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Research Article

Upregulation of rat P23 (a member of the YjgF protein family) by fasting, glucose diet and fatty acid feeding

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Abstract. In a previous study, we identified and purified a 99-amino-acid rat liver-kidney perchloric-acid-soluble 23-kDa protein (P23) which displays 30% identity with a highly conserved domain of heat shock proteins (HSPs), as well as an AT-rich 3′ untranslated region, which has also been described to play a role in H70 mRNA life span and protein expression. An identical perchloric-acid-soluble protein inhibiting protein synthesis in a rabbit reticulocyte lysate system was also found 2 years later by another group. More recently, the novel, the YjgF, protein family has been described, comprising, 24 full-length homologues, including P23, highly conserved through evolution, and consisting of approximately 130 residues each and sharing a common ternary structure. Independent studies from different laboratories have provided various hypothetical functions for each of these proteins. The high degree of evolutionary conservation may suggest that these proteins play an important role in cellular regulation. Although the function of none of these proteins is known precisely, we present experimental evidence which, combined with the relationship to glucose-regulating protein revealed here, and the relationship to fatty-acid-binding protein revealed by others, allow us to propose a role for P23. In rat liver, P23 expression is developmentally regulated and modulated by dietary glucose, and its mRNA is induced by starvation, in the presence of fatty-acids and in 3-MeDAB-induced hepatomas. The mRNA encoding mouse liver P23 is also hormonally modulated in a mouse line AT1F8. These data indicate that P23 protein might be a key controller of intermediary metabolism during fasting.

Key words. P23; YjgF; dietary glucose; development; regulation.

There are many genes and gene families that are highly conserved among distantly related organisms, yet whose functions are totally unpredictable by computer analyses of their sequences.

The gene family YERO57C/YjgF/UK114 (referred to as YjgF) is one such gene families; it is composed of 24 genes from 23 species including archaebacteria, eubacteria and eukaryotes that code for small proteins of 130 amino acids on average and whose sequences are highly conserved throughout the molecule. The molecular and biochemical functions of these genes are unknown, although three dimensional structures of Yjgfp from *Escherichia coli* and YabJp from *Bacillus subtilis* have been determined [1, 2]. However, these investigations have suggested that these homologues could be involved in essential processes for maintaining life, such as metabolism and cell proliferation.

Among mammalian species, the P23 cDNA nucleotide sequence [3] turned out to be identical to a cDNA described

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by Oka et al. [4], the product of which, perchloric-soluble protein (PSP), was characterized as an inhibitor of protein synthesis in vitro. The same authors have also shown an inhibitory activity of PSP on cell proliferation [5]. A murine homologue described as a novel heat-responsive phosphorylated protein was only 12 kDa in size, but possessed sequence similarity to PSP/P23 [6]. A human homologue named p14.5 isolated from monocytes was described as a translational inhibitor which undergoes up-regulation during cellular differentiation [7]. Other groups have reported a goat liver homologue protein named UK114 that is a tumour antigen expressed by malignant neoplasm [8, 9]. A separate group observed that a bovine brain calpain activator is nearly identical to UK114 protein [10]. A yeast mitochondrial matrix factor (Mmf1p) has also been recently identified as a member of the YjgF family and is reported as being involved in maintenance of the mitochondrial genome [11]. As previously described [1, 6], P23 shares similarities with heat shock proteins (HSPs), the function of which is to solubilize aggregated heat-damaged proteins in an ATP-dependent manner, particularly in the nucleus and nucleoli [12]. Glucose-regulated-proteins (GRPs) are also part of the highly conserved stress protein system. GRPs perform the same function as HSPs, but do so within the endoplasmic reticulum (ER) [13–15]. Two major grps, grp94 and grp78, have been well studied [16–18]; grp78 is about 60% homologous to hsp70, and grp94 is about 50% homologous to yeast hsp90 and *Drosophila* hsp83 [19–21]. During cell transformation, both are synthesized and expressed in increased amounts [22]. Thus, grp78 may bind to the abnormal, underglycosylated proteins in glucose-starved cells and help to solubilize them and change their conformation in the same way that hsp70 acts on heat-denatured nuclear proteins. The existence of related proteins in normal cells suggests that they are important for normal cellular metabolism. Since we had observed variations in P23 expression between starved and fed rats, we explored this phenomenon under conditions known to modulate glucose metabolism such as starvation, pregnancy, birth, fatty acid ingestion, as well as within 3-MeDAB-induced hepatic tumours. We show that P23 protein, like GRPs, is induced during these conditions.

Materials and methods

Animals

Male Wister rats (300 g) were either maintained on a normal diet, or starved for 48 h to 4 days, with or without addition of glucose on the 4th day. Other animals were fed with a carcinogenic diet (3′methyl-dimethyl amino azobenzene, 3-MeDAB). Pregnant females were obtained from Iffa Credo with all data regarding mating, and fetuses were removed at the 15th, 17th and 20th day of prenatal life. Animals were sacrificed at birth (neonates), at day 2, 5, 9 and 18 after birth or at the 3rd month of life (adults). Other male weaned rats were fed from day 21 to 40 with a commercial diet (10% fat) consisting of peanut-rapeseed or sunflower oils.

Immunization procedure

P23 antibodies were generated in rabbits using purified rat liver P23 as antigen [1]. P23 protein (0.25 mg) was emulsified in 1 ml saline/Freund's adjuvant system for each rabbit. Four white rabbits were injected with the immunogen in multiple intradermal, intramuscular and subcutaneous sites. Booster injections that contained the same dose of immunogen were administered at monthly intervals. The blood was taken from the rabbits and left to coagulate, and antibodies were purified from the plasma by affinity chromatography. The antiserum was precipitated by ammonium sulphate (80%) and extensively dialysed against 0.05 M pH 8 sodium phosphate buffer before being loaded on a DEAE 52 cellulose column washed and equilibrated in the same buffer. The flow-through fraction containing anti-P23 antibodies was collected and coupled to CNBr-activated Sepharose 4B previously washed with 1 M NaCl, equilibrated in 0.05 M pH 8 phosphate buffer and then saturated by a perchloric acid extract from rat spleen which does not express P23 protein.

RNA isolation and Northern blot analysis

Total RNA was prepared from different rat tissues using a single-step procedure [23]. Samples of RNA (20 µg/lane) were resolved by 0.8% agarose/formaldehyde denaturating gels, transferred to a Nylon membrane (Hybond N+; Amersham, Little Chalfont, UK) and hybridized at 65 °C under standard conditions with 10^8 cpm/ml of random-primed (Boeringer Mannheim, Mannheim, Germany) $\alpha[^{32}P]$dCTP-labelled probe (Amersham). The probe obtained with 30 ng of P23 cDNA [1] hybridizes to both the specific 0.9 kb mRNA and the internal 28S RNA. This characteristic was utilized for the choice of 28S RNA as a quantitative standard in rat experiments, except for the AT1F8 cell line experiment for which the cDNA encoding the polyadenylate binding protein (polyA-BP) was used.

Before transfer, gels were stained with ethidium bromide. Differences in the amounts of 18S and 28S were found with the different preparations, indicating experimental variations [24]; percentages of mRNA levels were determined in separate experiments by densitometry analysis of Northern blot autoradiographs performed with a Bio 1D system (Vilbert Lourmat, Marne-la-Valle, France). The equivalence of the inputs was then controlled for either 28 SRNA or polyA-BP mRNA abundance.

Gel electrophoresis and Western immunoblot analysis

Prior to electrophoresis, the different studied tissues were weighed and polytroned with 10 vol of solubilization buffer (SB: 62 mM Tris HCl pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycerol, 0.002% bromophenol blue), incubated for 5 min at 100 °C and centrifuged for 10 min at 12,000 rpm. Ten microlitres were resolved by SDS polyacrylamide gel electrophoresis in 15% acrylamide slab gels, as described by Läemmli [25]. After electrophoresis, the proteins were transferred onto nitrocellulose membranes for immunoblot analysis.

After transfer, the nitrocellulose membranes were incubated overnight at 4 °C with a polyclonal anti-rat P23 antibody (1/400), 10 mM Tris-HCl (pH 8), 0.15 M NaCl and 5% non-fat dry milk. The primary antibody was washed away and peroxidase-conjugated anti-rabbit antibody was applied (1/2000) for 2 h at room temperature. Again, the blots were washed and the immunoreactive proteins were visualized by exposure to the ECL Western blotting analysis system (Amersham). The nitrocellulose membranes were exposed to Kodak film.

Hormonal regulation studies

The cell line used was AT1F8, a subclone of hepatocyte-like cell lines (mhAT**)** derived from liver of transgenic mice expressing the SV40 early genes under the direction of the liver-specific antithrombin III promoter [26]. Cells were cultured in HamF12-DMEM (v/v) medium supplemented with 200 U/ml penicillin, 50 µg/ml streptomycin, 0.1 µM insulin, 1 µM dexamethasone, 1 µM triiodothyronin and 5% fetal calf serum (FCS).

When regulation of gene expression was examined, cells were first grown in the presence of FCS. Twenty-four hours before the experiment, cells were cultured in a serum-free, glucose-free medium supplemented with 100 µg/ml albumin, 10 mM lactate and 2 mM oxaloacetate.

The various induction studies were then performed for 24 h without FCS but with 100 µg/ml albumin under the following concentrations according to the different incubations: 17 mM glucose, 10 nM insulin, 1 µM glucagon, 1 mM 8Br-cAMP and 0.1 mM CPT-cAMP.

Results

Expression of P23 mRNA in liver is induced by starvation and modulated by dietary glucose

In eukaryotic cells, the ER contains a number of chaperones and folding catalysts that assist in the folding/assembly processes of newly synthesized proteins during their transit in the secretory pathway. When various treatments, including glucose starvation, lead to the accumulation of unfolded proteins in this compartment, ER-resident stress proteins are induced primarily at the transcriptional level. We compared P23 mRNA expression in livers from rats maintained on a normal diet with that of rats starved for 4 days and others for 3 days, then supplemented with dietary glucose on day 4. Enhanced expression of P23 mRNA in liver of starved animals (fig. 1B, lane 2) was observed compared to that of animals on a normal diet (fig. 1B, lane 3). After 3 days of starvation, glucose was added to the diet for 1 day before sacrifice. Under these conditions, we observed a decrease in P23 mRNA expression (fig. 1B, lane 1). These results are summarized in figure 1C.

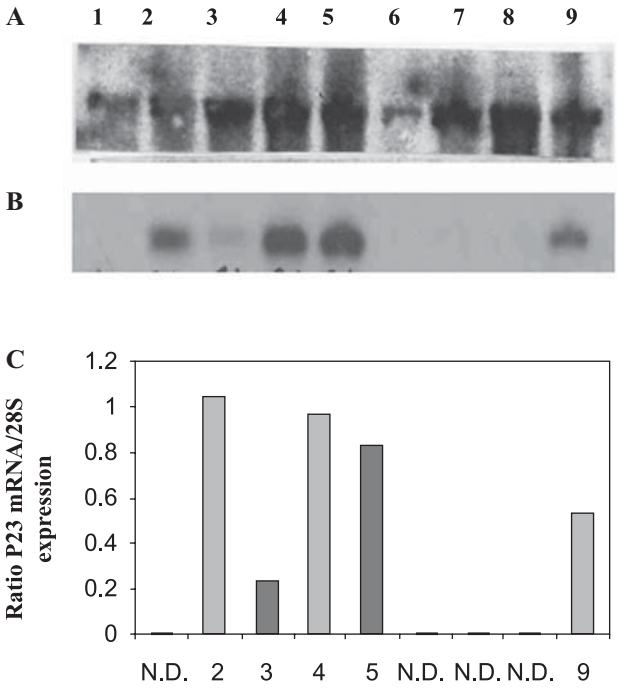

Figure 1. Northern blot analysis of rat P23 mRNA in different glucose diet conditions. Total RNA (20 µg) loaded per lane on a multiple rat tissue Northern blot probed with 860-bp cDNA encoding P23. 28S RNA (as internal control of the amounts of RNA loaded) (*A*) and a single 0.9-kb P23 band (*B*), corresponding to liver mRNA of starved rats (lane 2) and sugar supplied after fasting (lane 1), liver mRNA of rats with normal diet as control (lane 3), kidney RNA of fasted rats (lane 5) and sugar supplied after fasting (lane 4), brain mRNA of fasted rats (lane 7), intestine mRNA of fasted rats (lane 6), spleen RNA of fasted rats (lane 8) and RNA from 3MeDAB induced rat hepatoma (lane 9). The autoradiographs were scanned. The ratio of P23 mRNA/28S signals was determined and plotted (*C*).

Effect of dietary glucose on P23 expression in different tissues

The effects of dietary glucose on P23 expression in several tissues (kidney, intestine, brain, spleen) were also analyzed. As previously described [1], a high constitutive expression of kidney P23 mRNA was observed in starved animals (fig. 1B, lane 4) while in kidneys, repression of P23 mRNA expression by glucose was less evident (fig. 1B, lanes 4, 5). In other tissues, such as brain, spleen and intestine, constitutive expression was barely detectable

in starved animals (fig. 1B, lanes 6–8). In contrast, in a 3 MeDAB-induced hepatoma, increased expression of P23 mRNA was observed (fig. 1B, lane 9) as compared to normal liver (fig. 1B, lane 3). A histogram reflects more precisely different P23 expressions (fig. 2C) Interestingly, in tumours, accumulation of glucose-regulated proteins such as GRP78 appears to be not a consequence of transformation itself, but rather a consequence of the depletion of glucose in the tumoral microenvironment (rapidly growing transformed cells and poor vascularization) [18].

P23 expression in liver is developmentally regulated

We investigated whether developmental conditions could generate variations in P23 expression. Northern blot analysis of P23 mRNA from newborn and fetal rat liver revealed an increased amount of P23 mRNA in newborn rat liver compared with that of fetal rat liver at day 18 (fig. 2B, lanes 4, 5). These results were confirmed by a Western blot analysis where we noticed that P23 protein was very low in liver embryos on days 15, 17 and 20 (fig. 3 lanes 1–3) and then increased at birth (fig. 3, lane 4), due to lack of glucose associated with the extra-uterine environment. Indeed, gluconeogenesis starts very rapidly in the newborn rat as it represents the only metabolic pathway allowing long-lasting glucose production. We also observed an increased amount of P23 protein during suckling, due to glucose-free maternal milk (fig. 3, lanes 5–8). Alterations in the hormonal environment, associated with increased substrate availability, seem to favour P23 induction in the female rat during gestation, compared to a rat on a normal diet, in terms of both P23 mRNA expression (fig. 2B, lanes 3, 6) as in P23 protein expression (fig. 3, lanes 8, 9).

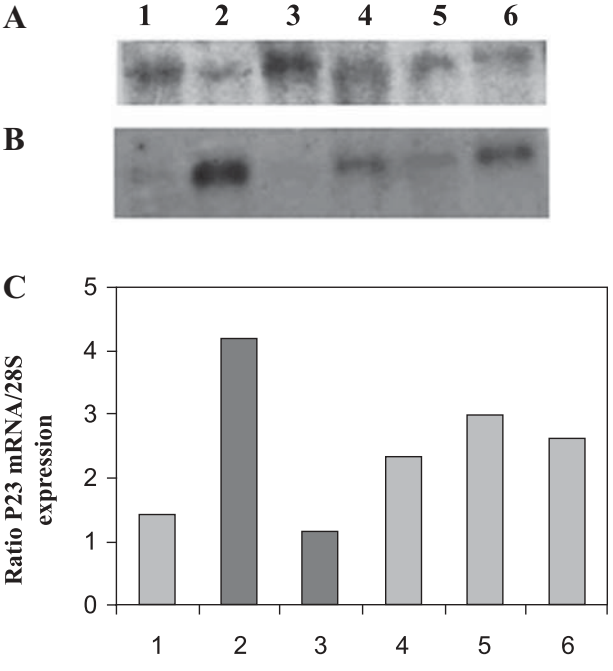

Figure 2. Northern blot analysis of rat liver P23 mRNA at different stages of development. Total rat liver RNA (20 µg) loaded per lane on a Northern blot was probed with 860-bp cDNA encoding P23, yielding an estimation of 28S RNA (as internal control of the amounts of RNA loaded) in all tissues (*A*) and a single 0.9-kb band in (*B*) corresponding to liver mRNA from adult rats after fasting (lane 2), diet supplemented with sugar (lane 1), normal diet (lane 3), from liver of newborn rat (lane 4), from fetal liver at day 18 (lane 5), from liver of pregnant rat (lane 6). The autoradiographs were scanned. The ratios of P23 mRNA/28S signals were determined and plotted (*C*).

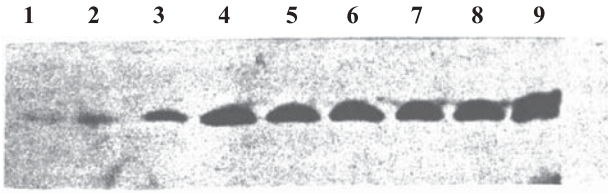

Figure 3. Western blot analysis of rat liver P23 expression during development. Each lane contains 5 µg of P23 protein from liver of newborn rat (lane 4), from fetal rat at 15, 17, 20 days of gestation (lanes 1–3), from liver of newborn suckling rats at 2, 5, 9, 18 days after birth (lanes 5–8) and from liver of pregnant rat (lane 9).

P23 mRNA is hormonally modulated

The liver plays a central role in maintaining blood glucose homeostasis. During fasting, gluconeogenesis consumes energy and is responsible for replenishing blood glucose functions at a high rate. The key hormonal regulators involved in this mechanism are glucagon and insulin, which act at different levels (pre- and post-translational) and in opposite directions on the activities of regulatory enzymes [27]. As reported by these authors, variations in glucagon and insulin secretion rates are primarily under dietary control. We observed that glucagon and insulin act differentially on P23 mRNA expression (fig. 4B, lanes 6 and 7 vs lane 5). We have often noticed in mouse only and never in rat, the presence of two transcripts (fig. 4B, lanes 5–7). We showed that glucose supply suppressed the expression of the lower band (lanes 2–4). These observations are underlined by plotted representation (fig. 4 C). Fluctuating P23 expression due to cAMP, insulin and glucagon hormones under dietary control lead us to hypothesize that the P23 gene might have a regulatory role in gluconeogenesis.

P23 is regulated by fatty acids

Prolonged deprivation of food induces dramatic changes in mammalian metabolism, including the release of large amounts of fatty acids from the adipose tissue, followed by their oxidation in liver. The nuclear receptors known as peroxisome-proliferator-activated receptor (PPARs) have been found to play a role in regulating mitochondrial and

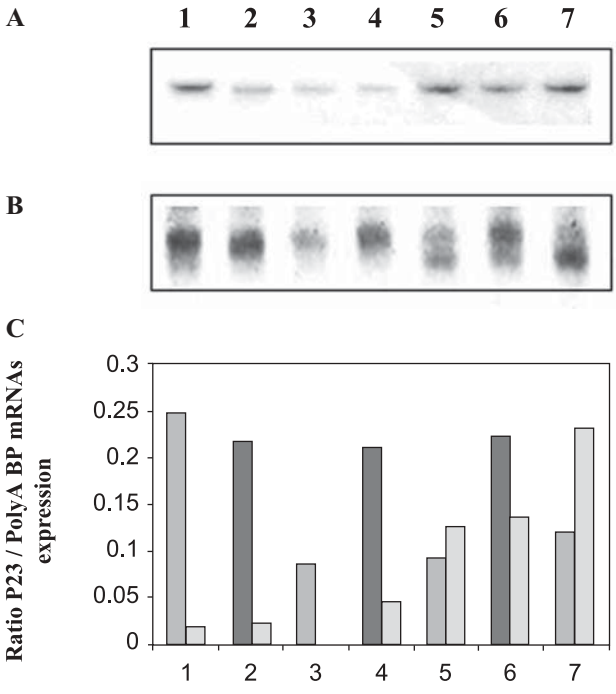

Figure 4. Effect of hormone treatment on P23 mRNA expression in a mouse AT1F8 cell line. Total RNA (20 µg) extracted from an AT1F8 cell line culture was loaded per lane on a Northern blot probed with 860-bp cDNA encoding P23. (*A*) the blot was rehybridized with polyA-BP cDNA used as a quantitative loading standard. (*B*) P23 mRNA expression in cells maintained in the absence of glucose for 24 h then supplied with glucose (lanes 1–4), plus insulin for 24 h (lane 2), plus insulin and cAMP (lane 3), plus insulin and glucagon (lane 4), with cAMP (lane 5), with glucagon (lane 6), with insulin plus cAMP (lane 7). (*C*) The autoradiographs were scanned. The ratio of P23 mRNA/polyA-BP signals were determined for each lower and upper band respectively.

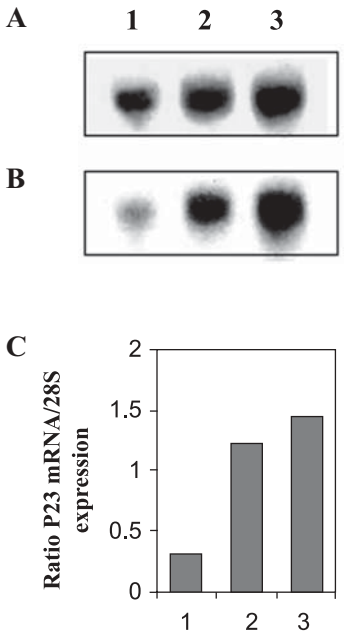

Figure 5. Effect of a fat-supplemented diet on rat liver P23 mRNA expression. Total RNA (20 µg) loaded per lane on an mRNA blot probed with 860-bp cDNA encoding P23, yielding an estimation of 28S RNA (as internal control of the amounts of RNA loaded) in rat liver (*A*) and a single 0.9-kb band (*B*), corresponding to RNA from liver of rats fed with a normal diet (lane 1), supplemented with a 10% peanut-rapeseed oil diet (lane 2) or supplemented with a 10% sunflower oil diet (lane 3). The autoradiographs were scanned. The ratio of P23 mRNA/28S signals was determined and plotted (*C*).

peroxisomal fatty acid oxidation, suggesting that PPARs may be involved in the transcriptional response to fasting. In fact, evidence is accumulating for considering that cellular lipid-binding proteins play central roles in cellular lipid uptake and metabolism [28, 29].

PSP1, the protein identical to P23 has been reported to be a fatty-acid-binding-protein (FABP)-like protein, which may be involved in the intracellular metabolism of fatty acids [30]. Indeed, FABP gene expression is transcriptionally upregulated by long fatty acid (LFA) regulation which is mediated by PPARs. After activation by fatty acid, PPAR binds as a heterodimer to the retinoid-X-receptor, to a specific responsive element, PPRE, generally located in target gene promoters [31]. We addressed the issue whether P23 might also be regulated by LFA, and examined P23 mRNA expression in rats on a fat diet (fig. 5B, lanes 2, 3). The amounts of P23 mRNA increased remarkably after administration of peanut-rapeseed or sunflower oils added to the diet compared with control rats undergoing a normal diet (fig. 5B, lane 1), as revealed by the histogram (fig. 5C). We conclude that the P23 gene might be specifically upregulated by fatty acids through a molecular mechanism similar to that of FABP, leading it to activate or inactivate target genes for the cell to adjust to metabolic changes.

Discussion

Taken together, our data suggest that P23 might be a new tissue-specific (liver-kidney) GRP-like protein; P23 is induced when animals are under starvation, at birth, during suckling and in MeDAB hepatomas where the growing transformed cells are known to consume the glucose from the medium. Like GRPs, the P23 protein is localized in the microsomal fraction and more precisely in the ER [2]. Synthesis of ER-resident proteins is regulated inside the organelle according to the demand for them. PSP protein, the P23 homologue described by Oka et al. [2] has been described as an inhibitor of protein synthesis. The presence of stress proteins that are involved in the initiation factor phosphorylation step of protein synthesis has been reported by many authors [32–34]. Translation inhibition by P23 results in an important decrease in amino acid consumption which could help the cell to maintain a sufficient pool of amino acids needed for neoglucogenesis. This implies a role for P23 in transducing dietary signals

for glucose metabolism regulation in liver. This hypothesis is corroborated by studies on metabolic phenotypes generated by mutants defective in the YER057c/YjgF protein family, which provide important clues as to the function of this highly conserved class of proteins [35]. The results presented by these authors suggest that YjgF protein or, alternatively, the product of its action, if it functions as an enzyme, is important for the appropriate function and/or regulation of the isoleucine biosynthetic pathway, and is required for the pentose phosphate pathway in thiamine synthesis.

Furthermore, evidence that PSP, the protein identical to P23, could be an FABP-like protein involved in the intracellular metabolism of fatty acids has been reported [30]. Intracellular binding proteins have been described as evolving from a common ancestor gene more than 900 million years ago [36]. Despite the high degree of diversification in amino acid sequence and ligand-binding specificity, a common three-dimensional fold has been conserved throughout the FABP protein family, as in the YjgF family. Unlike other tissues, the ability of the liver to oxidize fatty acids increases considerably when glucose availability is reduced (during e.g. birth, pregnancy, fasting, on a fatty acid diet). Liver FABP functions as a regulator of lipid metabolism, implying a direct interaction with PPARs. The expression of a number of genes involved in hepatic amino acid metabolism was recently shown to be regulated by PPARs [37]. Furthermore, cytosolic peroxisome-proliferator-binding protein was recently identified as a member of the H70 family [38]. Consequently, P23 protein can also be regarded as functioning as a cellular binding protein, given that a conserved active site common to members of the YjgF protein family has also been described for YabJ, a purine regulatory protein of *B. subtilis*, whose regulation of transcription is sensitive to levels of some nutrients [4]. These authors propose that YabJ and its high-identity homologues use the conserved, deep, narrow cleft for catalysis or binding of a common chemical entity which implies recognition of an appropriately shaped small-molecule ligand or a single-amino-acid residue in a protein ligand. Recently, functional ligand screening of HI0719, another member of this YER057c/YjgF/UK114 protein family, identified compounds such as α,β-unsaturated acids that bind at the described putative active site [39]. Furthermore, the oligomeric assembly and ligand-binding structure of UK14 and p14.5, both members of YjgF protein family, proved essential for the stoichiometric hydrophobic ligand (e.g. fatty acid)-binding activity of the two proteins [40].

There is different protein, also called P23, which is a regulatory co-chaperone of hsp90 but which can also act as a general molecular chaperone by binding to unfolded polypeptides. The contributions of these two biochemical activities to the function of P23 have been recently reported [41].

Finally, in light of all the results provided by different studies on the unknown role of the proteins of the YER057c/YjgF/UK114 family, we have demonstrated for the first time that a member of this family, the rat P23 protein, might be a novel tissue-specific GRP-like protein, with a general role in gluconeogenesis and in maintenance of glucose homeostasis.

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