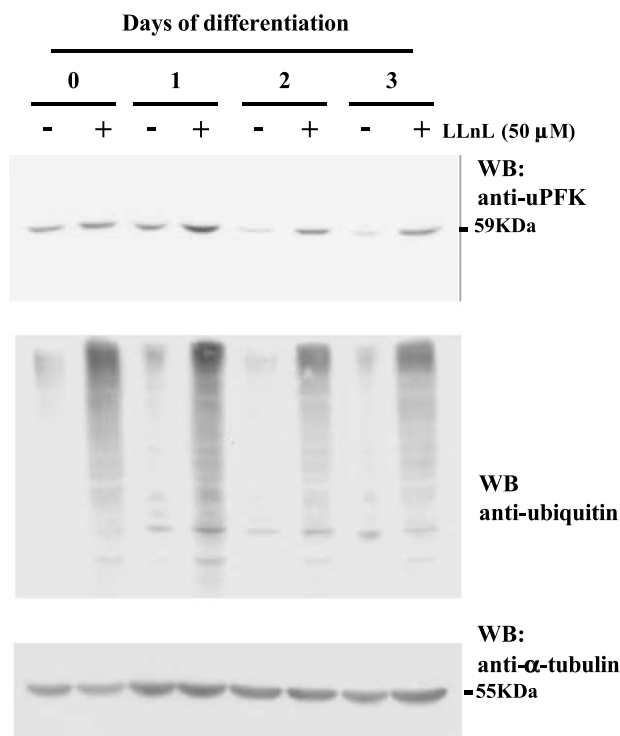


Fig. 4. The proteasome pathway is involved in the regulation of uPFK-2. Whole extracts from C2C12 at different days of differentiation, incubated with (+) or without (-) 50  $\mu$ M of LLnL for 8 h, were analyzed by Western blot against uPFK-2. Anti-ubiquitin immunodetection was made as a control of the action of the proteasome inhibitor and the immunoblot with anti-tubulin protein as load control. A representative experiment is shown.



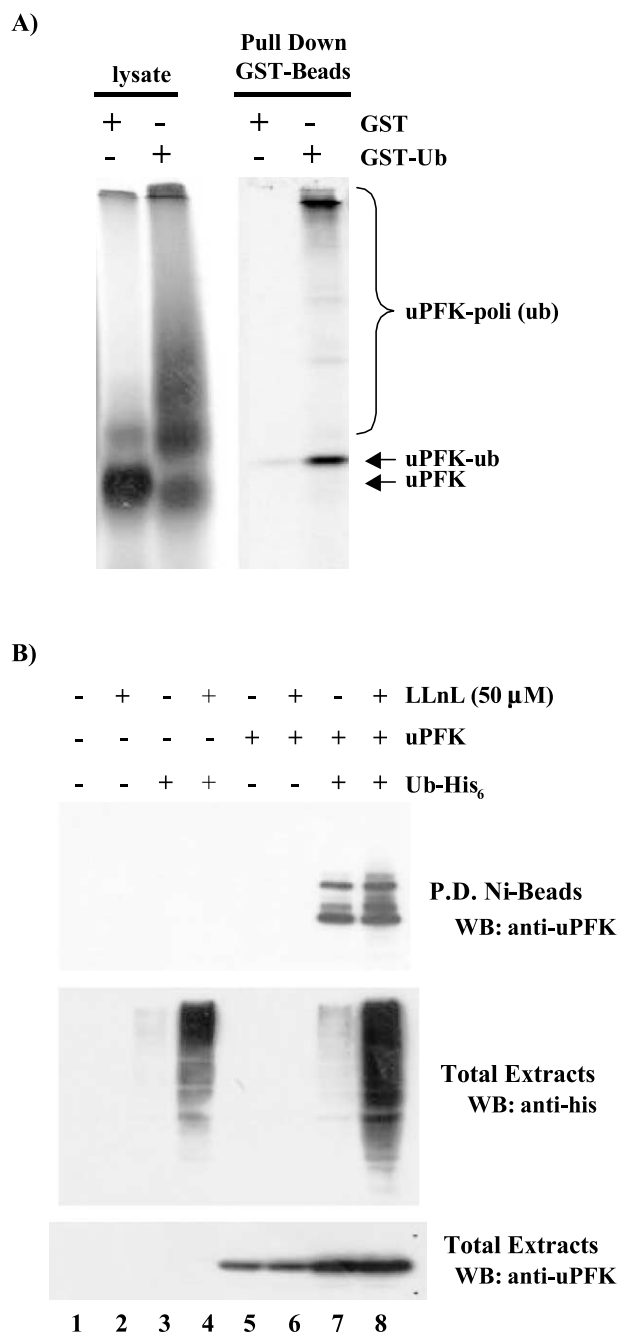


Fig. 5. Ubiquitination of uPFK-2. A: In vitro ubiquitination assay. Radiolabeled uPFK-2 by in vitro translation was incubated with either GST or GST-ubiquitin (GST-ub). After pull-down with glutathione beads, protein were eluted from the beads with sample buffer containing dithiothreitol (DTT) and resolved by SDS-PAGE in parallel with rabbit reticulocyte lysate containing the incubation mix before the pull-down (lysate lanes). uPFK-2 was detected by autoradiography of the gel. B: In vivo ubiquitination of uPFK-2. HEK-293T cells were transiently transfected with pcDNA3 vector alone (lanes 1 and 2), with uPFK-pc3 (lanes 5–8) or with pMT107 (lanes 3, 4, 7 and 8), in the presence (+) or absence (–) of 50  $\mu$ M of LLnL. The amount of protein of each transfection was normalized before the pull-down with Ni beads. Proteins were eluted from beads with sample buffer and electrophoresed by SDS-PAGE. uPFK-2 present in the pull-down samples was detected by immunoblot with a specific antibody. The middle and lower panels show the overexpression of ubiquitin and uPFK-2 in the normalized extracts before the pull-down by immunoblotting with anti-his6 for ubiquitin or with anti-uPFK-2 for uPFK-2. Both panels are representative of two independent experiments.

cells before differentiation. Moreover, uPFK-2 in these extracts was processed and appeared as higher molecular weight bands (\* in Fig. 3A), suggesting a post-translational modification of the protein. A post-translational modification that could produce this characteristic shift of the molecular weight is protein ubiquitination and this modification is related to protein degradation by the proteasome complex. We also determined uPFK-2 half-life in C2C12 either exponentially, growing in high serum media, or after 3 days of myogenic differentiation, induced by serum deprivation. Results shown in Fig. 3C indicate that myogenic differentiation decreased uPFK-2 half-life almost to 50% of that of exponentially growing cells, further suggesting an increased degradation in differentiating C2C12 cells.

### 3.4. Implication of the ubiquitin-proteasome proteolytic pathway in the degradation of uPFK-2

Due to the importance of the ubiquitin-proteasome proteolytic pathway during muscle differentiation [28], we studied the implication of this pathway in the specific degradation of uPFK-2 protein. C2C12 cells were cultured for 0–3 days in differentiation media and 8 h before harvesting 50  $\mu$ M LLnL, a proteasome inhibitor, was added and the levels of uPFK-2 analyzed in cell extracts by Western blot analysis. As shown in Fig. 4, uPFK-2 protein decreased in control cells whereas cells treated with the proteasome inhibitor maintained uPFK-2 protein levels. These results demonstrate the involvement of ubiquitin-proteasome proteolytic pathway in the decrease of uPFK-2 during myogenic differentiation.

In order to determine whether uPFK-2 could be ubiquitinated in vitro, we performed pull-down experiments with glutathione beads incubated with [<sup>35</sup>S]methionine radiolabeled uPFK-2 protein, synthesized using a rabbit reticulocyte system, and GST or GST-ubiquitin. Pull-down pellets were resolved by SDS-PAGE and the ubiquitinous isozyme detected by autoradiography (Fig. 5A). uPFK-2 incubated with GST-ubiquitin was resolved in multiple bands of high molecular weight after the pull-down due to the ubiquitination of uPFK-2 in the reticulocyte extracts, whereas uPFK-2 incubated with GST alone was not ubiquitinated. The bands of higher molecular weight corresponding to uPFK-2 are consistent with the covalent cross-linking of multiple GST-ubiquitin moieties (35 kDa) to uPFK-2 protein. After pull-down we could observe monoubiquitinated as well as several polyubiquitinated forms of uPFK-2. The ubiquitination of uPFK-2 might target the protein to proteasome-mediated degradation. To confirm this hypothesis, we tested the effect of the proteasome inhibitor LLnL on uPFK-2 levels in vivo. As shown in Fig. 5B, treatment of HEK-293T cells with LLnL resulted in an increase of uPFK-2 levels and accumulation of ubiquitinated uPFK-2 in pull-down extracts. In conditions where uPFK-2 cDNA and ubiquitin His6-tag were overexpressed, we could only detect uPFK-2 after the pull-down Ni bead extracts when cells were coexpressing both constructs. In these conditions we detected higher molecular weight bands showing that uPFK-2 is ubiquitinated in vivo.

## 4. Discussion

A remarkable property of cultured C2C12 cells is the activation of the myogenic program upon changes of culture conditions (serum withdrawal), characterized by a cell cycle exit

and muscle differentiation [1–3]. Since the isolation of the *pkfkb3* gene from brain [16,29], placenta [30] and several cell lines [17,18,31], this gene has been characterized by its ubiquitous distribution and its presence in proliferating and transformed cell lines, as well as in tumors [16–19]. The high kinase/bisphosphatase activity of this gene product can explain the high Fru-2,6-P<sub>2</sub> concentration found in the cells where it is present, which in turn sustains high glycolytic rates [15]. There is evidence of an upregulation of its expression in response to different stimuli such as progesterone [31], serum, insulin [17], proinflammatory molecules [18] or hypoxia [20]. The results presented in this study demonstrate that *pkfkb3* gene is expressed in C2C12 cells and it is downregulated during myogenic cell differentiation, correlating with a decrease in the amount of the enzyme. We also demonstrate that the decreases in uPFK-2 protein levels are due to its enhanced degradation through the ubiquitin-proteasome proteolytic pathway.

In adult muscle, a muscle-specific PFK-2 isozyme derived from *pkfkb1* gene is present. Vandoolaeghe et al. [32] investigated at what stage of differentiation the *pkfkb1* muscle-specific (M) promoter became functional, showing that muscle PFK-2 did not appear during differentiation of L6 myoblasts into myotubes induced by growth factor withdrawal and hormonal treatment, and demonstrating that this isozyme is a marker of mature skeletal muscle. There is no evidence for the presence of *pkfkb1* gene products (liver or muscle isozymes) or inducible PFK-2 in differentiated C2C12 cells. In contrast, the expression of *pkfkb3* decreases and the uPFK-2 protein gene product is degraded during myogenesis.

In order to confirm that this decline in *pkfkb3* gene expression and uPFK-2 protein levels are due to differentiation and not to serum starvation, we determined the levels of uPFK-2 on NIH3T3 fibroblast cells that do not differentiate when cultured in 2% FBS medium. As observed in Fig. 2C, the levels of uPFK-2 protein were kept constant during the 3 days of culture. In addition, we accelerated differentiation of C2C12 in 2% FBS medium using desIGF (a myogenic differentiation factor) [26], observing a more pronounced decrease in uPFK-2 protein levels 24 h after addition of the differentiation factor (Fig. 2D). Altogether, these observations confirmed the hypothesis that myogenic differentiation in C2C12 cells parallels a decrease in *pkfkb3* gene expression. However, BMP-2 which promotes transdifferentiation from the myogenic to the osteogenic lineage [27] also induced a pronounced decrease in uPFK-2 protein levels. This fact suggests that likely the decreases in uPFK-2 levels are more related to the exit from the cell cycle than to the commitment to a specific cell fate.

In the past few years, there has been an increasing interest for the ubiquitin-proteasome pathway of protein degradation [33]. This proteolytic pathway is essential for the myogenic differentiation and inhibitors of the proteasome, such as lactacystin, reversibly block both the fusion of L6 myoblasts and the accumulation of muscle-specific proteins [28,34]; moreover, the subunit composition of proteasomes changes when the dividing C2C12 myoblasts fuse into myotubes [35] and this proteolytic pathway is also implicated in the degradation of MyoD during the G1 phase of the cell cycle [36]. The results presented in the current study are the first direct demonstration of the implication of the ubiquitin-proteasome proteolytic pathway in the degradation of uPFK-2. In vivo, the

ubiquitous isozyme has a half-life of 6 h at the third day of differentiation as compared to more than 10 h in undifferentiated cells (Fig. 3B). These differences in the half-life of uPFK-2 can be explained by the enhanced degradation of this isozyme during myogenesis.

Amino acid sequence analysis reveals that uPFK-2 contains one putative PEST sequence in its C-terminal, sequences that are common to many short-lived proteins and reported to be motifs for rapid degradation [37]. Furthermore, uPFK-2 also contains a DSGYXS motif that is present in many proteins, such as  $\kappa$ B,  $\beta$ -catenin or Vpu, and whose phosphorylation on the two serines is required for ubiquitin-dependent degradation [38]. Although the presence of these motifs in the uPFK-2 sequence, their relative relevance in uPFK-2 degradation needs further investigation.

The expression and activity of the *pkfkb3* gene are highly regulated during proliferation and differentiation processes at multiple levels. This gene can be regulated by different growth factors or hypoxia [17–20] and herein we show an additional control via its degradation by the ubiquitin-proteasome proteolytic pathway. In conclusion, *pkfkb3* gene seems to play an important role sustaining the high glycolytic flux of proliferating cells, while its expression is downregulated and the corresponding protein degraded through the ubiquitin-proteasome proteolytic pathway during C2C12 cell differentiation.

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