

Characterization of a new liver- and kidney-specific *pfkfb3* isozyme that is downregulated by cell proliferation and dedifferentiation

Joan Duran^a, Marta Gómez^a, Aurea Navarro-Sabate^a, Lluís Riera-Sans^b, Mercè Obach^a, Anna Manzano^a, Jose C. Perales^c, Ramon Bartrons^{a,*}

^a *Unitat Bioquímica i Biologia Molecular, Departament de Ciències Fisiològiques, Campus de Ciències de la Salut, IDIBELL – Universitat de Barcelona, Spain*

^b *Laboratory of Epithelial Homeostasis and Cancer, Department of Differentiation and Cancer, Center for Genomic Regulation, Spain*

^c *Unitat de Biofísica, Departament de Ciències Fisiològiques, Campus de Ciències de la Salut, IDIBELL – Universitat de Barcelona, Spain*

Received 20 December 2007

Available online 10 January 2008

Abstract

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2) catalyzes the synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6-P₂), a signalling molecule that controls the balance between glycolysis and gluconeogenesis in several cell types. Four genes, designated *Pfkfb1–4*, code several PFK-2 isozymes that differ in their kinetic properties, molecular masses, and regulation by protein kinases. In rat tissues, *Pfkfb3* gene accounts for eight splice variants and two of them, ubiquitous and inducible PFK-2 isozymes, have been extensively studied and related to cell proliferation and tumour metabolism. Here, we characterize a new kidney- and liver-specific *Pfkfb3* isozyme, a product of the RB2K3 splice variant, and demonstrate that its expression, in primary cultured hepatocytes, depends on hepatic cell proliferation and dedifferentiation. In parallel, our results provide further evidence that ubiquitous PFK-2 is a crucial isozyme in supporting growing and proliferant cell metabolism.

© 2008 Elsevier Inc. All rights reserved.

Keywords: 6-Phosphofructo-2-kinase/fructose 2,6-bisphosphatase; Fructose 2,6-bisphosphate; Hepatocytes; Alternative splicing; Isoform; Isozyme; Dedifferentiation; Proliferation

Fructose 2,6-bisphosphate (Fru-2,6-P₂) was discovered in liver during research into the mechanism of glucagon-stimulated gluconeogenesis [1,2]. This metabolite is the most powerful allosteric activator of 6-phosphofructo-1-kinase, as well as being an inhibitor of fructose-1,6-bisphosphatase. Because of its antagonistic actions on these enzymes, Fru-2,6-P₂ plays a crucial role in governing flux along glycolytic and gluconeogenic pathways [1–3]. The Fru-2,6-P₂ cellular pull depends on the balance between the reciprocal reactions of 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase from the bifunctional enzyme PFK-2, which functions as a homodimeric protein with

these two activities located at separate sites on each protein subunit [1–3]. Different PFK-2 isozymes have been identified in mammals and are generated by alternative splicing of the transcribed RNA from four genes, designated *Pfkfb1–4* [4]. PFK-2 isozymes differ in their kinetic properties, molecular masses, and responses to phosphorylation by protein kinases, with the kinase:bisphosphatase activity ratio being the most important aspect of any given isozyme [5]. In the adult liver the main isozyme is L-PFK-2, a product of the *Pfkfb1* gene that is modulated by protein kinase A phosphorylation, which activates its bisphosphatase activity that breaks down Fru-2,6-P₂ [5]. In embryonic or proliferating cells, studies have observed the expression of the *Pfkfb3* gene [6–9]. This gene was originally cloned from a foetal bovine brain cDNA library [10,11], from placenta [12] and from a tumour cell line [13]. It has the highest

* Corresponding author. Fax: +34 934024268.

E-mail address: rbartrons@ub.edu (R. Bartrons).

kinase:bisphosphatase activity ratio and thus maintains elevated Fru-2,6-P₂ levels, which in turn sustain high glycolytic rates in the cell [14]; furthermore, its targeted disruption results in embryonic lethality [15]. Recently, using transgenic animals, we demonstrated that overexpression of *Pfkfb3* in mice liver sustains high Fru-2,6-P₂ levels in this tissue, resulting in animal weight gain [16]. The two prevailing forms of *Pfkfb3*-expressed isozymes are the ubiquitous (uPFK-2), which is induced by insulin [8], progesterone [13], and hypoxia [17,18], and the inducible (iPFK-2), which is induced by inflammatory stimuli [7]. Both these isoforms are generated through the alternative splicing of the *Pfkfb3* gene [7,19]. In addition, at the cDNA level, Watanabe et al. [20,21] have reported the occurrence of eight splice variants of the *Pfkfb3* gene in rat tissues, while Kessler et al. [22] have identified six in human brain, all of them designated as isoforms differing at their carboxyl termini. In rat liver, two *Pfkfb3* isoforms have been identified: RB2K6 and RB2K3 [21]. While the former was reported as the rat homologue to the human uPFK-2 isozyme, the other isoform (RB2K3) has so far not been linked to a translated protein. Here, we characterize the RB2K3 *Pfkfb3* splice variant isozyme that is present in adult rat liver and kidney. Our results demonstrate that its regulated expression in hepatocytes depends on cell proliferation and dedifferentiation processes, and that this occurs in the opposite direction to that observed for uPFK-2. Our observations support the idea that the tissue-specific *Pfkfb* isozymes are expressed to adapt their enzymatic properties to the metabolic demand of a particular tissue or cell status, with PFK-2 isoenzymatic switches being a crucial phenomenon in development, regeneration, and cell transformation events.

Materials and methods

Chemicals. Media, sera, and antibiotics were obtained from Life Technologies, Inc. (Grand Island, NY, USA). Dimethyl sulphoxide (DMSO) and epidermal growth factor (EGF) were obtained from SIGMA. Collagen type I was purchased from Biological Industries, and Matrigel from Becton–Dickinson. Insulin and dexamethasone were obtained from SIGMA. Restriction enzymes were purchased from Phermentas.

Animal care and treatment. Male Sprague–Dawley rats purchased from Harlan Interfarma IBERICA S.L (Spain) were maintained under a constant 12h light–dark cycle and fed a standard rodent chow and water *ad libitum*. All animal protocols were approved by the Ethics Committee of the University of Barcelona. For studies on liver development, caesareans were performed on timed-pregnant rats under pentobarbital anaesthesia (50 mg/kg body weight, administered by intraperitoneal injection) on embryonic days 18 and 20. In adult studies, male rats were anaesthetized and decapitated, and tissues were dissected, snap frozen in liquid nitrogen and stored at –80 °C until analysis.

Cell culture. Rat hepatocytes were obtained as in Bartrons et al. [23] and seeded for 4 h on collagen (4 µg/cm²) or Matrigel (prepared following manufacturer's protocol) coated plates with Williams E medium (Bio-Wittaker, Cambrex Bio Science, Verviers, Belgium) supplemented with 10% foetal bovine serum (Invitrogen), L-glutamine, gentamicin, insulin (1 nM) and dexamethasone (1 nM). After seeding, cells were cultured with or without foetal bovine serum, DMSO (1%) or EGF (20 ng/ml) for 24, 48

or 72 h. All incubations were performed in a humidified atmosphere of 10% CO₂ at 37 °C.

RT-PCR analysis. Total RNA from cultured hepatocytes was extracted using Ultraspec (Biotex) and treated with DNaseI (Phermentas) to eliminate genomic DNA contamination. Five micrograms of total RNA was reverse transcribed using a Ready-To-Go First Strand Isolation Kit (Amersham Biosciences) using random primers. PCR was performed using 10 µl of reverse-transcribed RNA with rat *Pfkfb3* specific primers P1 and P6 [21]. Amplification products were analyzed in 2% agarose gel stained with ethidium bromide.

Western blot. Western blot was performed with 50 µg of total tissue extracts prepared as in [16], or with 50 µg of total cellular protein from primary hepatocytes using a 50 mM Tris–HCl pH:6.8, 10% glycerol, and 2% SDS lysis buffer. Proteins were separated in 10% SDS–PAGE and transferred to an Immobilon membrane (Millipore Corp., Bedford, MA, USA). Membranes were probed with the following primary antibodies: a specific polyclonal antibody against the C terminus of uPFK-2 [8], and anti-LPFK-2, anti-C/EBPα, and anti-PCNA from Santa Cruz Biotechnology (Santa Cruz, California, USA). Bound antibody was visualized with horseradish peroxidase-conjugated sheep anti-rabbit or donkey anti-mouse antibody. The antigen–antibody complexes were developed by enhanced chemiluminescence using ECL (Amersham Bioscience). Bands were quantified by densitometric scanning using Quantity-One software (Bio-Rad).

Statistics. Results are expressed as the mean ± standard error. Statistical analysis was always performed by one-way analysis of variance and the Student's *t* test. Values of *p* < 0.05, *p* < 0.01, and *p* < 0.001 were considered significant.

Results and discussion

Pfkfb3 protein expression in rat tissues and primary hepatocytes

The *Pfkfb3* gene accounts for two reported isozymes, ubiquitous (uPFK-2) [11], and inducible (iPFK2) [7], and also for another six different splice variants [20,21], all of them generated through alternative splicing of the variable 3' region of the transcribed mRNA (Fig. 1A). The study of its protein expression profile in rat tissues, using a human antibody that recognizes the variable exon 16 (or exon G) of the translated isozyme [8] (Fig. 1A, in bold capitals), revealed the presence of two reactive bands. Whereas one of them corresponded to the reported 59 kDa uPFK-2 protein [8,18], the other, with a high molecular weight, was suspected to correspond to a new isozyme translated from the previously described RB2K3 isoform [21]. This splice variant differs from uPFK-2 in an 87 bp variable exon (exon B, in Figs. 1 and 2A), which adds 29 amino acids to the carboxyl terminus of the protein without affecting its reading frame. As shown in Fig. 1B, uPFK-2 was found constitutively expressed in all tissues, the highest levels being achieved in proliferating ones. In contrast, the high molecular weight band was only detected in kidney and liver. In freshly-isolated rat primary hepatocytes, we detected the same two-band profile as in whole liver (Fig. 1C, 0 h), but during a 72 h culture period on a collagen monolayer, a progressive loss of the undescribed protein was observed, accompanying hepatocyte proliferation and dedifferentiation processes (Fig. 1C).

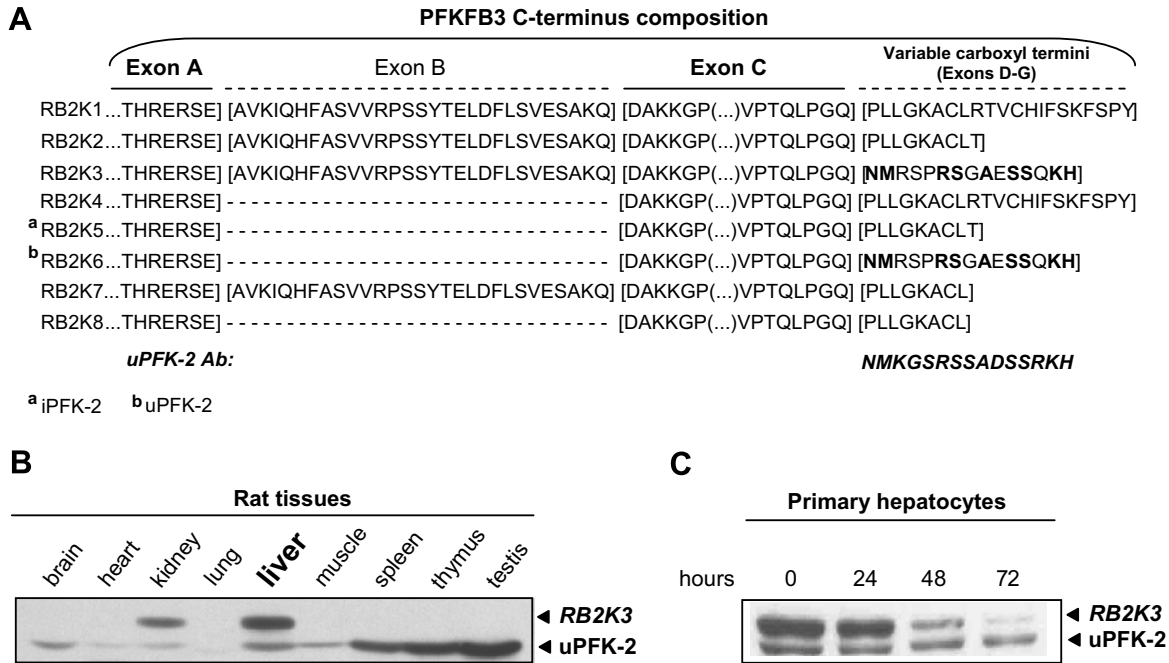


Fig. 1. (A) Schematic representation and comparison of the different PFKFB3 protein splice variants. All isoforms are named as in [20,21]. RB2K5 and RB2K6 correspond to iPFK-2 and uPFK-2, respectively. The human peptide used in uPFK-2 antibody development is shown in bold, italic capitals. RB2K3 and uPFK-2 splice variants are both specifically recognized by the uPFK-2 antibody. (B) One hundred micrograms of total protein extracts from various rat tissues, and (C) fifty micrograms from rat primary hepatocytes cultured on a collagen monolayer at different times were analyzed by Western blot using the described antibody.

The expression of more than one isozyme in the same tissue suggests that different isoforms play key roles in different physiological conditions. As reviewed in [3–5], each PFK-2 monomer comprises a catalytic core (with the kinase and bisphosphatase activities) and two regulatory regions at the amino and carboxyl termini. Although, the core structure is highly conserved among isoforms, there are important structural variations in their terminal regions. Consistent with the divergence of the amino-acid sequences in these regions, it is the use of alternative exons in processing the primary transcript to generate various mRNAs that gives rise to the numerous isoforms. The variable terminal regions between uPFK-2 and RB2K3 can elicit a regulatory function in the enzyme through changes in its tertiary and quaternary conformations in response to the multiplicity of effectors, perhaps in a similar manner to that reported for the different *Pfkfb1* splice isoforms [4,5].

Pfkfb3 alternative splicing characterization in primary cultured hepatocytes

Retrotranscribed cDNA from total hepatocyte RNA at different collagen culture stages was amplified by PCR and analyzed in 2% agarose gel. Specific primers P1 and P6 [21], designed on the two 3' cDNA constant exons A and G (or 13 and 16) (Fig. 2A), were used and the expected 371 and 284 bp products, corresponding to RB2K3 and RB2K6 (uPFK-2) splice variants, respectively, were detected (Figs. 2B and C). In addition, we performed a

restriction analysis using the *Eam 1105I* enzyme, with a single cut site at the alternative exon B, and this showed that the 371 bp RT-PCR fragment corresponded to the RB2K3 isoform. Finally, the two PCR products were sequenced, confirming the hypothesis that two different splice variants of the *Pfkfb3* gene are present in hepatocytes, and that they correspond to uPFK-2 and RB2K3 isoforms.

uPFK-2 and RB2K3 isoforms are differentially expressed in hepatocytes depending on cell differentiation and proliferation

As has been reported, *Pfkfb3* gene product is ubiquitously expressed in proliferating tissues, transformed cell lines, and in various tumours [7–9]. In addition, uPFK-2 has, when compared to the other PFK-2 isoforms, the highest kinase:bisphosphatase activity ratio; it thus maintains elevated Fru-2,6-P₂ levels, which in turn sustain high glycolytic rates in the cell [14]. As a result, this gene has been closely linked to cell proliferation. Rat primary hepatocytes, following tissue disruption of cell–cell contact during isolation and during primary culture, exhibit rapid proliferation and dedifferentiation, progressing in G1 phase independently of growth factor stimulation, up to the restriction point located in the mid-late G1 phase of the cell cycle [24]. However, the amount of time hepatocytes can maintain their differentiated and quiescent state depends on cell culture conditions and media [25]. Previous studies have demonstrated that adhesion to collagen matrix inhib-

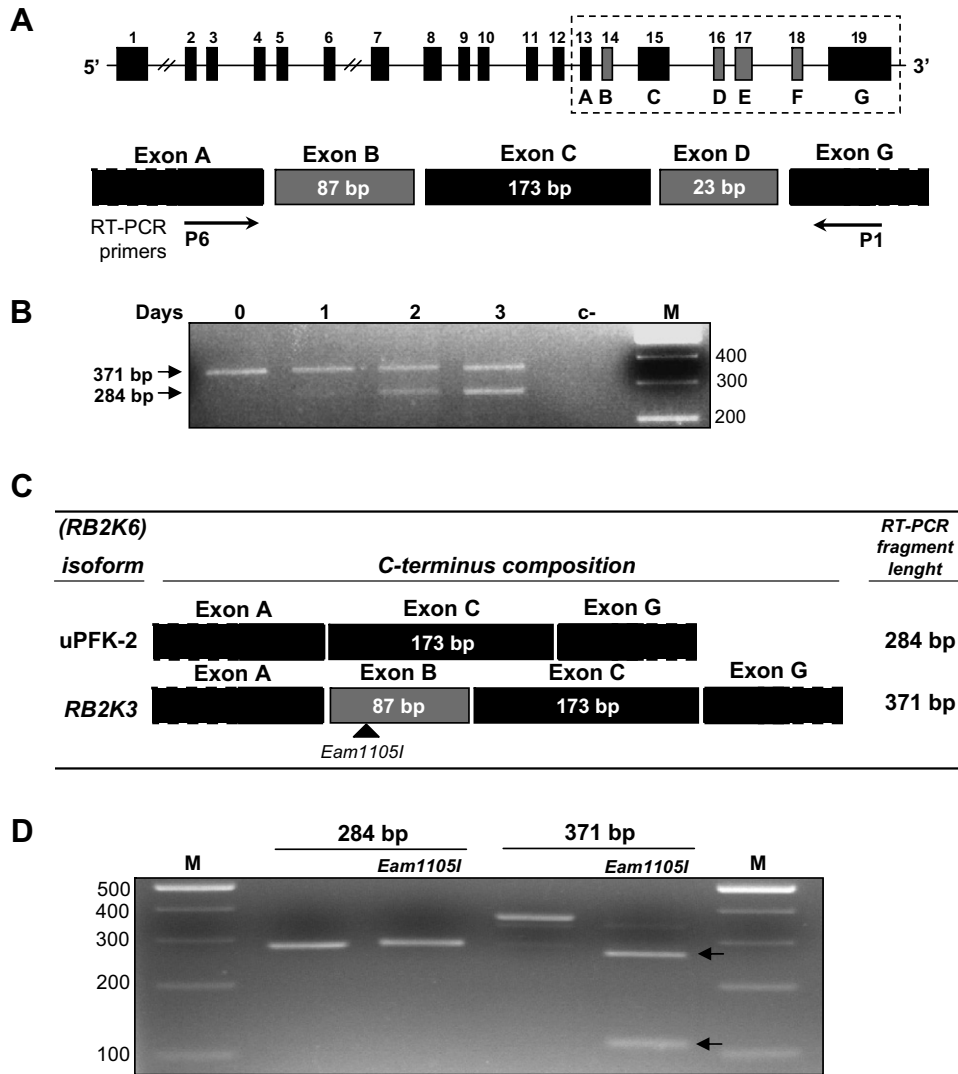


Fig. 2. (A) Upper panel: Diagram of *Pfkfb3* gene organization. Exons on variable region are named from A to G. Lower panel: Schematic representation of 3' variable region organization (without exons E and F). Alternative spliced exons are shown as grey boxes. Lengths of relevant fragments are expressed as base pairing (bp). Primers P1 and P6 [21] used in RT-PCR amplification are indicated. (B) RNA from primary hepatocytes at different times was retrotranscribed and amplified by PCR using P1–P6-specific primers. RT-PCR products were analyzed in 2% agarose gel and stained with ethidium bromide. Fragments obtained corresponded to RB2K3 (371 bp) and uPFK-2 (284 bp) cDNA amplification, respectively. (C) Comparison of the different C-terminal exon composition between uPFK-2 and RB2K3 cDNAs. *Eam 1105I* restriction enzyme cut site is shown. (D) Two hundred and eighty-four and three hundred and seventy-one base pair RT-PCR fragments were isolated, purified, and analyzed by *Eam 1105I* restriction. Reaction products were analyzed in 2% agarose gel.

its hepatocyte cell spreading and cell cycle progression, while adhesion to collagen film allows cell spreading and G1-S progression in response to growth factors [25]. On the other hand, after mitogenic stimulation (with sera, insulin or growth factors) primary hepatocytes progress in late G1 and undergo DNA synthesis [26].

As shown in Fig. 3A, hepatocytes cultured on a collagen matrix (or gel), which maintains cells in a quiescent and differentiated state, exhibited a constant RB2K3/uPFK-2 expression ratio across the culture. This feature is especially consistent during the first 48 h after cell seeding. In contrast, hepatocytes cultured on collagen monolayer (or film) showed a progressive loss in RB2K3 protein levels, which was reflected by the rapid decrease of the RB2K3/uPFK-

2 expression ratio shown in the graph. When these cultures were supplemented with media containing 10% FBS as a mitogenic factor, the decrease in RB2K3 protein content was accelerated, becoming total at 48 h. This feature was accompanied by both a rapid induction of uPFK-2 protein levels and a progressive loss of the adult liver L-PFK-2 isozyme. In order to assess hepatocyte differentiation in each experimental condition, levels of the typical liver marker C/EBP α , which regulates many liver-specific genes and is essential for maintenance of the quiescent and differentiated tissue [27], were monitored in all blots.

A suitable *in vivo* model for studying hepatocyte maturation and terminal liver differentiation could be the transition between foetal and adult rat liver. Previous research

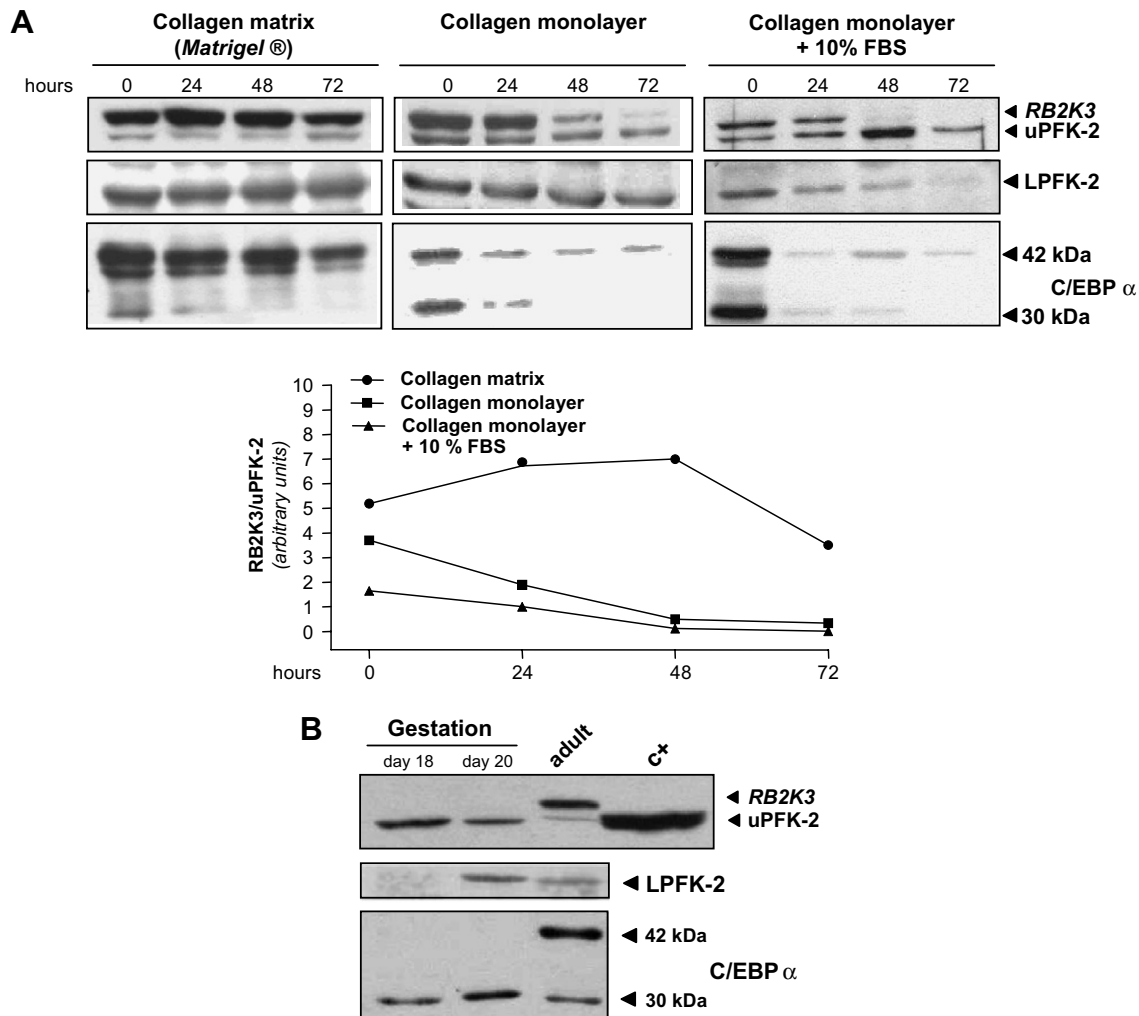


Fig. 3. (A) uPFK-2 and RB2K3 protein levels in different hepatocyte culture systems (collagen matrix, collagen monolayer and collagen monolayer with 10% FBS supplemented media) over 72 h. Fifty micrograms of total protein extracts were analyzed by Western blot against uPFK-2, L-PFK-2, and C/EBP α in order to assess cell differentiation. For each point, ratios between RB2K3 and uPFK-2 protein levels, quantified by densitometric scanning, were represented in the graph. (B) Fifty micrograms of total protein extracts from rat livers on gestation days 18 and 20 were analyzed and compared to adult levels by Western blot against uPFK-2, L-PFK-2 and C/EBP α . Extracts from Hela cells overexpressing human uPFK-2 [11] were used as positive control (c+).

on *Pfkfb1* isozymes [28] has demonstrated that foetal rat liver contains a form of PFK-2 that exhibits marked differences from the enzyme expressed in adult liver. In accordance with the results presented above, the experiment shown in Fig. 3B illustrated the robust link between RB2K3 isozyme expression and the hepatic differentiated and quiescent cell state (as shown in adult liver). In contrast, the uPFK-2 isozyme was found to be highly expressed, preferentially, in more undifferentiated and immature hepatic cells (as can be observed in rat foetal liver samples), and with a similar profile to that detected in proliferant hepatocytes on collagen monolayer cultures.

Foetal liver contains certain isozymes of carbohydrate metabolism which differ from those found in adult tissue, and this is the case of PFK-2. *Pfkfb1* has been reported to account for at least three isoforms (named Foetal, Liver, and Muscle) that are expressed during the perinatal transition [28]. In the present study, we demonstrate that *Pfkfb3*

isozymes are also expressed in liver cells, and that their switch is specifically regulated depending on hepatocyte faces.

DMSO and EGF modulate RB2K3 and uPFK-2 expression in primary cultured hepatocytes

When added to culture media, DMSO is reported to induce hepatocyte redifferentiation and helps to maintain normal liver transcription factors (C/EBP α and HNF4). Therefore, DMSO exhibits a protective effect against dedifferentiation on collagen monolayer-coated hepatocytes [29]. On the other hand, EGF stimulates hepatocytes to progress in late G1 phase of the cell cycle and to undergo DNA synthesis [24–26]. As Fig. 4A shows, the protective effect of DMSO on hepatocytes was assessed by the maintenance of C/EBP α levels at 24 and 48 h, as observed in samples treated with this compound, whereas the EGF

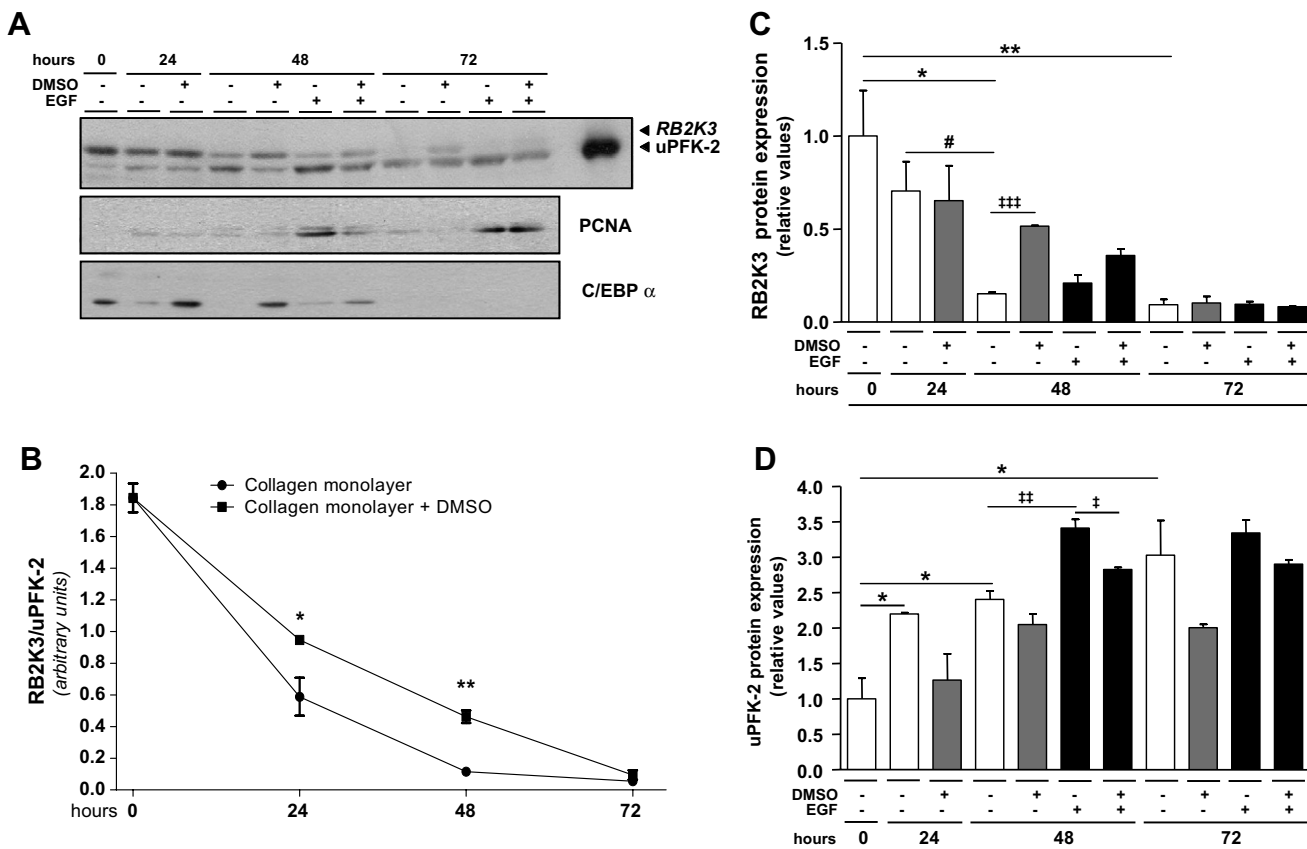


Fig. 4. (A) Primary rat hepatocytes cultured on collagen monolayer and treated with/without 1% DMSO and/or 20 ng/ml EGF. Fifty micrograms of total protein extracts from hepatocyte cultures at 0, 24, 48, and 72 h after treatments were analyzed by Western blot against uPFK-2, PCNA, and C/EBP α . (B) Ratios between RB2K3 and uPFK-2 protein levels at each culture point. Significant differences were observed when comparing control and DMSO treated cells at 24 and 48 h. (C) RB2K3 and (D) uPFK-2 protein expression, expressed as relative values versus 0 h, at 24, 48, and 72 h after each culture condition. All graph points and bars represent the mean \pm SEM of the data obtained by densitometric scanning from three independent experiments ($^*p < 0.05$ versus 0 h; $^{**}p < 0.01$ versus 0 h; $^{\#}p < 0.05$ between 24 and 48 h; $^{\ddagger}p < 0.05$; $^{\ddagger\ddagger}p < 0.01$; $^{\ddagger\ddagger\ddagger}p < 0.001$).

mitogenic effect was monitored by detecting the proliferating cell nuclear antigen (PCNA), a nuclear protein required for DNA synthesis and repair that is detected in cells in the S-phase.

Our results show that in the presence of DMSO, the RB2K3/uPFK-2 expression ratio decreased more slowly, it being significant at 24 and 48 h when compared to control cultured hepatocytes (Fig. 4B). Densitometric analysis of RB2K3 protein levels, expressed as relative values versus the start point (0 h), revealed significant decreases at 48 and 72 h, and also between 24 and 48 h (Fig. 4C), thus demonstrating that the hepatocyte dedifferentiation process in collagen monolayer culture is sufficient for RB2K3 isozyme regulation. When DMSO was added to cells the RB2K3 downregulation profile was delayed, with significant differences being detected at 48 h. No significant effects on its expression were found under EGF mitogenic stimulation. In contrast, uPFK-2 protein levels were significantly increased both in response to cell culture progression (that underlies dedifferentiation and proliferation) and, to a greater extent, after EGF mitogenic stimulation (Fig. 4C). In DMSO treated conditions, the increase in

uPFK-2 protein was partially inhibited, with significant differences at 48 h under EGF treatment.

These findings provide the first demonstration of the up-regulation of the uPFK-2 isozyme in hepatic cell growth and proliferation. Previous results corroborated its role in sustaining high glycolytic flux in proliferative cells and its involvement in cell-cycle progression and anchorage-independent growth [30]. Taken together, these results provide new insights into the crucial role of *Pfkfb3* gene expression in cell proliferation and tumour transformation, and reveal the part played by PFK-2 isoenzymatic switching in cell plasticity and metabolic adaptive responses.

Acknowledgments

We are grateful to E. Adanero, Alvaro Gimeno, and Dr. M. Molas for skilful technical assistance. We also thank Robin Rycroft for revising the English. J.D. was recipient of a research fellowship from the Generalitat de Catalunya, and M.G and M.O. from the Ministerio de Educación y Ciencia. This work was supported by the Ministerio de Educación y Ciencia (BFU 2003/01442 and BFU2006/

02412/BMC) and the Generalitat de Catalunya (2005/SGR/0022).

References

- [1] E. Van Schaftingen, Fructose 2,6-bisphosphate, *Adv. Enzymol. Relat. Areas Mol. Biol.* 59 (1987) 315–395.
- [2] S.J. Pilkis, T.H. Claus, I.J. Kurland, A.J. Lange, 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme, *Annu. Rev. Biochem.* 64 (1995) 799–835.
- [3] C. Wu, S.A. Khan, L.J. Peng, A.J. Lange, Roles for Fructose 2,6-bisphosphate in the control of fuel metabolism: beyond its allosteric effects on glycolytic and gluconeogenic enzymes, *Adv. Enzyme Regul.* 46 (2006) 72–88.
- [4] D.A. Okar, A. Manzano, A. Navarro-Sabaté, L.I. Riera, R. Bartrons, A.J. Lange, PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose 2,6-bisphosphate, *Trends Biochem. Sci.* 26 (2001) 30–35.
- [5] M.H. Rider, L. Bertrand, D. Vertommen, P.A. Michels, G.G. Rousseau, L. Hue, 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis, *Biochem. J.* 381 (Pt 3) (2004) 561–579.
- [6] N. Goren, A. Manzano, L. Riera, S. Ambrosio, F. Ventura, R. Bartrons, 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase expression in rat brain during development, *Brain Res. Mol. Brain Res.* 75 (1) (2000) 138–142.
- [7] J. Chesney, R. Mitchell, R. Benigni, M. Bacher, L. Spiegel, Y. Al-Abed, J.H. Han, C. Metz, R. Bucala, An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect, *Proc. Natl. Acad. Sci. USA* 96 (1999) 3047–3052.
- [8] L. Riera, A. Manzano, A. Navarro-Sabaté, J.C. Perales, R. Bartrons, Insulin induces PFKFB3 gene expression in HT29 human colon adenocarcinoma cells, *Biochim. Biophys. Acta* 1589 (2) (2002) 89–92.
- [9] T. Atsumi, J. Chesney, C. Metz, L. Leng, S. Donnelly, Z. Makita, R. Mitchell, R. Bucala, High expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (iPFK-2; PFKFB3) in human cancers, *Cancer Res.* 62 (20) (2002) 5881–5887.
- [10] F. Ventura, S. Ambrosio, R. Bartrons, M.R. el-Maghrabi, A.J. Lange, S.J. Pilkis, Cloning and expression of a catalytic core bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, *Biochem. Biophys. Res. Commun.* 209 (3) (1995) 140–148.
- [11] A. Manzano, J.L. Rosa, F. Ventura, J.X. Perez, M. Nadal, X. Estivill, S. Ambrosio, J. Gil, R. Bartrons, Molecular cloning, expression, and chromosomal localization of a ubiquitously expressed human 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene (PFKFB3), *Cytogenet. Cell Genet.* 83 (1998) 214–217.
- [12] A. Sakai, M. Kato, M. Fukasawa, M. Ishiguro, E. Furuya, R. Sakakibara, Cloning of cDNA encoding for a novel isozyme of fructose 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase from human placenta, *J. Biochem. (Tokyo)* 119 (1996) 506–511.
- [13] J.A. Hamilton, M.J. Callaghan, R.L. Sutherland, C.K. Watts, Identification of PRG1, a novel progesterin-responsive gene with sequence homology to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, *Mol. Endocrinol.* 11 (1997) 490–502.
- [14] R. Sakakibara, M. Kato, N. Okamura, T. Nakagawa, Y. Komada, N. Tominaga, M. Shimojo, M. Fukasawa, Characterization of a human placental fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase, *J. Biochem. (Tokyo)* 122 (1997) 122–128.
- [15] J. Chesney, S. Telang, A. Yalcin, A. Clem, N. Wallis, R. Bucala, Targeted disruption of inducible 6-phosphofructo-2-kinase results in embryonic lethality, *Biochem. Biophys. Res. Commun.* 331 (1) (2005) 139–146.
- [16] J. Duran, A. Navarro-Sabate, A. Pujol, J.C. Perales, A. Manzano, M. Obach, M. Gómez, R. Bartrons, Overexpression of ubiquitous 6-phosphofructo-2-kinase in the liver of transgenic mice results in weight gain, *Biochem. Biophys. Res. Commun.* 365 (2) (2008) 291–297.
- [17] A. Minchenko, I. Leshchinsky, I. Opentanova, N. Sang, V. Srinivas, V. Armstead, J. Caro, Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect, *J. Biol. Chem.* 277 (8) (2002) 6183–6187.
- [18] M. Obach, A. Navarro-Sabaté, J. Caro, X. Kong, J. Duran, M. Gómez, J.C. Perales, F. Ventura, J.L. Rosa, R. Bartrons, 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia, *J. Biol. Chem.* 279 (51) (2004) 53562–53570.
- [19] A. Navarro-Sabaté, A. Manzano, L. Riera, J.L. Rosa, F. Ventura, R. Bartrons, The human ubiquitous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene (PFKFB3): promoter characterization and genomic structure, *Gene* 264 (1) (2001) 131–138.
- [20] F. Watanabe, A. Sakai, E. Furuya, Novel isoforms of rat brain fructose 6-phosphate 2-kinase/fructose 2,6-bisphosphatase are generated by tissue-specific alternative splicing, *J. Neurochem.* 69 (1) (1997) 1–9.
- [21] F. Watanabe, E. Furuya, Tissue-specific alternative splicing of rat brain fructose 6-phosphate 2-kinase/fructose 2,6-bisphosphatase, *FEBS Lett.* 458 (3) (1999) 304–308.
- [22] R. Kessler, K. Eschrich, Splice isoforms of ubiquitous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in human brain, *Brain Res. Mol. Brain Res.* 87 (2) (2001) 190–195.
- [23] R. Bartrons, L. Hue, E. Van Schaftingen, H.G. Hers, Hormonal control of fructose 2,6-bisphosphate concentration in isolated rat hepatocytes, *Biochem. J.* 214 (3) (1983) 829–837.
- [24] A. Coutant, C. Rescan, D. Gilot, P. Loyer, C. Guguen-Guillouzo, G. Baffet, PI3K-FRAP/mTOR pathway is critical for hepatocyte proliferation whereas MEK/ERK supports both proliferation and survival, *Hepatology* 36 (2002) 1079–1088.
- [25] J.T. Fassett, D. Tobolt, C.J. Nelsen, J.H. Albrecht, L.K. Hansen, The role of collagen structure in mitogen stimulation of ERK, cyclin D1 expression, and G1-S progression in rat hepatocytes, *J. Biol. Chem.* 278 (34) (2003) 31691–31700.
- [26] C.J. Band, C. Mounier, B.I. Posner, Epidermal growth factor and insulin-induced deoxyribonucleic acid synthesis in primary rat hepatocytes is phosphatidylinositol 3-kinase dependent and dissociated from protooncogene induction, *Endocrinology* 140 (1999) 5626–5634.
- [27] R.C. Wilkinson, A.J. Dickson, Expression of CCAAT/enhancer binding protein family genes in monolayer and sandwich culture of hepatocytes: induction of stress-inducible GADD153, *Biochem. Biophys. Res. Commun.* 289 (5) (2001) 942–949.
- [28] M. Casado, L. Boscá, P. Martín-Sanz, Multiple forms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase are expressed in perinatal rat liver, *Am. J. Physiol.* 270 (2 Pt 1) (1996) E244–E250.
- [29] T. Su, D.J. Waxman, Impact of dimethyl sulfoxide on expression of nuclear receptors and drug-inducible cytochromes P450 in primary rat hepatocytes, *Arch. Biochem. Biophys.* 424 (2) (2004) 226–234.
- [30] M.N. Calvo, R. Bartrons, E. Castano, J.C. Perales, A. Navarro-Sabate, A. Manzano, PFKFB3 gene silencing decreases glycolysis, induces cell-cycle delay and inhibits anchorage-independent growth in HeLa cells, *FEBS Lett.* 580 (2006) 3308–3314.